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EPIDEMIOLOGY

SERO-EPIDEMIOLOGY OF THE EPSTEIN-BARR VIRUS: PRELIMINARY ANALYSIS OF AN INTERNATIONAL STUDY - A REVIEW

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The Epstein-Barr virus (EBV) is associated with three human diseases: infectious mononucleosis (IM), Burkitt's lymphoma (BL), and nasopharyngeal carcinoma (NPC), the latter two being malignant. When considered on a world basis, these three diseases have a different distribution and are to a certain extent mutually exclusive.

The restricted geographical distribution of BL (Burkitt, 1962) suggests the intervention of an environmental factor, whereas the epidemiological characteristics of NPC suggest genetic factors (Ho, 1972; de-Thé, 1972). The prevalence of IM in upper socio-economic groups in temperate zones reflects a delayed primary infection by EBV, as compared to early childhood infection in most parts of the tropical zones, where Paul Bunnell positive IM syndrome is very rare, if not absent.

The first question is: what is the relation between the epidemiological characteristics of EBV and the distribution of the three EBV-associated diseases? Knowledge of the epidemiological behaviour of EBV in populations at risk to a varying degree is essential if a testable hypothesis regarding the nature of the association (causative or non-causative) between EBV and these diseases is to be proposed, and also for an understanding of the pathogenesis of such an association, and is a prerequisite to proposals for any type of control of EBV infection; such control might be the only way of establishing the role of this virus in human malignancies.

Antibodies to EBV have been found in every population tested so far in Africa, America, Asia, Australia, Europe (Deinhardt et al., 1969), and even the North Pole (Tischendorf et al., 1970). Some Indian tribes in the remote plateau of Matto Grosso in Brazil, in whom measles, mumps and respiratory virus antibodies were practically absent, showed EBV antibodies (Black et al., 1970).

Whereas, in tropical countries, children are infected very early in life (Kafuko et al., 1972), the hygiene conditions prevailing in developed countries appear to influence the age of primary infection, since up to 40 or 50% of the adolescents entering some colleges in the USA have no detectable antibody to EBV (Wahren et al., 1970; Evans, 1974).

We present here a preliminary analysis of an epidemiological study on EBV carried out in different populations and conducted in collaboration with a number of institutions in Hong Kong, Singapore, Uganda and France. This will mainly be concerned with the situation in Singapore and Uganda. Data from Hong Kong are not yet complete; detailed reports now in preparation will cover all the areas and ethnic groups studied. The situation in Singapore is unique in as much as three different ethnic groups are represented there: Chinese, Malays and Indians, with pronounced differences in NPC risk (very high, medium and nil, respectively). Because these ethnic groups live in similar environments, the differences between them in the characteristics of EBV infection and immune response should improve our understanding of the relationship between EBV and NPC.

MATERIALS AND METHODS

Population samples were taken in Hong Kong, Singapore, the West Nile District of Uganda, and the city of Nancy in France. In Singapore, a new housing development was chosen with a population of some 150 000. Census

lists were used to obtain a random sample of households, and all family members were included. The sample was selected to ensure equal numbers of Chinese and Indians. A similar procedure was used in Hong Kong. In Uganda, in the ongoing prospective BL study (de-Thé & Geser, 1973), which involves 35 000 children aged 2-5 from a population of 160 000 inhabitants, a census of the population had been made, and from this census eight families were chosen at random, the nearest 75 neighbouring families of each of these eight forming the sample. In Nancy, the "Centre de Médecine Préventive" sees a sample of families on a routine basis, half volunteers, half randomly selected.

The main interest of the present study lies in the comparison of two anti-EBV antibody activities against the viral capsid antigen (VCA) and soluble complement-fixing antigen (CF/S). The number of sera titrated for antibodies to the EBV VCA and to the CF/S from each area is given in Table 1. The CF/S titres of sera from Hong Kong having not yet been determined, the preliminary analysis presented here does not discuss these sera in detail.

Testing procedures included the manipulation of all sera by only one laboratory, and the mixing of sera from each area for each testing experiment, so as to reduce as far as possible the variability in the titres observed as between different antigen batches and different laboratories (Geser et al., 1974). The VCA titration was carried out according to Henle & Henle (1966), using Jijoye cell line having approximately 5% immunofluorescent cells at the time of harvest. Series of 400-600 sera were tested with every antigen batch. Titration for CF/S antibodies was carried out using a soluble antigen prepared from the NC-37 cell line as the source of antigen (Vonka et al., 1970), except for sera from Nancy, France, where the QIMR/WIL cell line was used as the source of soluble antigen (Sohier & de-Thé, 1972; de-Thé et al., 1973).

Table 1. Epidemiology of EBV in various populations: number of sera tested

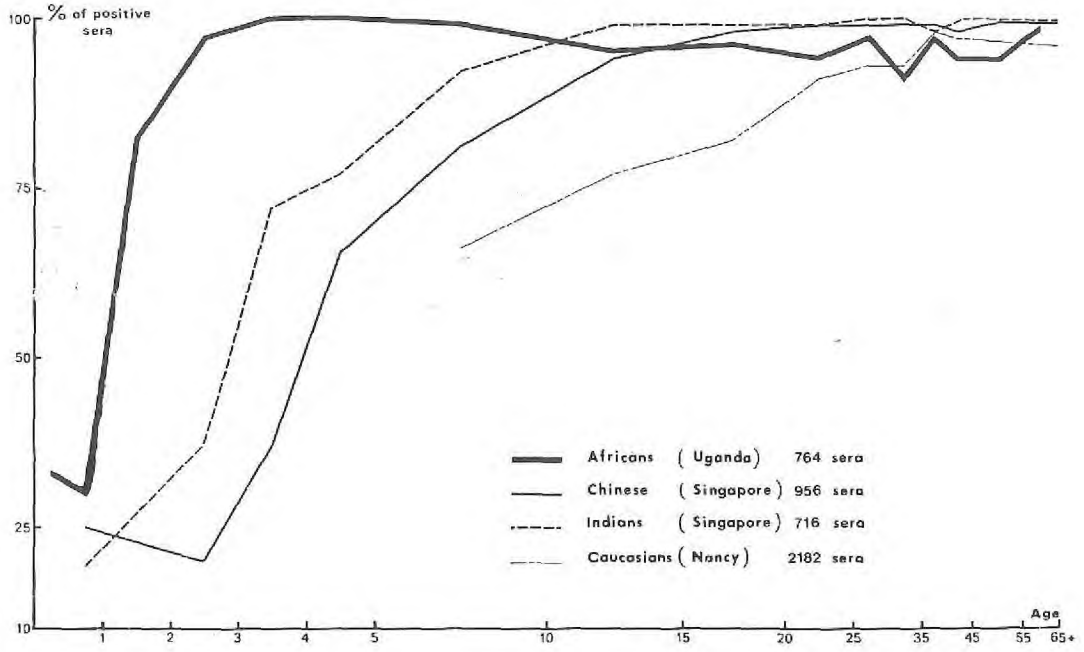
Place of origin and ethnic group		Number of sera tested for VCA	Number of sera tested for CF/S	Total
Hong Kong	(Chinese)	888	-	888
Singapore	(Chinese)	956	676	1 632
Singapore	(Indians)	716	304	1 020
Singapore	(Malays)	251	170	421
East Africa - Uganda, West Nile District	(Sudanic tribes)	764	357	1 121
Nancy	(Caucasians)	2 182	609	2 791
Total		5 757	2 116	7 873

RESULTS

Prevalence of VCA antibodies according to age and ethnic group

The age-specific prevalence of VCA antibodies in the four ethnic groups studied is shown in Fig. 1. Up to five years of age, large differences in the percentage of individuals with antibodies against VCA were observed between the four groups. As an example, 97% of the Ugandan children aged two to three years were found to be VCA-positive, whereas the same age-group in Singapore showed only 20% of the Chinese and 37% of the Indians to be positive. These large differences tapered off progressively in older age-groups. All Ugandan children aged three to five years (151 were tested) were found to be VCA-positive. During that period, the Chinese children in Singapore experienced a moderate rate of infection with percentages of VCA-positive reaching 65% in the fifth year of life. The Indian children appeared to be exposed to a higher risk of infection, with a proportion of VCA-positive children of 72% between three and four years of age, and reaching nearly 90% at five years. In Hong Kong, the infection rate in the one-to-four-years age-group appears higher than that in Chinese Singaporians, and a higher proportion of VCA-positives in females

FIG. 1. AGE-SPECIFIC PREVALENCE OF VCA ANTIBODIES IN FOUR POPULATIONS (BOTH SEXES)



as compared to males was found in both areas.

In Uganda, an unexpected *decline* in the percentage of VCA-positive individuals was observed. As will be seen from Table 2, *none* of the 151 sera from children aged three to five was found to be negative, whereas in the age-groups 5-9 and 10-14 years, 1% and 5% respectively were found to be VCA-negative. This phenomenon was more pronounced in males than in females, and was not observed to the same extent in any other population studied (Sohier et al., 1974).

Geometric mean titres (GMT) of VCA antibodies in various age and ethnic groups

The GMT of the VCA antibodies of seropositive sera in the four ethnic groups concerned are shown in Fig. 2. High VCA GMT were observed in individuals in the four populations.

The most dramatic situation concerns the Ugandans, who exhibited a very high humoral response to VCA early in life, with a GMT reaching *423* in the one-to-two-years age-group. After this high peak, the VCA titres of Ugandan children sharply and steadily decreased to a GMT of 61 at ten years of age, this level being maintained up to the age of 65.

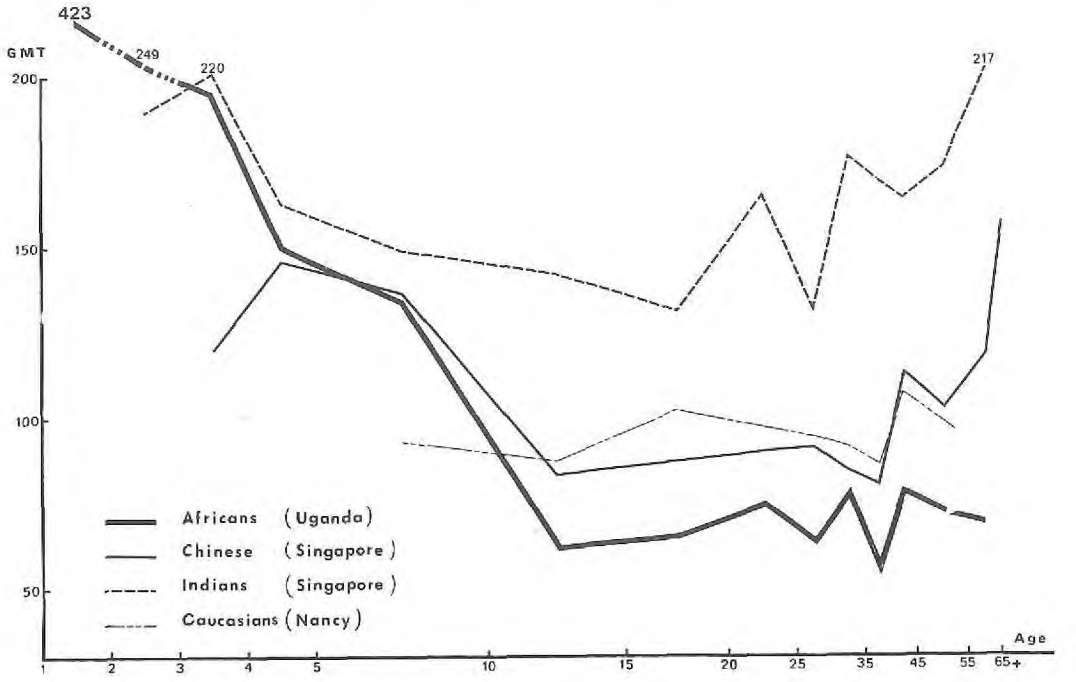
Singaporian Indians showed a very different pattern of immune response to VCA. As seen in Fig. 2, the VCA GMT were relatively *high* and *stable* from 2 to 52 years of age, varying around 150 (the value for all ages taken together being 154.9).

Table 2. Distribution of viral capsid antigen (VCA) titres in Uganda

Age	VCA titre (both sexes)										Total positives	Total	% Negatives (<10)	% High titres (≥640)	GMT of sera +	Standard error[a]	GMT all sera	Standard error[a]
	<10	10	20	40	80	160	320	640	1280	≥2560								
Months:																		
0-5	8	-	1	-	2	-	1	-	-	-	4	12	67	0	67.3	0.7	11.9	0.6
6-11	16	-	-	3	1	2	1	-	-	-	7	23	70	0	84.1	0.5	11.8	0.4
12-23	7	-	-	1	1	4	8	11	4	2	31	38	18	39	423.2	0.3	186.8	0.5
24-35	2	-	1	3	13	11	16	9	7	4	64	66	3	24	249.4	0.2	221.6	0.2
36-47	-	-	2	12	13	23	13	13	11	1	88	88	0	27	196.4	0.2	196.4	0.2
48-59	-	-	3	8	12	16	15	4	4	1	63	63	0	13	149.8	0.2	149.8	0.2
Years:																		
5-9	1	7	10	21	41	44	36	11	13	6	189	190	1	13	134.2	0.1	131.9	0.1
10-14	3	3	4	18	14	7	6	1	-	-	53	56	5	2	61.6	0.2	53.8	0.2
15-19	1	2	2	7	5	7	3	-	-	-	26	27	4	0	64.6	0.3	58.8	0.3
20-24	2	4	1	10	7	6	2	2	2	-	34	36	6	11	74.5	0.3	64.1	0.3
25-29	1	1	5	9	8	7	4	-	-	-	34	35	3	0	62.6	0.2	58.3	0.3
30-34	3	1	2	7	10	8	1	2	-	-	31	34	9	6	77.4	0.2	60.8	0.3
35-39	1	4	4	6	8	4	4	-	-	-	30	31	3	0	54.6	0.3	50.6	0.3
40-44	1	1	2	2	2	6	2	-	-	-	15	16	6	0	78.2	0.4	65.8	0.4
45-54	2	2	3	8	4	5	7	-	-	-	29	31	6	0	71.8	0.3	60.5	0.3
55-64	-	1	2	2	3	1	3	-	-	-	12	12	0	0	69.2	0.5	69.2	0.5
65+	1	-	-	1	1	-	2	1	-	-	5	6	17	17	183.8	0.7	100.8	1.1
Total:																		
No.	49	26	42	118	145	151	124	54	41	14	715	764	6	12	121.1	0.1	98.7	0.1
%	6	3	5.5	15	19	20	16	7	5	2								

[a] In dilution.

FIG. 2. AGE-SPECIFIC GEOMETRIC MEAN TITRES OF VCA ANTIBODIES IN FOUR POPULATIONS (BOTH SEXES)



Singaporian Chinese showed peak values of VCA titres in the three-to-nine-years age-group (GMT of 120-140). Later in life, the level of VCA GMT decreases to 80 and then rises again around 40 years of age (the value for all age groups taken together was 95). The values for Hong Kong, to be reported in detail later, are similar to those for Chinese Singaporians.

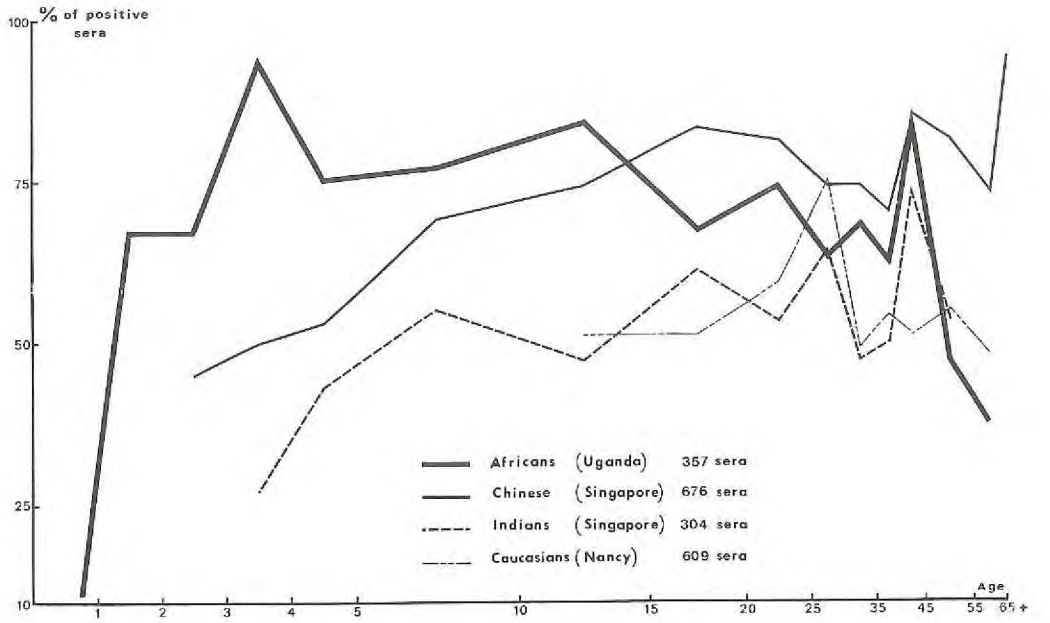
Caucasians in Nancy showed quite a stable immune response to VCA with the GMT fluctuating around 90-95 in all age-groups observed.

Comparison between GMT in males and females in all age and ethnic groups showed that, in general, females respond more to VCA antigen(s) than males.

Prevalence of CF/S antibodies according to age and ethnic group

The data on the proportion of individuals with CF antibodies against EBV soluble antigen in the various age and ethnic groups are shown in Fig. 3. Ugandans develop CF antibodies earlier than any other group, since more than 60% of babies aged one to two have CF/S antibodies, and by the age of three to four years, 94% are CF/S-positive. The proportion of positive sera for CF/S antibodies decreases

FIG. 3. AGE-SPECIFIC PREVALENCE OF CF/S ANTIBODIES IN FOUR POPULATIONS (BOTH SEXES)



thereafter to 75% and remains around this level up to 45 years of age, when the proportion decreases again to 50%.

Chinese Singaporians are next with regard to the prevalence of CF/S antibodies. Between two and five years of age, approximately 50% of the children have CF/S antibodies. The proportion then increases to 70% by ten years, and subsequently fluctuates around 70-80% up to 65 years of age.

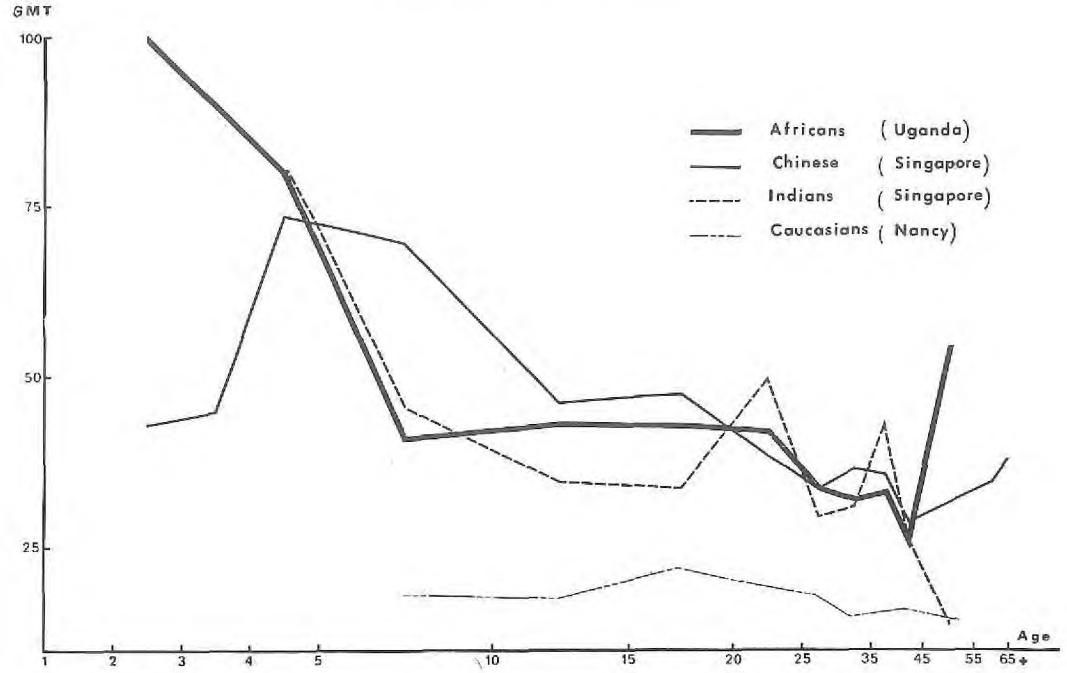
Indian Singaporians have a slightly lower prevalence of CF/S antibodies than Chinese, with only 30-40% having antibodies up to five years of age, 50% at ten years, and the level then remaining stable up to 55 years of age.

Caucasians are quite stable, with 50% of individuals having CF/S antibodies in all age-groups, starting at five years.

Geometric mean titres (GMT) of CF/S antibodies in various age and ethnic groups

The age-specific GMT for CF/S antibodies of the positive sera in the four ethnic groups are shown in Fig. 4. Again, the Ugandans show a peculiar pattern: between one and four years of age, more than one-third

FIG. 4. AGE-SPECIFIC GEOMETRIC MEAN TITRES OF CF/S ANTIBODIES IN FOUR POPULATIONS (BOTH SEXES)



of the children have CF/S titres greater than or equal to 160, with the GMT reaching 100 in the 2-3 years age-group. The level of immune response to CF/S decreases sharply thereafter, and in the five-to-nine-years age-group, the CF/S GMT decreases to 50, remaining at that level until 40 years of age.

Chinese Singaporians also respond well to the soluble antigen, although later in life and at a lower level than Ugandans. Between five and nine years of age, 27% of the sera had a titre of ≥160, with the GMT at 70. Later on, the number of high titres decreased as well as the GMT, which fluctuated around 40.

In contrast, Indians responded poorly to the soluble antigen. Between five and nine years of age, 9% of the sera had a titre ≥160, with the GMT at 45.6. The GMT of the older age groups fluctuated around 35.

Caucasians' CF/S titres are difficult to compare with the above data, since sera were not titrated beyond 1:64, using a different cell line as source of antigen. However, the distribution of titres seems to be quite similar to that observed in Indians.

Differences exist between sexes in the humoral response to the soluble antigen. These are most apparent in Uganda, where boys under seven years of age appear to react significantly more than girls to the S antigen. The Chinese show a similar trend, though in a milder way and later in life.

DISCUSSION

The historical development of research in poliomyelitis provides an example of the unique value of sero-epidemiology in understanding the pathogenesis and achieving the control of virus-induced disease (Paul, 1973). The development of the Lansing, mouse-adapted, strain of polio virus allowed the age-specific prevalence of antibodies in various parts of the world to be established, indicating that countries with warm climates and poor socio-economic conditions suffered earlier and higher rates of infection than countries having temperate climates and developed economies. The contrasting epidemiological distribution of the paralytic disease and of early infection by the polio virus led to the understanding of the pathogenesis of poliomyelitis.

In a similar way, much of the distribution of infectious mononucleosis (IM) in different populations can be explained by the distribution of age at first infection with EBV in populations at risk. When EBV primary infection takes place early in life and is holoendemic, there is no "susceptible" population available in the 15-25-year age-group for IM syndromes to occur in.

The relationship between a malignant disease and viral infection is unlikely to be as simple as the relationship between poliomyelitis and such infection. Nevertheless, it is instructive to examine the extent to which the serological results described in the previous sections can be related to the epidemiology of BL or NPC, and whether testable hypotheses can be rejected or others suggested.

Burkitt's lymphoma

The main epidemiological features of Burkitt's lymphoma are: (1) the age distribution of the tumour, rising from a very low incidence below the age of four to a peak at ages 5, 6 and 7, then falling rapidly, with few cases over age 10; (2) the sex difference, with a preponderance of males (2.5:1); (3) the geographical distribution, the high-incidence areas coinciding with areas of holoendemic malaria; and (4) the clustering phenomena, including both the space-time clustering and the seasonal effect (Williams et al., 1974). This last aspect strongly suggests a short interval (6-18 months) between some necessary triggering environmental factor and the clinical development of BL.

The following comments can be made with regard to the sero-epidemiological behaviour of EBV in the communities studied.

The environmental load of EBV in the pre-BL age group (0-3 years) is far higher in Uganda than in any of the other environments considered in the present study. All children aged three to five tested in Uganda were found to be infected, whereas in Singapore 20-50% of children of the same age had not been exposed. It is unlikely that this greater load of microbiological infection is restricted to EBV.

The high humoral response to both VCA and CF/S antigen in Ugandan children around three years of age is probably related to early primary infection, occurring in infants whose reticulo-epithelial system is

immature, thus allowing a heavier and more prolonged viral multiplication to take place and leading to massive production of antigen. The effect of early infection on cell-mediated response to EBV is unknown but is probably important.

During the period of peak BL incidence and thereafter, an appreciable proportion of African children, especially boys, have undetectable levels of VCA antibodies. A similar phenomenon was observed by Kafuko et al. in 1972, during a health survey of the same population of the West Nile. A cohort phenomenon due to epidemics of EBV infection was proposed as one alternative to explain such a phenomenon. The fact that the same phenomenon, involving the same age-groups, was observed a few years later makes a cohort effect very unlikely.

During the follow-up of the population surveyed in the prospective BL project (de-Thé & Geser, 1973), regular rebleeding of some randomly selected groups is being carried out. In this follow-up study, four children out of 446 individuals have showed a decrease in VCA antibodies over the 10-15 month period from titres at 40-80 to 10, and for two children to undetectable levels. This loss of detectable VCA antibodies can result either from the formation of antigen-antibody complexes or from a severe immune impairment. Early and massive primary viral infections are known to lead to persistent infection, allowing immune complexes to be formed.

In this context, it is important to remember that most cases of subacute sclerosing panencephalitis (SSPE) are seen in individuals infected early in life by measles virus, and that persistent viral infection and impaired cell-mediated immunity (CMI) appear to be critical for disease development (Brody & Detels, 1973).

Impairment of the immune defence mechanisms, possibly due to malaria, may lead in part to depression of humoral immunity. Detection and titration of antibodies to other viruses and parasites, such as cytomegalovirus and malaria, will indicate whether the lack of antibody is restricted to EBV.

In view of the probable short latent period of BL, and because a 100% prevalence of EBV was observed between 3 and 5 years of age in Uganda, it is unlikely that BL could follow *shortly* (6-12 months) after primary EBV infection, as is the case for infectious mononucleosis. The oncogenic potential of EBV, if it is to be expressed, may need to be enhanced by other factors. Reactivation is one of the characteristic properties of herpesviruses, and more research is needed on EBV reactivation; this may help to solve the problem.

Nasopharyngeal carcinoma

Two aspects of the epidemiology of NPC are relevant here. The first is the age distribution of the disease: among Chinese populations there is an incidence peak in the 45-54 age group and then a fall; in the intermediate risk areas (North and East Africa), there is a second peak between 10 and 20 years of age. This behaviour would suggest that exposures to environmental factors during teenage life, or perhaps earlier, are important in the etiology of NPC. The second aspect is the role of genetic factors in Chinese and related populations for NPC risk (Ho, 1972).

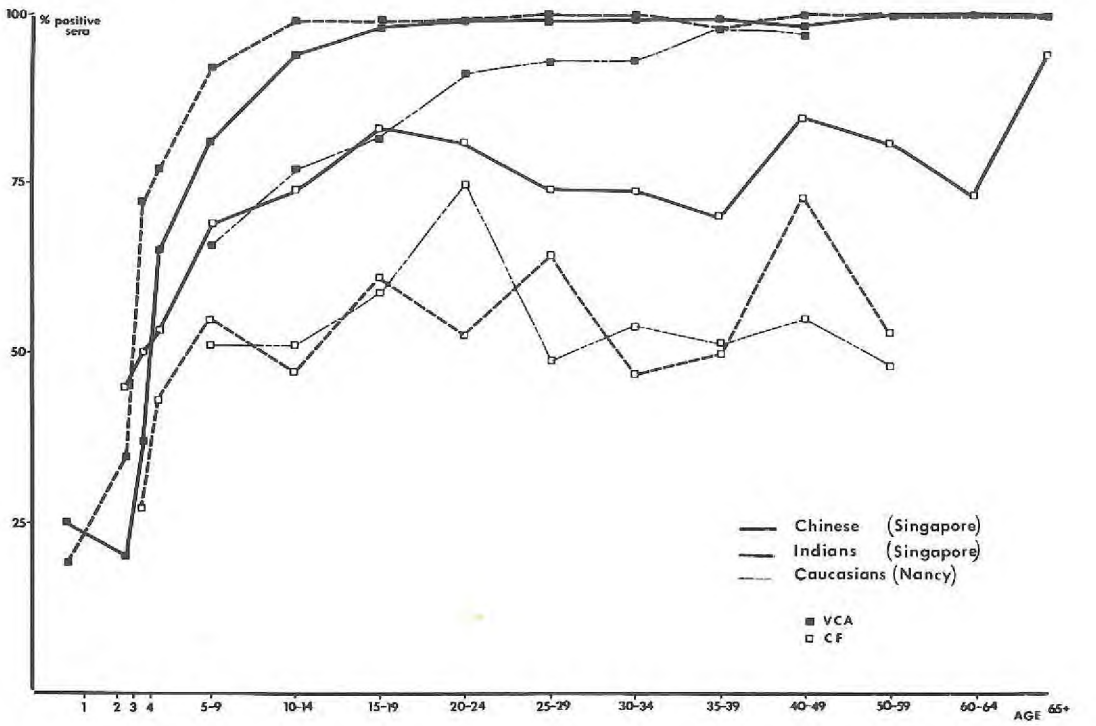
The question that obviously arises is whether the sero-epidemiological pattern observed, particularly before the age of twenty, can be linked to

NPC risk. The answer appears to lie in the differential evolution of the anti-VCA and CF/S antibody response with age among the Chinese and non-Chinese populations. The anti-VCA antibody is often taken as a measure of exposure and, for this activity, it can be seen from Fig. 5 that the Singapore Chinese lie between the Singapore Indians and the French Caucasians.

In contrast, the CF antibodies evolve with age in a very different way. As shown in Fig. 5, both the Indian and Nancy populations show a prevalence of approximately 50% of CF antibodies in all age-groups over five. Among the Chinese, the prevalence of CF antibodies rises steadily from the 5-9-year age-group, reaching 85% by 40 years of age. It may be speculated that the difference in CF prevalence is a specific Chinese response to EBV. The question that must be answered next is whether serological responses can be associated with certain genetic markers, such as HL-A profile, for which a characteristic pattern is now being linked with high NPC risk (Simons et al.[1]).

[1] See p.252.

FIG. 5. AGE-SPECIFIC PREVALENCE OF VCA AND CF ANTIBODIES (BOTH SEXES)



SUMMARY

Samples of Chinese, Indian, African and Caucasian populations, randomly selected in Hong Kong, Singapore, the West Nile District of Uganda, and Nancy, France, were titrated for antibodies to EBV, viral capsid (VCA) and complement-fixing soluble (CF/S) antigens. The age-specific prevalence of infection (as reflected by the proportion of VCA-positive individuals) varied greatly up to the age of 10 years in the four populations studied, the West Nile District of Uganda being outstanding in having an early and massive infection rate. Differences were also observed between ethnic groups in Singapore, where the Chinese appeared to have a delayed infection rate compared to the Indians.

Immune response, as measured by the prevalence of CF/S antibodies and by the geometric mean titres (GMT) of VCA and CF/S antibodies, differed significantly between ethnic groups, Ugandan infants (1-3-year age-groups) having a humoral response to VCA and CF/S antigens as high as or higher than that of Burkitt's lymphoma patients, decreasing thereafter to levels lower than those of any other ethnic group observed. Indians in Singapore, known to be at no risk for NPC and BL, exhibited a higher and steadier immune response to VCA in going from young to old age-groups than did the Chinese.

ACKNOWLEDGEMENTS

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EXPERIMENTAL EVIDENCE FOR INFLUENCE OF TYPE-C VIRUSES ON MAREK'S DISEASE VIRUS-ASSOCIATED ONCOGENESIS

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Marek's disease (MD) is a highly contagious lymphoproliferative disease of chickens with which a Group-B herpesvirus is associated (Churchill & Biggs, 1967; Nazerian et al., 1968). Enhancement of an avian leukosis virus (ALV) by interaction with Marek's disease virus (MDV) was demonstrated *in vitro* (Frankel & Groupé, 1971). The following report summarizes experimental evidence that interactions between type-C viruses and MDV may influence the responses of chickens initially free of active ALV infection.

MATERIAL AND METHODS

Viruses

Cell-free MDV pools were extracted (Calnek et al., 1970) from skin and feather follicle epithelium (FFE) of four-week-old SPAFAS chickens. The chickens, housed in isolators, were inoculated intra-abdominally (i.a.) at four days of age with a duck embryo fibroblast (DEF) cell suspension infected with the Georgia (GA) isolate (Eidson & Schmittle, 1968) of MDV. ALV was not detected in this inoculum, or in any of the MDV pools, during direct examination by the complement-fixation test for avian leukosis (COFAL) (Sarma et al., 1964), simultaneous detection (Schlom & Spiegelman, 1971), radioimmune precipitin (Suni et al., 1973) and 60-70 S RNA assays. Chicken embryo fibroblast (CEF) cultures into which the original MDV inoculum and pools were inoculated did not contain ALV by these techniques. Resistance-inducing factor (RIF) tests (Rubin, 1960) were also negative. Viable mycoplasmas, bacteria, or other adventitious avian viruses were not detected. For certain experiments (see Table 1), cell-free MDV was similarly produced in Cornell S-line, Athens-Canadian (A-C) or LSI-SPF chickens. The number of focus-forming units (FFU) in each MDV pool was determined (Adldinger & Calnek, 1971) in chicken kidney (CK) cultures (Churchill & Biggs, 1967). The kidneys were obtained from strictly quarantined LSI-SPF chickens 10 days of age. In other studies (see Table 1), the CEF cultures (Kottaridis et al., 1968) used were derived from LSI-SPF embryos.

Table 1. Characteristics of cell-free MDV pools contaminated with an ALV during skin extraction

Preparation		Characteristics										
Chickens[a]		MDV pools	Focus formation and plaques		LSI-SPF chickens[b] (%)				S-line chickens[b] (%)			
					Cumulative mortality		Tumour[c]		Cumulative mortality		Tumour[c]	
Type	ALV	ALV	CEF	CK	3 weeks	8 weeks	3 weeks	8 weeks	3 weeks	8 weeks	3 weeks	8 weeks
S-line	+	+	0	Small	100	-	0	-	12	72	16	96
A-C	+	+	0	Small	100	-	0	-	ND[d]	ND[d]	ND[d]	ND[d]
SPAFAS	0	0	Large	Large	18	40	8	35	9	87	22	91
LSI-SPF	0	0	Large	Large	11	56	11	67	ND[d]	ND[d]	ND[d]	ND[d]

[a] Isolator-held four-day-old chickens inoculated intra-abdominally with 100 FFU of cell-associated MDV; ALV not detected in this inoculum.

[b] 100 FFU per chicken.

[c] Gross lymphoid tumours in visceral and/or neural tissues.

[d] Not determined.

The Rous associated viruses (RAV-1, RAV-2, RAV-7, RAV-50; ALV Subgroups A-D, respectively) were supplied in tissue-culture fluids by Dr E. Bernstein, University Laboratories, Highland Park, N.J., and Dr P. Vogt, University of Southern California School of Medicine, Los Angeles. Fluid from reticuloendotheliosis virus (REV)-infected CEF cultures was obtained from Dr H. Bose, University of Texas, Austin.

Chickens

Embryonated eggs and chickens from the LSI-SPF flock (Frankel et al., 1974) were supplied by Dr W. Farrow, Life Sciences Research Laboratories. Tests with trap-nested LSI-SPF chickens revealed that 95% were of the C/E phenotype. SPAFAS embryonated eggs, obtained from SPAFAS, Inc., Norwich, Conn., were also predominantly of the C/E phenotype. Neutralizing antibodies (NAs) to ALV, MDV and a variety of other avian viruses were not demonstrated in serum samples from newly-hatched LSI-SPF and SPAFAS chickens. COFAL and RIF tests were consistently negative. Cornell S-line (Hutt & Cole, 1947) embryonated eggs were supplied by Dr R. Cole, Cornell University, Ithaca, N.Y. Three-day-old A-C chickens were obtained from Dr C. Eidson, University of Georgia, Athens. With both S-line and A-C chickens, embryonic expression of ALV gs antigen was detected by COFAL tests, and RIF assays were frequently positive for ALV.

Contact-exposure and inoculation

As donors in viral exposure experiments, groups of 20 three-day-old LSI-SPF and SPAFAS chickens were distributed every two weeks for three months into each of four widely separated areas. These chickens were inoculated with 20-150 FFU of MDV (area A), 10^5 $TCID_{50}$ of RAV-2 (area B), MDV, RAV-2 or both viruses together (area C), or diluent (area D). Experimental LSI-SPF and SPAFAS

chickens were subsequently introduced at three days of age into each of the four areas. Studies involving parenteral administration of virus were conducted in isolators with LSI-SPF or SPAFAS chickens inoculated i.a. at four days of age. At necropsy, tissues were collected and fixed in Petrunkevitch cupric-paranitrophenol (Petrunkevitch, 1933), embedded in paraffin, sectioned, and stained with haematoxylin and eosin.

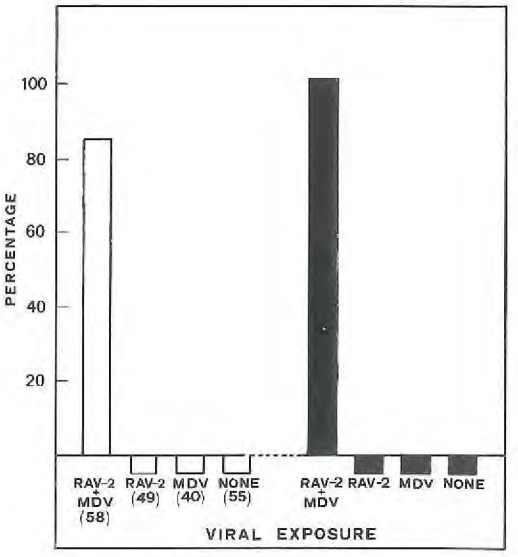
RESULTS

Mortality (81%) and gross tumour (100%) development occurred in LSI-SPF chickens during eight weeks of concurrent exposure to MDV and RAV-2 (Fig. 1). In this representative study, neither mortality nor gross tumour development was observed in chickens exposed to this ALV or MDV alone; however, histological examination revealed lymphoid cell infiltration in visceral organs and peripheral nerves of the latter group. Although it was not possible to control the infecting virus dose, since the levels of virus contamination may have varied in each of the environments, periodic sampling of contact-exposed chickens revealed the presence of ALV and/or MDV. ALV is not readily transmitted horizontally in nature but, under the

FIG. 1. MORTALITY AND TUMOUR INCIDENCE FOLLOWING VIRAL EXPOSURE OF LSI-SPF CHICKENS

Cumulative mortality and gross tumour incidence at eight weeks among LSI-SPF chickens housed from three days of age in a RAV-2 + MDV environment. The tumour incidence reflects gross lymphoid tumours in visceral and/or neural tissues. Figures in parentheses denote number of chickens per group.

☐ Mortality. ■ Tumour response.



conditions of these experiments, was detected during monitoring of sera from randomly selected, appropriately exposed chickens. Although these samplings were positive, it is conceivable that ALV could not have been isolated from all chickens in these groups. MDV was demonstrated in homogenates of skin from representative chickens housed in environments contaminated with MDV, and specific NAs developed.

In another experiment, six-day-old SPAFAS embryonated eggs were inoculated via the yolk sac with RAV-2 (10^5 $TCID_{50}$) or diluent. The chickens were placed in either an MDV or RAV-2 environment. Table 2 shows that the frequency of MDV recovery during 3-5 weeks was 54% from the RAV-2 + MDV group, although NAs to MDV were detected in each serum sampled at eight weeks. However, MDV was isolated from all chickens in the diluent + MDV group. Enhanced mortality rates (33% by six weeks; 71% by eight weeks) were exhibited in the RAV-2 + MDV group, compared with the diluent + MDV chickens (16% and 42%, respectively). Although the gross tumour incidence was similar in both groups throughout this experiment, differences in tumour characteristics were observed during necropsy of survivors at eight weeks. The appearance of extensive nodular lymphomatous masses replacing the normal architecture of one or more organs was noted in 16% of the RAV-2 + MDV chickens, compared with 54% of the diluent + MDV group. Chickens that did not show this type of response exhibited only moderate focal or diffuse tumour involvement.

Table 2. Response of SPAFAS chickens infected *in ovo* with RAV-2 and housed[a] in an MDV environment

Inoculum (yolk sac)	Viral exposure	No.	Response (%)			
			MDV[b] isolation	Cumulative mortality		Tumour[c]
				6 weeks	8 weeks	
RAV-2[d]	MDV	34	54	33	71	16
RAV-2[d]	RAV-2	10	0	0	0	0
Diluent	MDV	45	100	16	42	54
Diluent	RAV-2	15	0	0	0	0

[a] From three days of age.

[b] In skin homogenates from five chickens per group individually examined weekly (3-5 weeks).

[c] Extensive nodular lymphomatous masses completely replacing normal architecture of one or more organs in survivors necropsied at eight weeks.

[d] 10^5 $TCID_{50}$ per egg (tissue culture infectious dose, 50%).

Differences in response between the two groups were also revealed in *in vitro* cell-mediated immunity studies with washed lymphoid cells and sera randomly collected from 10 survivors (Fig. 2). Microcytotoxicity tests (Mitchen et al., 1973) showed that autologous sera conferred a killing effect on lymphoid cells from the RAV-2 + MDV chickens when the mixtures were added to MDV-infected CEF target cells. Lymphoid cells mixed with autologous sera from the diluent + MDV group did not show the killing effect, although lymphoid cells alone were active.

Other avian type-C viruses affected the response of chickens infected with MDV (Table 3). Inoculation of LSI-SPF chickens with MDV (50-150 FFU) combined with ALV, Subgroups A-D (10^5 $TCID_{50}$), or REV resulted in enhanced mortality rates, compared to chickens infected with MDV alone.

MDV pools contaminated with an ALV (MDV-ALV) during the extraction process from skin of chickens naturally infected with ALV (S-line, A-C) were distinguished from ALV-free MDV produced in chickens without ALV infection (SPAFAS or LSI-SPF). Table 1 shows that MDV-ALV, in contrast to MDV, did not induce characteristic focus formation or any other cytological changes in CEF cultures. MDV-ALV produced smaller foci than MDV in CK cultures.

FIG. 2. MICROCYTOTOXICITY TESTS WITH SERA FROM SPAFAS CHICKENS INFECTED IN OVO

Microcytotoxicity tests with lymphoid cells and sera from SPAFAS chickens infected with RAV-2 *in ovo* (10^5 $TCID_{50}$) and housed from three days of age in an MDV environment. The ordinates represent the difference between MDV-infected and uninfected CEF target cells in two studies with 10 chickens randomly selected at eight weeks.

□ Lymphoid cells without autologous serum.

■ Lymphoid cells with autologous serum.

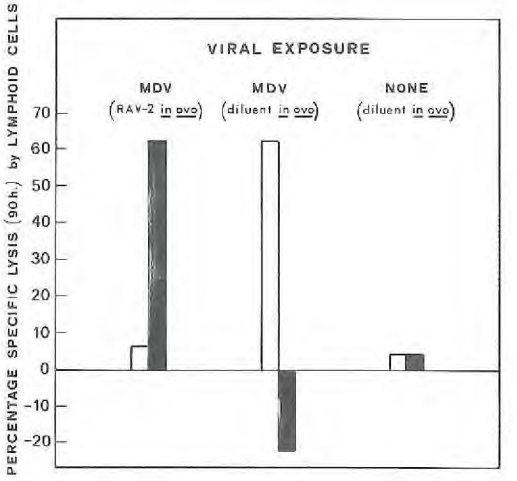


Table 3. Mortality rate in LSI-SPF chickens inoculated with MDV combined with a variety of type-C viruses

Inoculum[a]	No.	Cumulative mortality (%)				
		Weeks: 2	3	4	5	6
RAV-1	18	0	6	6	6	6
MDV	19	0	0	0	0	5
RAV-1 + MDV	18	6	6	11	33	50
RAV-2	14	0	0	0	0	0
MDV	40	0	0	0	5	5
RAV-2 + MDV	40	3	3	13	22	33
RAV-7	20	0	5	5	5	5
MDV	18	11	17	28	33	44
RAV-7 + MDV	18	5	35	72	78	83
RAV-50	16	6	19	25	25	25
MDV	18	11	17	28	33	44
RAV-50 + MDV	12	0	50	75	75	75
REV	35	49	61	68	78	81
MDV	32	3	3	3	3	3
REV + MDV	27	78	100	-	-	-

[a] Administered intra-abdominally to four-day-old chickens.
RAV: 10^5 $TCID_{50}$; REV: titre unknown; MDV: 50-150 FFU per chicken.

Further, inoculation of LSI-SPF chickens with 100 FFU of MDV-ALV resulted in an early, high mortality (100% by three weeks), compared with chickens infected with MDV (11-18%). Although gross tumour development was not observed in chickens inoculated with MDV-ALV, histological examination revealed extensive lymphoid cell infiltration in visceral organs and peripheral nerves by three weeks. LSI-SPF chickens injected with MDV exhibited 35-67% gross visceral and/or neural tumour incidence at eight weeks. In contrast, inoculation of S-line chickens with MDV-ALV did not result in an early, high mortality (12%) by three weeks. During necropsy of S-line survivors at eight weeks, it was observed that MDV-ALV and MDV produced similar tumour responses (96% and 91%, respectively).

Preliminary experiments indicate that significant levels of ALV-specific mRNA can be found in tissues from isolator-held SPAFAS chickens four weeks after inoculation with MDV. A DNA probe (Kufe et al., 1972), prepared from RAV-2 60-70 S RNA, was used to detect the ALV-specific

mRNA, and hybridization was analysed by means of thermal elution from hydroxyapatite (Kohne & Britten, 1971). Table 4 shows that the extent of hybridization with the DNA probe was significantly greater with polysomal RNA (pRNA) (Axel et al., 1972) extracted from tissues of chickens infected with MDV compared with pRNA from tissues of control chickens. Since ALV was not detected in the MDV inoculum, and infectious ALV was not isolated from any of the tissues, the ALV-specific mRNA synthesis may represent expression of endogenous type-C virus. It is conceivable, however, that MDV and ALV may share some nucleic acid homology.

Table 4. ALV-specific RNA sequences in tissues from isolator-held SPAFAS chickens[a] inoculated with MDV[b]

Inoculum	Chicken No.	Tissue	Percentage ^{3}H-DNA probe hybridized[c]
Diluent	1	Kidney	0
	2	Kidney	0
		Liver	0
	3	Kidney	1.5
	4	Kidney	2.1
		Spleen	0
	5	Kidney	0
MDV	6	Kidney tumour	15.9
	7	Kidney tumour	7.3
	8	Kidney tumour	12.4
		Ovary tumour	8.3
	9	Spleen tumour	5.7

[a] Four weeks of age.

[b] 100 FFU administered intra-abdominally at four days of age. ALV not detected in this inoculum.

[c] Approximately 200 µg pRNA (treated with deoxyribonuclease I) were annealed with 500 cpm of ^{3}H-DNA probe in 0.8 M phosphate buffer (pH 6.8) at 67°C. At a C_0t of approximately 3 000, the annealing mixture was adsorbed on to hydroxyapatite (Bio-Gel HT) and eluted with 0.12 M phosphate buffer. The amount of probe eluting at 85-98°C was considered hybridized to RNA.

CONCLUSIONS

The observation that the concurrent presence of an avian type-C virus and MDV produced additive effects *in vivo* is of general interest. Thus, type-C viruses that may be present in the host could conceivably influence herpesvirus-associated oncogenesis.

ACKNOWLEDGEMENTS

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ALTERATION IN EPSTEIN-BARR VIRUS-HUMAN LYMPHOID CELL INTERACTIONS CAUSED BY THE PRESENCE OF TYPE-C VIRAL GENOME

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It appears of importance and interest in relation to the problem of human carcinogenesis to investigate dual infections of human cells with Epstein-Barr virus (EBV) and type-C virus (Hampar et al., 1970; Maruyama & Dmochowski, 1971; Essex et al., 1972; Miyoshi et al., 1972; Hampar et al., 1973). In order to provide an experimental model for investigating the possibility of co-carcinogenesis in man by two viral agents, particularly a latent dual infection, we attempted several years ago to establish a cell line of human lymphoid origin neither producing EBV nor type-C virus, by infecting NC-37 cells (Jensen[2]) with Friend murine leukaemia virus (FLV) (Friend, 1957). As a result, a cell line (FVNC) has been established and maintained for three years which is free of detectable EBV- and FLV-related immunofluorescent antigens, but which has both viral genomes in all individual cells in a repressed form (Osato et al., 1975). This paper describes some distinct properties of such FVNC cells as compared with the original NC-37 cells. In view of our findings, the possible effects of a co-existing type-C viral genome on EBV-human cell interactions will be discussed. In addition, the possibility of type-C virus co-carcinogenesis will also be described.

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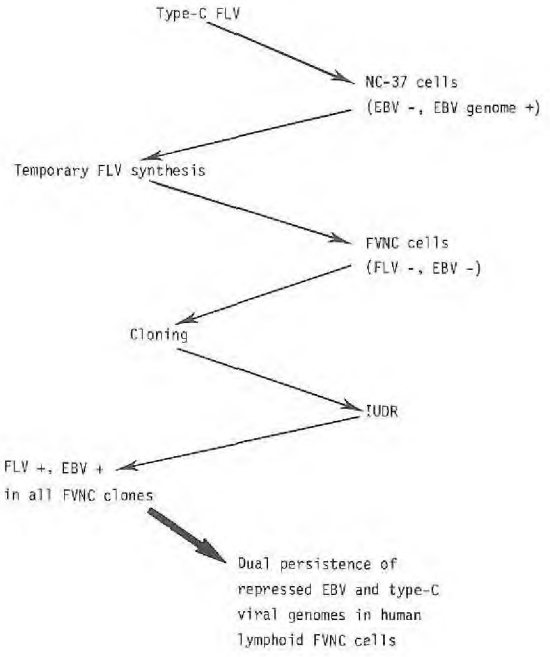
[2] Unpublished data.

OUTLINE OF FVNC EXPERIMENTAL SYSTEM

The FVNC experimental system is shown in Fig. 1. Human lymphoid NC-37 cells not producing EBV (Durr et al., 1970) but harbouring the repressed viral genome in each individual cell (Sugawara et al., 1972, 1973) were exposed to type-C FLV. FLV synthesis was clearly demonstrated by immunofluorescence, but the antigen disappeared in the weeks following exposure to the virus. Such FLV-infected NC-37 cells (designated FVNC) grew well and have been maintained for three years; both EBV and FLV markers were barely seen by immunofluorescence and electron microscopy. The establishment of the non-productive FVNC cell line has prompted us to examine whether the EBV genome is involved in FVNC cells as in NC-37 (Sugawara et al., 1972, 1973; Sugawara & Osato, 1973a, 1973b) and whether the FLV genome co-exists in the cells along with the resident EBV genome in a repressed form. A striking induction of both EBV and FLV occurred when FVNC cells were exposed to 5-iododeoxyuridine (IUDR). We subsequently examined a number of FVNC clones for the dual induction. Both EBV and FLV antigenic markers were evident in all IUDR-treated FVNC clones tested. We therefore feel that our FVNC experimental system may be unique, as EBV and type-C viral genomes are present together in each individual cell in a repressed form (Osato et al., 1975).

FIG. 1. FVNC EXPERIMENTAL SYSTEM



MORPHOLOGY AND CHROMOSOMES OF FVNC CELLS

FVNC cells were of lymphoid morphology, similar to the original NC-37 cells. However, when examined chromosomally (Osato et al., 1969), one D-group chromosome was consistently missing (Fig. 2). This change was first noted several weeks after exposure to FLV as the viral immunofluorescence (Osato et al., 1964) disappeared rapidly from the infected NC-37 cells (Osato et al., 1975) (Table 1).

GROWTH CHARACTERISTICS OF FVNC CELLS

FVNC cells grew slightly faster than uninfected NC-37 cells in fluid culture, the latter having a doubling time of 24 hours compared to 22 hours for the former. However, when grown in semi-solid agar (Hinuma & Grace, 1968), a remarkable difference in colony-forming capacity was noted between the two cell lines, as shown in Table 2. In contrast to the growth in fluid culture, only a small number of FVNC colonies could be seen, namely about 1/10 of those formed by NC-37 cells.

SUPERINFECTION OF FVNC CELLS WITH EBV

When FVNC and the original NC-37 cells were exposed to EBV, FVNC cells were three times more sensitive than NC-37, as shown by immunofluorescence using sera from a nasopharyngeal carcinoma patient. Many more early antigen (EA)-positive (Henle et al., 1970) cells with intense fluorescence were seen. In addition, an obvious

FIG. 2. KARYOTYPE OF FVNC CELL

One of the D-group chromosomes is missing (arrow).

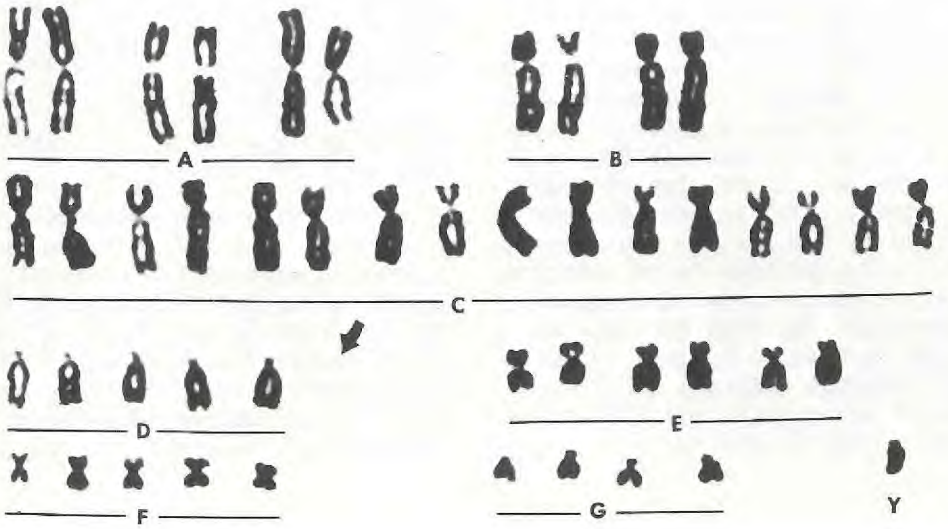


Table 1. Decrease in D chromosomes in FVNC cells

Time after FLV infection (days)	NC-37 with 6 D chromosomes (%)	FVNC with 5 D chromosomes (%)
27	93	60
36	90	63
41	97	90
58	100	83
80	97	93
94	93	93

FLV induction occurred in FVNC cells following the superinfection, as demonstrated by murine type-C virus group-specific antigen (Geering et al., 1966) immunofluorescence, using anti-Gross lymphoma serum. These results are summarized in Table 3.

NUMBER OF EBV GENOMES IN FVNC CELLS

In view of these results, the question arose as to whether the remarkable differences between FVNC and NC-37 cells can be attributed to an unstable interaction between the repressed EBV and FLV genomes in FVNC cells. The number of EBV genomes was therefore measured by a nucleic acid hybridization technique (Nonoyama & Pagano, 1971). A striking decrease in the number of resident EBV genomes was noted in FVNC cells. There were too few viral genomes to be detected, as seen in Table 4, but the immunofluorescence investigations showed that EBV-associated nuclear antigen (EBNA) (Reedman & Klein, 1973) was intensely positive in all FVNC cells as well as NC-37 cells.

POSSIBLE EBV-TYPE-C VIRUS COCARCINOGENESIS IN MAN: ITS SIGNIFICANCE AND MECHANISM

Our findings strongly suggest that EBV-human lymphoid cell interactions can be markedly modified by the co-existence of type-C viral genomes.

Although the significance and mechanism of the consistent D chromosome change in FVNC cells are not known, this change may be primarily due to a close association of the exogenous FLV genome with the human lymphoid NC-37 cell genome and a subsequent competitive and unstable interaction between the resident EBV genome and the newly associated FLV genome in FVNC cells.

FVNC cells grew rather faster than NC-37 in fluid culture but much slower in medium with semi-solid agar. A possible explanation for this difference is that NC-37 cells, which normally contain about 80 EBV genomes and are capable of growing well in agar medium, may revert to "normal" as the number of viral

Table 2. Colony formation by NC-37 and FVNC cells

Colony formation (%)[a]	
NC-37	FVNC
41.2	4.1
51.7	7.8
53.7	5.8

[a] In semi-solid agar medium.

genomes decreases. Alternatively, EBV induction may occur in FVNC cells in agar, leading eventually to cell degeneration. This is plausible, as EBV induction by IUDR occurred more readily in FVNC cells than in the original NC-37 cells (Osato et al.[1]).

It appeared of particular importance that FVNC cells were much more sensitive to EBV superinfection than NC-37 cells and that, in addition, an obvious induction of the repressed FLV genome occurred simultaneously. Our preliminary data showed, in contrast, that FLV superinfection resulted in a high frequency of induction of an EBV-related early product, early nuclear antigen (ENA) (Sugawara & Osato, 1973b), in FVNC cells (Osato et al.[2]). Thus, the exposure to "human cancer viruses" of cells primarily harbouring both EBV and type-C viral genomes appears to be particularly important among many oncogenic environmental factors.

We have not yet tried to determine the number of FLV genomes; however, the striking decrease in EBV genomes in FVNC cells (though the mechanism of this elimination is not clear), strongly suggests that the interaction between the repressed EBV and FLV genomes may be unstably competitive. It is therefore conceivable that FVNC cells themselves may be in an unstable state, and that the remarkable differences in properties between FVNC cells and the original NC-37 may primarily be attributed to an unstable competitive interaction between the two repressed viral genomes.

In view of our present and previous findings, the significance of dual EBV and type-C viral infection in relation to human carcinogenesis and the mechanism of the possible co-carcinogenesis by the two viral agents are illustrated in Fig. 3. When man is exposed to both EBV and type-C virus, these viral genomes may be closely associated with the genomes of some cells, possibly of lymphoid origin. Such cells harbouring the two repressed viral genomes might appear to be normal but could perhaps be in an unstable state and highly sensitive to various stimuli. Above all, exposure to exogenous EBV or type-C viruses, which may often occur

[1] Unpublished data.

[2] Unpublished data.

Table 3. Responsiveness of NC-37 and FVNC cells to EBV superinfection

Time after superinfection (days)	EA-positive cells (%)[a]		GSA-positive cells (%)[b]	
	NC-37	FVNC	NC-37	FVNC
2	6.9	28.2	-	-
4	13.4	32.6	0	12.4
10	2.9	6.4	0	4.4
15	0.6	3.5	-	-

[a] Immunofluorescence, stained with serum from nasopharyngeal carcinoma patient.

[b] Immunofluorescence, stained with anti-Gross lymphoma serum.

Table 4. Number of EBV genomes in NC-37 and FVNC cells

Cells	No. genomes/cell[a]	EBNA-positive cells (%)[b]
NC-37	80	100
FVNC	Not detectable	100

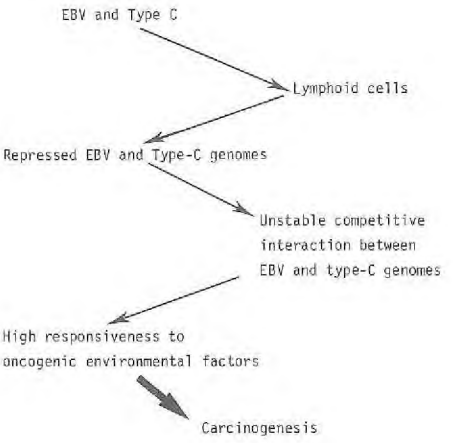
[a] By cRNA-DNA hybridization.

[b] By anticomplement immunofluorescence.

during aging, may be particularly important. By exposure to such exogenous "human cancer viruses", the production of superinfecting viruses and activation of their resident viral genomes could occur in the unstable "normal" cells described above. Such an unstable state and high sensitivity of cells harbouring both EBV and type-C viral genomes could be responsible for a potent human viral carcinogenesis, although a dual effect of EBV transformation and type-C virus transformation is also worth consideration.

Our findings thus suggest that a single infection by either EBV or type-C virus is not necessarily enough on its own to bring about viral carcinogenesis in man.

FIG. 3. POSSIBLE MECHANISM OF EBV-TYPE-C VIRUS CO-CARCINOGENESIS IN MAN



SUMMARY

We have recently established a cell line, designated FVNC, by infection of non-EBV-productive human lymphoid NC-37 cells with the type-C virus FLV. The FVNC cell line has been maintained free of detectable EBV- and FLV-related immunofluorescent antigens for three years but both viral genomes exist in the cells in a repressed form. The FVNC cells were morphologically similar to the uninfected NC-37 cells but a D-group chromosome change was consistently seen. The colony-forming capacity of FVNC cells in semi-solid agar medium was about 1/10 of that of NC-37, although the former grew rather faster than the latter in fluid culture. FVNC cells were three times more sensitive to EBV superinfection than NC-37, as shown by immunofluorescence. In addition, an obvious induction of FLV antigenic markers occurred in FVNC cells on exposure to EBV. Nucleic acid hybridization experiments showed a striking decrease in the number of EBV genomes in FVNC cells. The number of genomes was too small to be measured, but EBNA was clearly present in all FVNC cells as well as in NC-37 cells. The implications of these findings were discussed from the point of view of a possible co-carcinogenesis in man by EBV and the type-C virus.

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ASSOCIATION OF EPSTEIN-BARR VIRUS AND AMERICAN BURKITT'S LYMPHOMA

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Some lymphomas in the USA have similar clinical features, response to treatment (Levine & Cho, 1974) and relationship to Epstein-Barr virus (EBV) (Levine et al., 1972; Ablashi et al., 1974) as found in African Burkitt's lymphoma (AfBL). Although Burkitt-type lymphomas are rare in the USA as compared to Africa, comparative studies in various parts of the world may lead to a better understanding of the etiology of this disease, and in particular its possible relationship to EBV. In this paper, we present serological studies using soluble antigen derived from an American Burkitt's lymphoma (AmBL) patient, and review some of the serological data relating to EBV and Burkitt's lymphoma.

MATERIALS AND METHODS

Patients described in this paper were registered in the AmBL registry (Levine, 1971). The soluble (S) antigens used in this study were prepared for complement fixation (CF) according to the methods of Sohier & de-Thé (1972). The cell lines from which the four S antigens were derived include NAB-1 (an EBV non-producer line from a case of AmBL (Gravell et al.[1]), HKLy-28 (a low EBV producer from a case of nasopharyngeal carcinoma), Raji (an EBV non-producer line from a case of AfBL), and P3HR-1 (an EBV producer line from a case of AfBL). Four other assays performed in these studies included measurements of fluorescent antibody directed against the viral capsid antigen (VCA), early antigen (EA), and EBV-associated nuclear antigen (EBNA), utilizing methods described previously (Ablashi et al., 1974). Complement fixation was also performed against a

[1] Unpublished data. The NAB-1 cell line was derived from a histopathologically confirmed malignant retroperitoneal mass.

particulate CF-associated antigen, as previously described (Ablashi et al., 1974). All lymphoblastoid cell lines were grown in the same manner. Comparability of CF antigens was assured by using in each test exactly four units of antigen titrated against the same serum.

RESULTS

Studies using the S antigen in the CF test showed close correlations between the assays using each of the four cell lines. Although there were occasional discordant sera (Fig. 1 A-C), indicating that the antigens may not be identical, similar geometric mean titres (GMT) suggest that similarities are more numerous than differences (Table 1). The relationship of the soluble antigens to the particulate CF antigen (Fig. 2) was not as close as between the different S antigens. Discordant sera included two with high S antibody titres and low particulate CF antibody titres, as well as two with the reverse, suggesting two separate antigen-

FIG. 1. COMPARISON OF ANTIBODIES TO EBV SOLUBLE ANTIGENS FROM VARIOUS SOURCES Sera from AmBL patients.

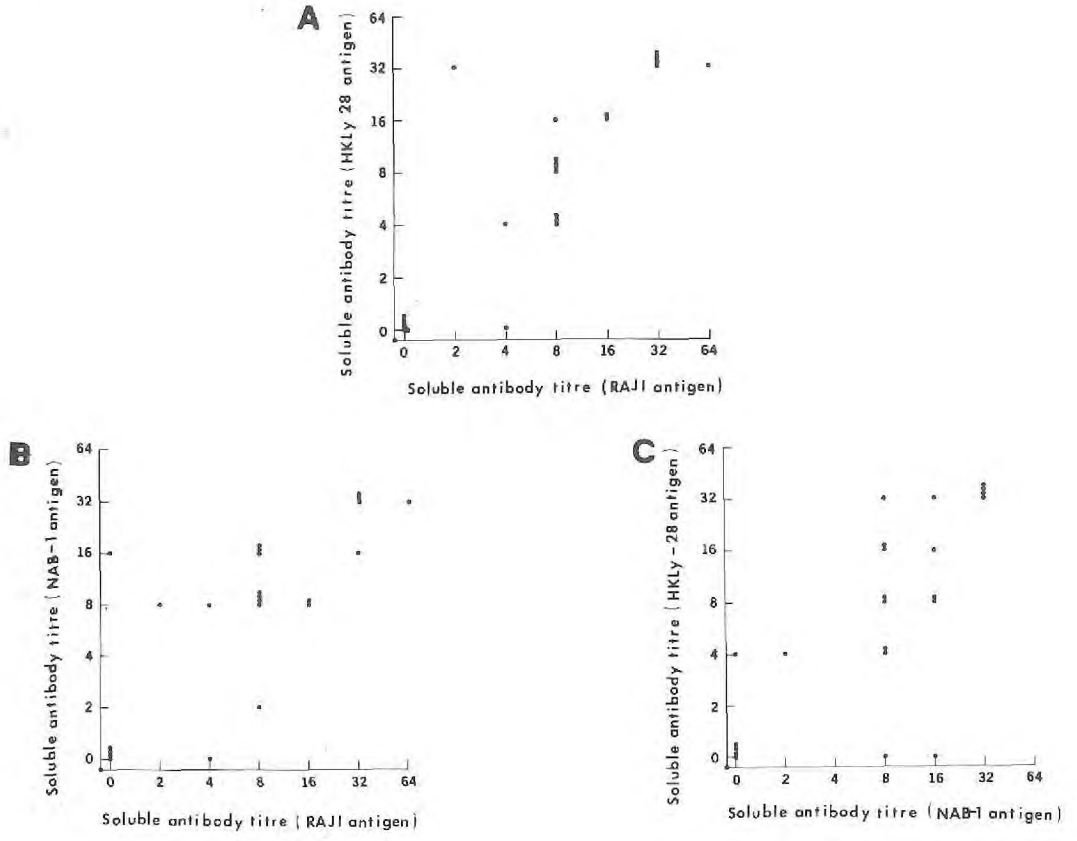


Table 1. Geometric mean titres of 23 American Burkitt's lymphoma sera against EBV soluble antigen prepared from various EBV cell cultures[a]

S Antigen and source			
HKLy-28 (NPC)	NAB-1 (AmBL)	Raji (AfBL)	P3HR-1 (AfBL)
6.479	7.091	6.677	6.677

[a] Four units of antigen were used in the microtitre CF test. All sera were diluted 1:2, tested for anticomplementary activity, and found to be negative.

antibody systems. Similar conclusions can be drawn regarding the relationship between the VCA titre and antibody to the CF particulate antigens (Fig. 3).

Analysis of the titres in the American Burkitt's patients and controls indicated that, in each of four test systems, titres in American Burkitt's patients were higher than those in the control groups (Table 2). In addition, the geometric mean titre of 10 patients who were still alive on 31 December 1973 (three of these patients were long-term survivors) suggested a correlation between antibody levels and survival.

On occasional patients, serial serum samples could be studied. Different patterns were observed and will be described more fully in a subsequent communication. One patient was shown to seroconvert in the course of her disease to four EBV-associated antibodies (Fig. 4).

DISCUSSION

A major problem in laboratory studies in Burkitt's lymphoma is the diagnosis and characterization of patients. In their original study on the serology of EBV and AmBL, Levine et al. (1972) graded the AmBL cases according to the availability of pathological material; they noted that all cases with typical histological sections and satisfactory imprints were EBV VCA-positive, and that the percentage of EBV-negatives increased as the technical problems of making the diagnosis increased. A similar rating was not available for all of these cases, so that it is possible that our AmBL cases do not form a completely homogeneous group. This is not unique to BL in the USA, since studies of AfBL also refer to cases with atypical features, and it is uncertain whether this relates to etiological factors, histological preparations or biological factors relevant to the age at the time of diagnosis (Lindahl et al., 1974). Biological differences are apparent in AfBL, since most tumours contain the EBV genome but occasional typical AfBL cases do not (Lindahl et al., 1974).

While multiple etiologies and difficulties in making a specific diagnosis can cause some confusion in studies of BL, it is still possible to state that the American cases share

FIG. 2. COMPARISON OF ANTIBODIES TO EBV SOLUBLE ANTIGEN WITH THOSE TO EBV PARTICULATE CF ANTIGEN

Sera from AmBL patients.

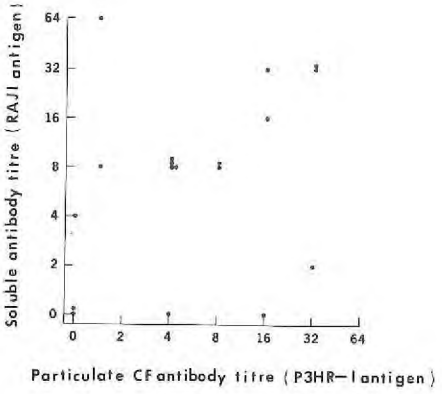


FIG. 3. COMPARISON OF ANTIBODIES TO EBV VIRAL CAPSID ANTIGEN WITH THOSE TO EBV PARTICULATE CF ANTIGEN

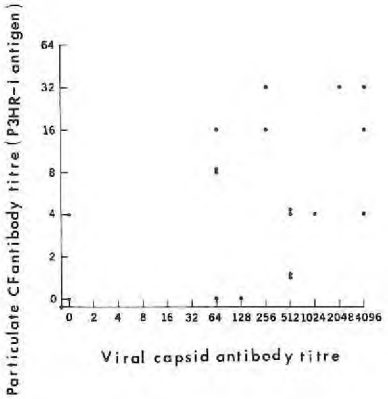


Table 2. Geometric mean titres against four EBV antigens in sera from American Burkitt's lymphoma patients believed alive and known dead, and from controls

Patients	Geometric mean titres[a]			
	anti-EBNA	anti-CF[b]	anti-EA	anti-VCA
AmBL - alive	81.8 (11) [9]	6.13 (13) [10]	19.7 (13) [10]	158 (13) [10]
AmBL - dead	44.4 (9) [8]	2.94 (9) [8]	4.77 (9) [8]	59.3 (9) [8]
Controls[c]	1.55 (9) [9]	2.94 (9) [9]	2.68 (7) [7]	20.2 (9) [9]

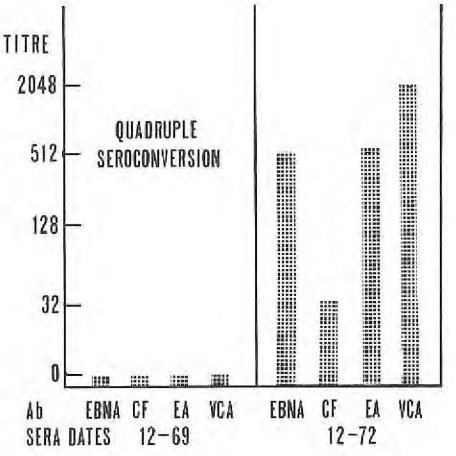
[a] Figures in parentheses are numbers of sera, those in square brackets are numbers of subjects.

[b] Particulate.

[c] Source: Ablashi et al. (1974).

FIG. 4. ANTI-EBV ANTIBODY LEVELS IN AN AmBL PATIENT WHO WAS AN ANTI-EBV SEROCONVERTER

Patient details: sex: female; registry No.: 00032; date of birth: 3.51; date of diagnosis: 12.69; age at diagnosis: 18; site of onset: colon, mesenteric lymph-nodes; initial clinical stage: 2.



many of the clinical and laboratory features of AfBL. In this study, it was again noted that antibodies to EBV are higher in AmBL patients than in controls. The finding that the immune response to the EBV S antigen is quite similar, although the antigens are prepared from different cell lines, suggests that the viruses associated with AfBL and AmBL are similar, but the discordancy in some sera between S antigen obtained from the NAB-1 line and the Raji line indicate that there may be some differences. Biochemical identification of these differences, if they do exist, may be possible when the antigens are studied in the purified form.

The observation of EBV seroconversion in the course of disease indicates that further studies are needed before EBV can be excluded as a cause of most cases of AmBL. At the present time, data from African and American cases suggest the possibility of more than one etiology for this syndrome.

ACKNOWLEDGEMENTS

We thank Mrs Barbara Cho, Mrs Janette Taylor and Mrs Karen Adelsberger for help in the preparation of this manuscript, and Miss Carolyn Meyers for help in performing the complement-fixation tests. This study was supported in part by NCI contract CO-25423 with Litton Bionetics, Inc.

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ANTIBODIES TO HERPESVIRUSES IN PATIENTS WITH CERVICAL CANCER AND CONTROLS

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Herpes simplex virus type 2 (HSV-2) has been intensively studied as a possible etiological factor in cervical cancer (Nahmias & Roizman, 1973; Muñoz, 1973). Most of the sero-epidemiological studies aimed at demonstrating an association between the presence of HSV-2 antibodies and the risk of developing cervical cancer have used neutralization tests. Although, in most studies, higher frequencies of HSV-2 antibodies have been found in women with cervical cancer than in control women, there are some interesting exceptions. In Cali, Colombia, which has one of the highest incidence rates for cervical cancer, and in Israel, with the lowest incidence rate, no difference was observed between cases and controls and a similar prevalence of HSV-2 antibodies was observed in both countries (Rawls et al., 1972; Pridan & Lilienfeld, 1971). Besides HSV-2, cytomegalovirus (CMV) and human papilloma virus are known to cause infections of the uterine cervix. The following study was carried out to study further the relationship of HSV-2 and CMV to cervical cancer, using the indirect immunofluorescence test (IF), and to compare their antibody pattern with that of herpes simplex virus type 1 (HSV-1) and Epstein-Barr virus (EBV), which have not been related to cervical cancer.

MATERIALS AND METHODS

Patients

Serum samples were obtained from 259 patients distributed as follows: 57 patients with cervical cancer (22 carcinomas *in situ* and 35 invasive carcinomas) diagnosed histologically at the University Hospital of Cali, Colombia, from 1970 to 1972; 58 patients with condyloma acuminatum diagnosed at the venereal diseases clinic of Cali from 1970 to 1972; 109 control patients matched by age, sex and socioeconomic status without cytological evidence of carcinoma or clinical evidence of condyloma; 35 blood donors at the National Cancer Institute, Bethesda, USA in the same age range as the controls from Cali.

Viruses

A plaque-purified syncytial substrain of the MS strain of HSV-2 and the 11124 strain of HSV-1 were grown and titrated in the Vero line of African green monkey kidney cells. The McKinley strain of CMV obtained from Dr C. Alford was grown and titrated in WI-38 cells. The HR1K lymphoblastoid cell line was used for EBV studies.

Viral capsid antigens (VCA) and early antigens (EA)

For the VCA of HSV-1 and HSV-2, Vero cells were inoculated with 1 plaque-forming unit (PFU) virus/cell, absorbed for 1 hour at 36°C and incubated for 24 hours at 36°C. Smears were prepared on Teflon-coated slides, fixed for 10 minutes in acetone and stored at -70°C. For the VCA tests with CMV, WI-38 cells were inoculated with approximately 1 PFU virus/cell, absorbed for 1 hour at 36°C and incubated for 72 hours at 36°C. For the early antigens of CMV, WI-38 cells were inoculated, absorbed and incubated as above, but 40 µg/ml of cytosine arabinoside was added to the medium. EBV-VCA titres were determined on acetone-fixed smears of the HR1K cell line, while EA titres were determined on Raji cells infected with EBV concentrates.

Immunofluorescence tests

The basic IF tests used to detect antibodies to VCA of HSV-1, HSV-2, CMV and EBV, and antibodies to early antigens of CMV and EBV, have been described elsewhere (Rajčáni et al.,1973; The et al., 1974; Pearson et al., 1970)

RESULTS

The slides were read with 98% agreement between two independent examiners. Table 1 shows the distribution of the antibody titres against VCA of HSV-2 and CMV in patients and controls. No significant difference in the distribution of these titres among the three groups from Cali, Colombia, was observed. All of them were positive, but high titres were more frequent among the patients with cervical carcinoma and condyloma than among the control group, while 23% of the controls from the USA did not have HSV-2 antibodies. No differences were observed, however, in the distribution of antibody titres against VCA of CMV (Table 1) and HSV-1 (Table 2). It is interesting to note that 80-90% of the patients from the three groups from Cali have EBV-VCA antibody titres higher than 1:160 (Table 2) and that a considerable proportion of these patients have antibodies against EBV early antigens, with titres higher than 1:40 in 20% of patients with cervical cancer and condyloma (Table 3). Of the patients with cervical cancer, 36% have antibodies against early antigens of CMV, as do 18% of the

Table 1. Distribution of antibody titres against viral capsid antigens of HSV-2 and CMV in patients and controls

Antibody titres	VCA - HSV-2 (%)				VCA - CMV (%)			
	Cervical cancer	Condyl-oma	Control (Cali)	Control (USA)	Cervical cancer	Condyl-oma	Control (Cali)	Control (USA)
<10	-	-	-	22.9	-	-	-	-
10-80	10.5	17.3	32.1	25.7	3.5	-	-	-
160-640	82.5	81.0	65.1	51.4	5.3	3.5	5.8	2.9
≤1280	7.0	1.7	2.8	-	91.2	96.5	94.2	97.1
No. of cases	57	58	109	35	57	58	104	35

Table 2. Distribution of antibody titres against viral capsid antigens of HSV-1 and EBV in patients and controls

Antibody titres	VCA - HSV-1 (%)				VCA - EBV (%)		
	Cervical cancer	Condyl-oma	Control (Cali)	Control (USA)	Cervical cancer	Condyl-oma	Control (Cali)
<10	-	-	-	-	-	2.4	1.3
10-80	1.7	3.5	1.0	8.6	13.8	9.5	20.0
160-640	38.6	29.8	32.4	31.4	65.5	69.1	74.7
≤1280	59.7	66.7	66.6	60.0	20.7	19.0	4.0
No. of cases	57	57	105	35	29	42	75

patients with condyloma, 19% of the controls from Cali and 11% of the controls from the USA (Table 3). No differences in the HSV-2 or CMV antibody patterns were observed between patients with carcinoma *in situ* and patients with invasive cancer. The VCA of HSV-1 and HSV-2 appeared as a diffuse cytoplasmic and nuclear fluorescence, the VCA of CMV as diffuse cytoplasmic and nuclear fluorescence, and large cytoplasmic inclusion bodies were often observed.

The early antigens of CMV appeared as fine punctate structures in the nuclei. Attempts to demonstrate antibodies to early antigens of HSV-2, using cells harvested three hours after infection or cells infected in the presence of cytosine arabinoside, showed no differences in number of positive sera or titres from the standard VCA antibody determination.

DISCUSSION

A low prevalence and no difference in the frequency of HSV-2 antibodies between cases and controls have been reported in Cali, Colombia, using a microneutralization test (Rawls et al., 1972). In the present study, different series of sera from the same population were tested using the indirect immunofluorescent test. Although no significant differences between cases and controls were found, all Cali sera showed HSV-2 antibodies, and a higher frequency of high titres was observed in patients with cervical cancer and condyloma acuminatum than in the controls. The controls from the USA showed a lower prevalence of HSV-2 antibodies and a lower frequency of high titres than the three groups from Cali. These data suggest that the IF test is more sensitive than the microneutralization test, although it is certainly less specific for HSV-2. The association between the frequency of HSV-2 neutralizing or immunofluorescent antibodies and cervical cancer is difficult to interpret. Studies aimed at determining HSV-2 specific antibodies are more valuable in the understanding of this association: positive associations between HSV-2 early and "non-virion" antigens and cervical cancer have been described (Sabin & Tarro, 1973; Hollinshead & Tarro, 1973; Aurelian et al., 1973). Our attempts to demonstrate antibodies to early antigens of HSV-2 by the IF test were unsuccessful.

CMV is another virus that can be transmitted venereally, and has been isolated more frequently than HSV-2 from cervical swabs of patients attending venereal disease clinics (Jordan et al., 1973). It has also been shown to persist in high titre in the semen of asymptomatic carriers (Lang et al., 1974). *In vitro* transformation of hamster embryo fibroblasts after exposure to CMV and HSV-2 inactivated with ultraviolet irradiation has been reported (Albrecht & Rapp, 1973; Rapp & Duff, 1973). Few studies have looked into a possible association between CMV and cervical cancer, and the results have been "controversial" (Sprecher-Goldberger et al., 1971; Vestergaard et al., 1972). One of the reasons may be that the complement-fixation (CF) test, which has been shown to be less sensitive than the IF test, has been used (Tsiantos et al., 1974). However, in the present study, while no differences were observed in the frequency of CMV-VCA titres between cases and controls, a higher frequency of antibodies to early antigens of CMV was observed among patients with cervical cancer than among controls. This suggests that cervical carcinoma patients have a higher prevalence of active CMV infection or that

Table 3. Distribution of antibody titres against early antigens of CMV and EBV in patients and controls

Antibody titres	EA - CMV (%)				EA - EBV (%)		
	Cervical cancer	Condyl-oma	Control (Cali)	Control (USA)	Cervical cancer	Condyl-oma	Control (Cali)
<10	64.0	82.1	80.8	88.6	65.5	45.0	67.3
10-20	36.0	17.9	19.2	11.4	13.8	35.0	24.5
≥40	-	-	-	-	20.7	20.0	8.2
No. of cases	50	56	99	35	29	20	49

the virus is in some way related to the development of the tumour.

The large number of people with high titres of antibodies to EBV-VCA and EBV early antigens is surprising. None of these people were known to have malignant lymphoma or nasopharyngeal carcinoma. The results suggest that the Cali population is exposed to a high prevalence of active EBV infection that stimulates high EBV-VCA antibody titres as well as antibody to some of the early antigens of EBV. These results also bring out the difficulties of establishing etiological roles for viruses in cancer by sero-epidemiological methods.

SUMMARY

The indirect immunofluorescent test was used to detect antibodies to herpesvirus types 1 and 2, cytomegalovirus and EBV in sera from patients with cervical carcinoma, condyloma acuminatum and controls from Cali, Colombia, and a control group from the USA. No significant differences were found in the prevalence of antibodies to viral capsid antigens of HSV-1 and CMV among the groups studied. However, titres for HSV-2 were higher in the three groups from Cali (cervical carcinoma, condyloma and controls) than in the control groups from USA. High EBV antibody titres (VCA and early antigens) were found in the three groups from Cali. Antibodies to early antigens of CMV were detected in a subsample of the subjects and with higher frequency in patients with cervical cancer. The significance of these findings is discussed.

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SERO-IMMUNOLOGICAL INVESTIGATIONS IN PATIENTS WITH CERVICAL CANCER: HIGHER RATE OF HSV-2 ANTIBODIES THAN IN SYPHILIS PATIENTS AND EVIDENCE OF IgM ANTIBODIES TO AN EARLY HSV-2 ANTIGEN

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As herpes simplex virus type 2 (HSV-2) is predominantly transmitted by venereal contact, it was thought that antibodies to HSV-2 would be correlated with antibodies to other venereal diseases, such as syphilis. We therefore compared a group of patients who had fluorescent treponema antibodies (FTA-ABS test) with matched controls by the microneutralization test with HSV types 1 and 2. The neutralizing titres were adjusted to neutralizing potencies (p_N) (Fazekas de St. Groth, 1961) and the p_N of HSV-1 antibodies related to the p_N of HSV-2 antibodies by the p_N-difference (Δp_N = p_N type 1 - p_N type 2) (Pauls & Dowdle, 1967). Patients without any neutralizing HSV antibodies were rare among syphilis patients as compared to the controls; sera with a low p_N-difference were more frequent in the syphilis group. The p_N-values of type-1 antibodies were similar in both groups, whereas syphilis patients had higher p_N-values of type-2 antibodies and consequently a lower p_N-difference (Table 1). These findings are in agreement with earlier results by Dueñas et al. (1972) in young prostitutes, who were positive for syphilis by the Venereal Disease Research Laboratory (VDRL) test. They show clearly that promiscuity is an essential factor when the incidence of HSV-2 antibodies is analysed. In certain studies on the relationship between genital herpes and cervical cancer, the influence of promiscuity was eliminated by the comparison of carefully matched pairs (Adam et al., 1971, 1972a, 1972b, 1973). Our view was that it might also be useful to

Table 1. Microneutralization test with HSV types 1 and 2 in patients seropositive and seronegative for syphilis

Patients	No.	Mean age (years)	Negative for neutralizing HSV antibodies	P	Δp_N[a] <0.50	P	Geometric mean p_N type 1	P	Geometric mean p_N type 2	P	Geometric mean Δp_N	P
Syphilis-sero-negative	100	39	21	<0.001	28	<0.025 >0.005	2.67±0.32	<0.4 >0.3	2.09±0.33	<0.01 >0.001	0.57±0.27	<0.02 >0.01
Syphilis sero-positive	100	40	3		53		2.72±0.38		2.24±0.39		0.47±0.27	

[a] $\Delta p_N = p_N$ HSV type 1 - p_N HSV type 2, where p_N is neutralizing potency.

compare cervical cancer patients with the patient groups mentioned above (Fig. 1).

The lowest p_N-values of both types of antibodies were observed in the sera without syphilis antibodies. The results are not very different when men and women or women only are considered. There is an equal increase in both antibody types when the results are restricted to the older women of the group. The break in the curve shows that only type-2 antibodies increase when the curve changes from the syphilis-negative to the FTA-positive group. In this group, there is an equal increase in both antibody types in going from men and women to women only, and then to older women. The curve continues to rise as values for the sera of both younger and older patients with cervical cancer are reached, the increase in type-2 antibodies prevailing over that of type-1 antibodies.

These results suggest that, with increasing age, women in particular are exposed to a continuous or intermittent antigenic stimulus by HSV and that this is especially true of cervical cancer patients - apart from the higher risk of infection with HSV-2 in this group.

It was of interest to see whether the quantitative differences found with neutralizing antibodies were linked with qualitatively different reactions in tests involving special antigens or antibodies (Table 2). Complement-fixing (CF) antibodies to an early antigen (EA) of HSV-2, which was prepared from four-hour infected human-embryo lung cells, were equally frequent in both cases and controls, and seemed to correlate with neutralizing antibodies; the latter were present in the sera of all of 60 cancer patients and in 77 of 87 control sera. CF antibodies to an antigen derived from a cervical cancer cell line were found more often in control sera than in the cancer sera. Fluorescent IgM antibodies to an early antigen of HSV-2 were demonstrated in acetone-fixed preparations of four-hour infected cells mixed with equal numbers of non-infected cells (Fig. 2). They were found in 28% of the cancer sera, less frequently in the control sera.

The investigations reported here show that, in patients with cervical

FIG. 1. NEUTRALIZING ANTIBODIES TO HSV TYPES 1 AND 2

..... Sera without treponema antibodies; x 55 men + 45 women, mean age 39 years; o 45 women, mean age 40 years; ● 27 women, mean age 52 years. ------ Sera with treponema antibodies; x 55 men + 45 women, mean age 40 years; o 45 women, mean age 41 years; ● 27 women, mean age 52 years. ——— Sera of patients with cervical cancer (carcinoma *in situ* + invasive carcinoma); o n = 27, mean age 40 years; ● n = 90, mean age 54 years.

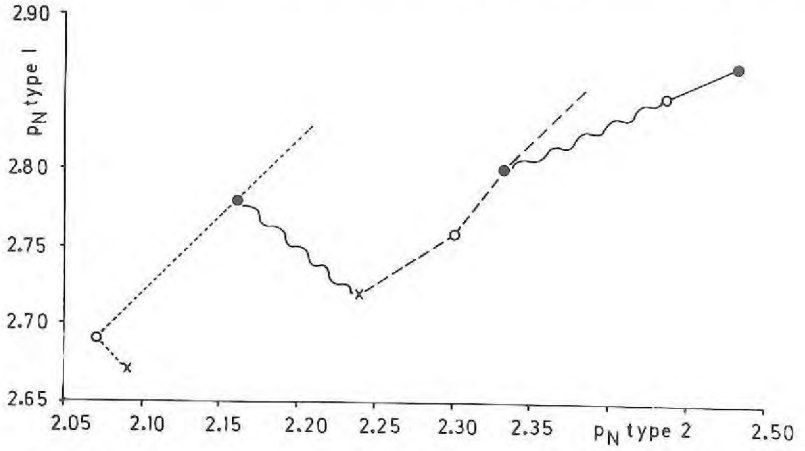


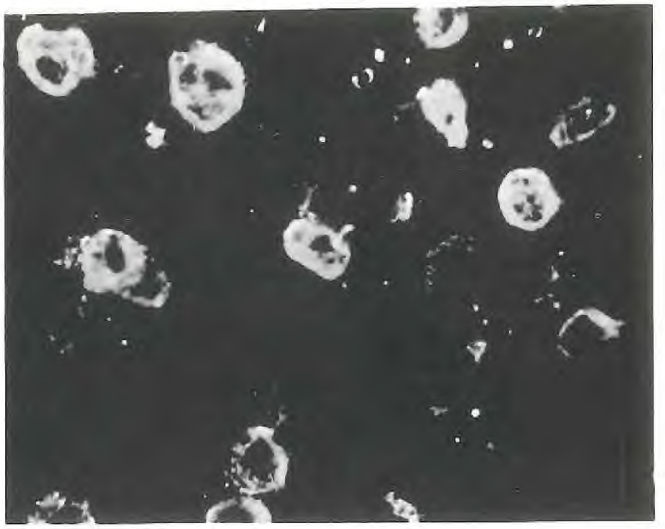
Table 2. Search for pathognostic antibodies in cervical cancer patients[a]

Patients	HSV-2 EA, CF antibodies	C-4-1 cells, CF antibodies	HSV-2 EA, fluorescent IgM antibodies
Cervical cancer with neutralizing HSV antibodies	55/60 (92%)	8/60 (13%)	16/56 (28%)
Controls with neutralizing HSV antibodies	73/77 (95%)	17/77 (22%)	4/66 (6%)
Controls without neutralizing HSV antibodies	0/10	1/10	0/10

[a] EA = early antigen, i.e., antigen prepared from human embryo fibroblasts, four hours after infection; C-4-1 = cell line derived from human cervical cancer (Auersperg & Hawryluk, 1962); CF: patient's sera 1:10, complement titrated. Values shown are ratios of numbers with CF or fluorescent IgM antibodies to numbers tested.

FIG. 2. INDIRECT IMMUNOFLUORESCENCE OF IgM ANTIBODIES IN THE SERUM OF PATIENTS WITH CERVICAL CANCER

Acetone-fixed preparation of four-hour HSV-2 infected cells, mixed with mock-infected cells. x 320



cancer, neutralizing HSV-2 antibodies can be found at a higher rate and with higher titres than in syphilis patients, who also have a higher risk of infection with HSV-2. The elevated titres of neutralizing antibodies are linked with the appearance of fluorescent IgM antibodies in some of the cancer patients. The results are in agreement with either the "preferential" or the "etiology hypothesis" but not with the "promiscuity hypothesis" (Aurelian, 1973).

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STUDIES ON THE SIGNIFICANCE OF EPSTEIN-BARR VIRUS-SPECIFIC IgM IN HUMAN SERA

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A modification of the indirect immunofluorescence test of Schmitz & Scherer (1972) was used to detect Epstein-Barr virus (EBV)-specific IgM. The main modification, made for reasons of supply of reagents, was the use of an antihuman IgM fluorescein-isothiocyanate (FITC) conjugate (Wellcome Research Laboratories) in place of an antihuman IgM rabbit serum followed by an anti-rabbit IgG/FITC conjugate. A full description of the test is given by Edwards & McSwiggan (1974).

Serum samples were obtained from a diagnostic laboratory to which they had been submitted for heterophil-antibody (HA) testing, and were examined for IgM to EBV without knowledge of the results of the HA tests. The results obtained on 166 sera are shown in Fig. 1. Both tests were positive in 51 sera and both negative in 108 sera. The EBV IgG test was also negative in 16 of the latter. In six sera, EBV IgM was detected but the HA test was negative, and in one serum the HA test was positive but EBV IgM was not detected. Both tests were repeated on the six sera in question and the results confirmed. In the 13 cases known to be under 10 years of age, no disagreements were found between the results of the two tests. A proportion of the sera were absorbed with the ox cell preparation used in the HA test. After absorption these sera were still found to be positive in the EBV IgM test, showing that the two tests involve reactions with distinct antigens.

The University Health Physicians and Public Health Laboratory Service Laboratories (1971) in the United Kingdom have made a six-year study

FIG. 1. COMPARISON OF RESULTS OF HETEROPHIL-ANTIBODY (HA) AND EBV-SPECIFIC IgM TESTS

Comparison of results of heterophil-antibody (HA) and EBV-specific IgM tests on 166 routine sera submitted for heterophil-antibody testing.

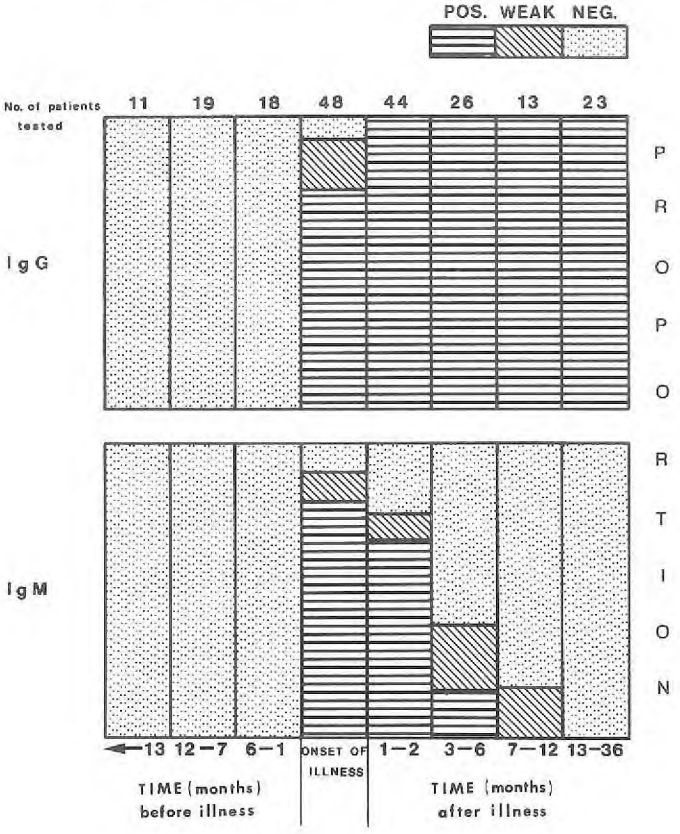
	HA Positive	HA Negative
EBV IgM Positive	51	6 (1 trace)
EBV IgM Negative	1	108

of infectious mononucleosis in five Universities. A full report is to be published shortly. From this study, serial sera were available for investigation of EBV IgM. All sera were also tested for EBV IgG by the method of Henle & Henle (1966). Sera from patients with illnesses resembling infectious mononucleosis were tested by the heterophil-antibody test described by Davidsohn & Henry (1969). Both these HA tests and those on the sera in Fig. 1 were performed by one of us (D.A.M.).

Fig. 2 shows the results of the EBV IgG and IgM tests on specimens from 48 cases of infectious mononucleosis from whom pre-illness and late post-illness sera were available, as well as an acute serum at onset and usually a convalescent serum one to two months after onset. With the exception of the onset specimens from two cases, all the acute and convalescent sera from these 48 cases were positive in the HA test. The respective proportions of sera reacting in the tests for EBV IgG and EBV IgM are shown at each time of sampling in relation to the onset of the illness, the time intervals being shown at the bottom of the diagram. All pre-illness specimens were negative for EBV IgG and IgM at a dilution of 1 in 8. All convalescent and post-illness specimens were positive for EBV IgG at a dilution of 1 in 32 or more. Weak reactions and negative reactions at a dilution of 1 in 8 occurred in either or both the IgG and IgM tests in a small proportion of cases at onset. Only two cases showed a

FIG. 2. RESULTS OF EBV-SPECIFIC IgM and IgG TESTS

Results of EBV-specific IgM and IgG tests on serial specimens from 48 confirmed cases of infectious mononucleosis.



negative heterophil-antibody test at onset, and in one of these a weak IgM reaction was detected. IgG was detected in the convalescent sera of all the cases whose specimens, taken at onset, gave negative results. IgM was detected in most convalescent specimens, but there was already a drop in the proportion of positive tests in 26 specimens taken between three and six months. Only weak reactions were detected in a small proportion of 13 specimens taken between seven and 12 months, and all specimens 13 months after illness were negative.

EBV IgM was not detected in later sera from 159 students whose sera had been positive for EBV IgG on arrival at University. These later sera were from: (a) 69 healthy students

on leaving University three years later; (*b*) 42 students from whom paired illness specimens were obtained during an influenza epidemic; and (*c*) 48 students from whom paired specimens were investigated during a "glandular fever"-type illness. This illness was not clearly distinguishable from infectious mononucleosis but all heterophil-antibody tests were negative.

In a small series of sera from 37 cases of Hodgkin's disease, EBV IgM was not detected.

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DISCUSSION SUMMARY

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Since tests for antibodies to viral capsid antigens (VCA) of Epstein-Barr virus (EBV) were considered subjective, the question was raised whether the drop in antibody titres to this antigen in older children was real. It was mentioned that, in sera collected from 1 200 children from the West Nile region of Uganda, the incidence of antibodies to EBV VCA followed a bell-shaped curve when high and low dilutions of sera were used. Sera from children that at one point had a titre of 40-80, had no detectable level of antibodies 12-18 months later. The specificity of these antibodies to EBV was not, however, quite clear, and they required further differentiation from antibodies to human cytomegalovirus. It was mentioned that, although there is no quantitative relationship between titre of antibodies to EBV VCA and EBV-determined nuclear antigen (EBNA), these two types of antibodies were qualitatively related and the EBNA test could be used in future epidemiological studies.

On the interaction between avian leukosis viruses (ALV) and Marek's disease virus (MDV), data were presented indicating that, contrary to some recent published reports, MDV alone is capable of producing a typical lymphoma in genetically susceptible chickens that were free from ALV group-specific antigens and chick helper factor. These chickens remained free from ALV group-specific antigens following the infection with MDV and development of the lymphoma. It was pointed out that the reported enhancement of MD lymphoma by Rouse-associated virus (RAV-2) could be both non-specific and mediated by several factors, such as the immunosuppression caused by either or both viruses. It was mentioned that both the genetic susceptibility of experimental chickens to infection with ALV and MDV and their susceptibility to tumour formation by these viruses must be carefully studied before they are used in studies on the interaction of viruses in oncogenesis.

The paradoxical situation that Friend virus (FV)-infected NC-37 human lymphoblastoid cells are positive for EBNA while negative for EBV DNA gave rise to several comments. It was mentioned that the results of DNA-DNA reassociation kinetics studies were not available yet, and that inducibility of the FVNC line by iododeoxyuridine (IUDR) indicated that the line must contain EBV DNA. Although the possibility of all EBV DNA having been associated with the lost chromosome in FVNC cells was excluded, it was suggested that non-integrated EBV DNA might have been eliminated by infection of NC-37 cells with Friend virus.

TRANSFORMATION IN VIVO AND LATENCY

HERPES SIMPLEX VIRAL LATENCY – A REVIEW

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HISTORICAL

The pathogenesis of recurrent herpetic disease has intrigued a diverse group of investigators for the past 75 years. The suggestion that there is a relationship between herpetic disease and the nervous system was one of the earliest to be put forward, and was made by Howard (1903, 1905) at the turn of the nineteenth century. At that time, he expanded upon the earlier observations of Head & Campbell (1900), which concerned zoster, and documented coincident herpetic lesions, trigeminal ganglionitis, and pneumonitis. At about the same time, Cushing (1905) noted that an intact trigeminal tract was necessary for the development of herpetic lesions (individuals in whom the trigeminal ganglion was removed as a treatment for trigeminal neuralgia subsequently developed herpetic lesions on the contralateral side of the face but not on the ipsilateral side). These observations, coupled with those of Howard, led Cushing (1904) to assert that "posterior-root ganglia lesions are responsible for the common forms of herpes about the eyes and nose". This assertion was obviously the result of considerable insight, and as will be seen, rigorous experimental confirmation is now almost complete.

Some 15 years after the observations of Howard and Cushing, Grüter (1920) and Löwenstein (1919) began the chain of experiments that now strongly support Cushing's statement. At that time, by transmitting herpetic keratitis, they established the rabbit as an important experimental animal in which to study herpetic infections. In this model, principally as a result of the later work by Goodpasture (1925a, 1925b) and Goodpasture & Teague (1923), the disease was unequivocally demonstrated to be infectious and viral-induced, and the role of the nervous system in pathogenesis was firmly established. When these results were coupled with the epidemiological data gathered by Andrewes & Carmichael (1930), which showed that individuals with recurrent herpetic disease possessed neutralizing antibody to the virus, it became clear that the natural history of herpetic disease was both complicated and unique.

Principally as a result of these

experimental and epidemiological considerations, and of observations concerning the outcome of various manipulations of the trigeminal tract in man (Carton & Kilbourne, 1952; Carton, 1953), a general hypothesis has been formulated to explain the pathogenesis of recurrent herpetic disease in man. Here, primary infection would result in viral replication in epithelial cells of the skin or mucous membrane, and subsequent invasion of superficial nerve endings. The virus would then travel intra-axonally in sensory nerves to the corresponding sensory ganglion (most often the trigeminal) where a latent infection would be established, most probably in neurons. Upon "reactivation", the virus would travel centrifugally from the neuronal soma in axons, ultimately reaching the epithelium where lesions would again be produced. If this hypothesis is a true one, then three phenomena obtain. Firstly, and most importantly, virus must be harboured in sensory ganglia. Secondly, the infection must travel to and from ganglia in associated nerve trunks. Thirdly, various "insults" must result in reactivation of the virus from the ganglion and reappearance of clinically apparent disease at the body surface. In the remainder of this communication, the evidence derived from experimental infections in animals and natural infections in man that support this hypothesis will be summarized. As will be shown, all phenomena, except reappearance of clinical disease following defined manipulation of experimental animals, have been demonstrated to occur. In these considerations, results obtained in experimental animals will be discussed interchangeably with those observed in man. At the moment, this decision is justified since, where phenomena have been defined in both animals and man, the results are similar.

ESTABLISHMENT AND CHARACTERISTICS OF THE LATENT INFECTION

It is now well established and appreciated that an infection of skin, cornea, or mucous membranes is followed by a latent infection of the corresponding sensory ganglia. The initial demonstration was made in a murine model established in our laboratories (Stevens & Cook, 1971, 1973a, 1973b). Here, herpes simplex virus was shown to remain latent for at least one year in the sacrosciatic spinal ganglia of mice previously infected in a rear foot-pad. In this system, virus could not be recovered directly, but was demonstrated only after the ganglia were removed and cultured as organs *in vitro*. Several days after the explant was made, infectious virus was replicated, and could be detected in supernatant fluids bathing the ganglia. Using similar techniques, we then succeeded in demonstrating latent virus in trigeminal ganglia and brainstems of rabbits inoculated on the cornea (Stevens et al., 1972; Knotts et al., 1973). Subsequently, we and others have established latent infections in sensory ganglia of mice inoculated by various other routes (Knotts et al., 1974; Walz et al., 1974). Finally, and most importantly, when the techniques were applied to human ganglia, natural herpetic infections in man were shown to induce latent infections in sensory ganglia (Bastian et al., 1972; Baringer & Swoveland, 1973; Rodda et al., 1973; Baringer, 1974). Here, particularly from the more extensive data presented by Baringer & Swoveland (1973) and Baringer (1974)[1], it now appears likely that half or more of the human population harbours latent herpes simplex virus in trigeminal ganglia.

The ganglionic cell involved in

[1] Personal communication.

maintainance of the latent infection, and the mechanism by which this infection is maintained have also been studied in some detail in our laboratories. At this time, the bulk of the experimental evidence is most consistent with the proposition that latent virus is associated with neurons (Cook et al., 1974), and that the viral genome is maintained in the neurons in a non-replicating state (Stevens, 1975). In addition, experiments in which latently infected ganglia were transplanted into latently infected mice, and into animals treated passively with antiviral IgG have led to the provocative, tentative conclusion that the latent infection is somehow modulated by antiviral IgG (Stevens & Cook, 1974). Here, we proposed that an interaction between the IgG and viral-induced membrane antigens on the surface of neurons may, through additional intracellular effector molecules, repress complete expression of the viral genome. This mechanism was modelled after the TL system of antigenic modulation that exists between murine lymphocytes and antibody (Old et al., 1968). There, exposure of lymphocytes possessing an organ-specific surface isoantigen to the corresponding antibody results in disappearance of the antigen.

EVIDENCE THAT VIRUS TRAVELS IN NERVES

The early work of Goodpasture suggested strongly that herpes simplex virus travels in nerve trunks. More recently, convincing experiments by Johnson (1964), Wildy (1967), and ourselves (Cook & Stevens, 1973) have made this concept one that is now generally accepted. Beyond this, it also seems likely that the virus travels in axons. The evidence that supports this latter conclusion is indirect and is summarized in detail elsewhere (Cook & Stevens, 1973; Stevens, 1975), but relates chiefly to the fact that axonal flow is now known to be bidirectional, and to the speed with which the infection moves in nerves, even in the presence of antibody.

REACTIVATION TO ACTIVE INFECTION

Soon after we had demonstrated that latent infections could be established in spinal ganglia of mice, we began an intensive study of reactivation. Until recently, our efforts in this direction were unrewarding. Despite the use of a variety of methods that are known to be associated with reactivation in man, we were completely unable to reactivate either infectious virus or clinically apparent disease in mice (Stevens & Cook, 1973b). However, very recent experiments have shown that the virus can be reactivated when mice are severely ill with pneumococcal pneumonia (Stevens et al, 1975). Here, mice with latent infections in sacrosciatic spinal ganglia were instilled intratracheally with pneumococci and given intensive penicillin therapy 24 hours later when they became very ill with lobar pneumonia. Analyses of ganglia indicated that viral DNA and infectious virus appeared 1-2 days after the bacteria were administered. In addition, proximal and distal sciatic nerve roots and the nerve trunk were co-cultivated with RK_{13} cells. The data derived from this procedure indicated that virus passed from ganglia to the nerve roots and then to the trunk itself, and that the greatest number of nerves and roots (50%) were positive (against a background of 5-10% in control animals) by four days after bacterial infection. It should be pointed out that the model is not a perfect one,

however, since clinically apparent disease was not produced, and virus was not recovered from the feet of the mice studied. Finally, it should also be noted that Walz et al. (1974) have also reactivated virus in this system by transecting the distal nerve roots at the point from which they leave the intervertebral foramina. There, infectious virus could be detected in the ganglia a few days after the roots were severed.

CONCLUSIONS AND PERSPECTIVES

From the evidence summarized here, it can be concluded that herpes simplex virus remains latent in sensory ganglia and travels in nerves. It is also likely that the endoneural route taken is axonal. In addition, the virus probably persists in some non-replicating state in neurons, and this interaction may be modulated by specific immune IgG. The virus can be reactivated from spinal ganglia by superimposed pneumococcal pneumonia or distal root section. However, induction of clinically apparent cutaneous disease has not yet been achieved. If this latter phenomenon can be accomplished experimentally, and surgical interruption of appropriate nerves then prevents the development of lesions, the last significant obstacle to acceptance of the general hypothesis presented earlier will have been removed. Beyond this, it is probable that a definition of the biochemical basis of latency and reactivation will depend upon development of a simpler system that can be readily manipulated *in vitro*.

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HERPES SIMPLEX VIRUS IN HUMAN SENSORY GANGLIA

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Herpes simplex virus is well known for its propensity to cause recurrent mucosal or cutaneous eruptions in man. A variety of clinical observations have suggested that the disorder may be related to involvement of the peripheral nervous system. Among these are the paraesthesias that commonly herald the eruption, and the less frequent neuralgic pain that may precede or attend the appearance of vesicles (Behrman & Knight, 1954). Further evidence that sensory ganglia might harbour the virus comes from the studies of Carton (1953) and Ellison et al. (1955), who demonstrated that injury to the centrally projecting fibres of the trigeminal ganglion resulted in herpes simplex lesions around the mouth in over 90% of patients. The eruption could not be provoked if the nerve between the skin and the ganglion had previously been cut. Rustigian et al. (1966) were unable to recover herpes simplex virus from cultured skin fragments at the site of recurrent eruptions, suggesting that the virus did not remain in latent form in the skin.

Recent studies in animals (Stevens et al., 1972; Baringer & Swoveland, 1974) have indicated that, when herpes simplex virus is inoculated at the periphery, it can travel to sensory ganglia, where it resides in latent fashion for prolonged periods and can be recovered only by maintaining the ganglia in explant culture or by co-cultivation techniques. These studies prompted a reinvestigation of the possibility that herpes simplex virus might reside in latent form in human sensory ganglia.

In previous studies (Baringer & Swoveland, 1973), trigeminal ganglia were removed from unselected cadavers and divided into the ganglionic mass, the root, and the divisions of the trigeminal nerve. These tissues were separately explanted and observed for the appearance of a viral cytopathic effect. In a series of seven patients, a cytopathic effect appeared within 10-45 days after the time of explantation. The virus recovered was identified as herpes simplex virus on the basis of fluorescent antibody test, electron microscopy, and neutralization by specific antisera. Explant cultures of the roots and trigeminal nerves did not yield virus after similar periods *in vitro*, but when deliberately exposed

to herpes simplex virus they did exhibit a cytopathic effect. Other studies (Bastian et al., 1972; Nahmias & Roizman, 1973; Plummer, 1973) indicated that the virus in trigeminal ganglia was a type-1 strain.

The studies reported here have attempted to assess more completely the frequency with which herpes simplex virus can be recovered from trigeminal ganglia and to see whether, in an analogous fashion, the virus may be recoverable from sacral ganglia.

METHODS

Ganglia were removed in sterile fashion from unselected cadavers undergoing autopsy less than 24 hours from the time of death. The trigeminal ganglia were removed as in previous studies (Baringer & Swoveland, 1973). By means of sterile instruments, the sacral and mid-thoracic spinal laminae were removed exposing the underlying ganglia, which appear as fusiform swellings on the roots. The second, third, and fourth sacral ganglia were removed bilaterally; in addition, mid-thoracic ganglia were removed. The ganglia were transported in sterile containers to the laboratory, where the connective tissue was removed, they were washed in Hanks' balanced salt solution, and minced finely. They were then re-washed twice in Hanks' solution, after which the tissues from the second and third sacral ganglia were divided into four portions and inoculated into 25-cm^2 plastic flasks; the smaller amounts of tissue from the fourth sacral and mid-thoracic ganglia were divided into two portions and similarly inoculated. To the tissues was added a suspension of approximately 500 000 freshly trypsinized human embryonic lung cells (Flow 2000) followed by 5 ml of Leibovitz L-15 media containing 10% fetal calf serum. The flasks containing the co-cultivated ganglia were incubated at 35°C and the media changed weekly. The cultures were observed for the development of a viral cytopathic effect. Presence of virus was confirmed by assay of the supernatant fluid using Flow 2000, Vero, or primary rabbit kidney cells. The identity of the viruses recovered was confirmed by fluorescent antibody test, neutralization test, or electron microscopy (Baringer & Swoveland, 1973). Typing of the viral isolates was performed by plaque reduction using rabbit antisera prepared against known type-1 and type-2 strains (Plummer et al., 1970) or in the laboratory of Dr A.J. Nahmias by fluorescent antibody technique (Nahmias et al., 1969).

RESULTS

The trigeminal ganglia of 71 cases have yielded herpes simplex virus in 31 or approximately 44% (Table 1). Of 26 positive cases in which both trigeminal ganglia were removed, the virus was recovered from both sides in 20. Typing of seven of the trigeminal isolates has revealed them to be of type-1 strain.

Sacral ganglia removed from 46 patients have yielded herpes simplex virus in five; in each case, the virus has been identified as a type-2 strain. One thoracic ganglia of 34 removed from patients has yielded virus that has been identified as a type-1 strain. Cell-free passage of this virus, the production of a cytopathic effect on rabbit kidney cells, and neutralization by anti-herpes sera indicated that the virus was herpes simplex rather than varicella-zoster virus. In two cases, a simultaneous infection by type-1 and type-2 virus was present in the trigeminal and sacral ganglia respectively.

Table 1. Recovery of herpes simplex virus from human sensory ganglia

Ganglia	Number of cases	Number positive
Trigeminal	71	31
Sacral	46	5
Thoracic	34	1

Viral cytopathic effect in trigeminal ganglia appeared as early as two weeks and as late as six weeks after explantation, and in sacral ganglia, from two to 13 weeks after explantation (Fig. 1). In nine of the trigeminal ganglia, all of the four flasks yielded virus.

DISCUSSION

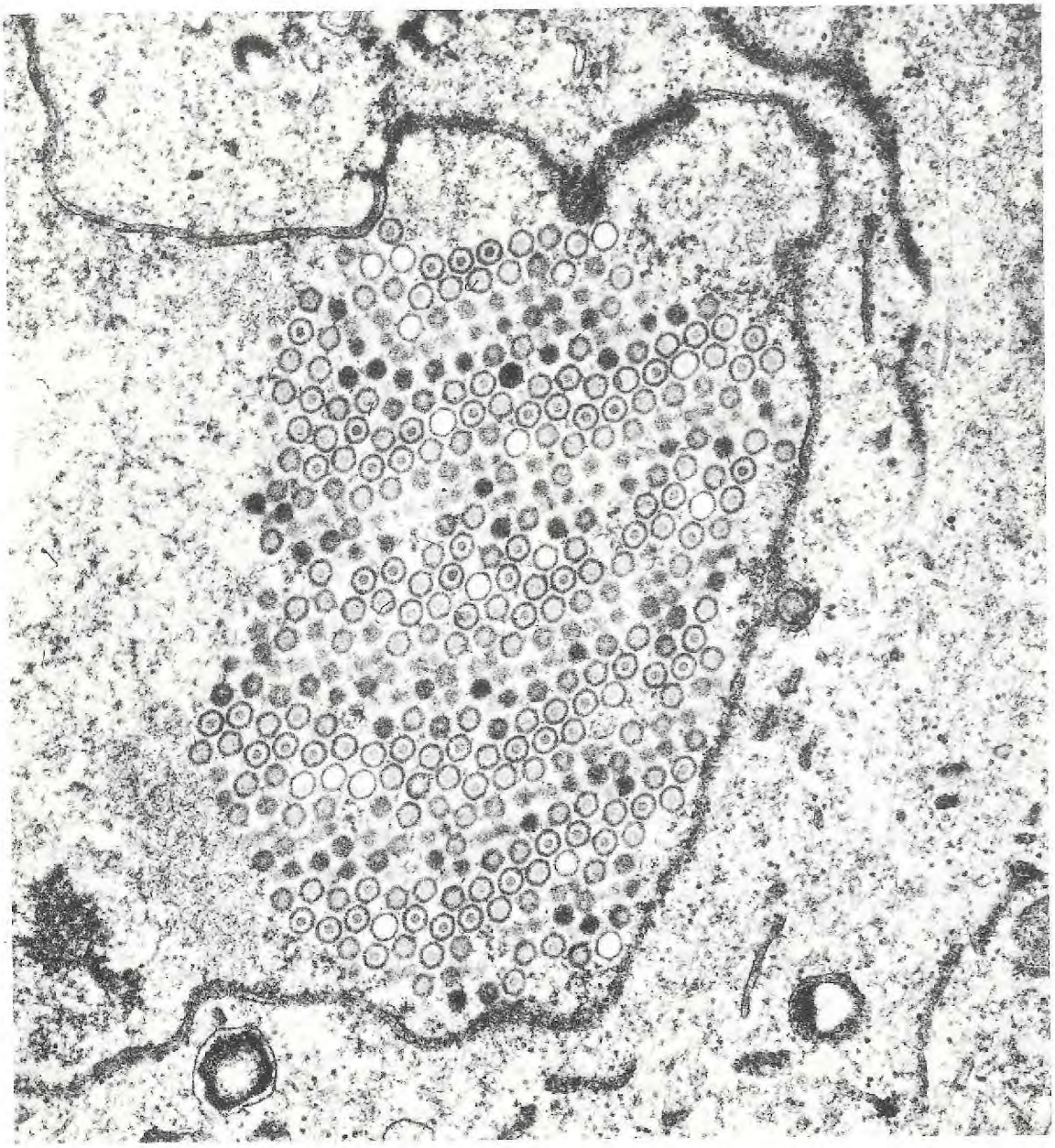
These studies indicate that herpes simplex virus is commonly present within the trigeminal ganglia of unselected cadavers, where it is most often present bilaterally. Preliminary data indicate that the virus from trigeminal ganglia is uniformly type-1 strain. Herpes simplex virus can be recovered less frequently from sacral ganglia. The virus appears to reside in the third or fourth sacral ganglia, and has not to date been recovered from the second sacral ganglia. The sacral isolates have uniformly been type 2. In two cases, a simultaneous infection by type-1 and type-2 virus at the trigeminal and sacral ganglia has been observed.

The finding of latent herpes simplex virus within sensory ganglia may afford an explanation for the paraesthesias or neuralgic pain that may be experienced in relation to recurrences of herpes simplex lesions. It is possible that either the reactivation process or phenomena associated with travel of virus down the nerve are important in the production of such symptoms. The activation of virus latent in the ganglia by damage to the sensory root can be readily understood on the basis of the reactivation of such latent virus in response to root injury.

The role of the ganglia in providing a safe harbour for the virus against the host's defence system or antiviral treatment remains unknown, as does the possible relationship of the residence in ganglia to the transforming and possible oncogenic role of the virus.

FIG. 1. HERPES SIMPLEX VIRUS PARTICLES IN CO-CULTIVATED SACRAL GANGLION EXPLANT

(x 37 800)



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HERPESVIRUS TYPE 2-INDUCED TUMOUR-SPECIFIC ANTIGEN (AG-4) AND SPECIFIC ANTIBODY IN PATIENTS WITH CERVICAL CANCER AND CONTROLS

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The association of herpesvirus type 2 (HSV-2) with squamous carcinoma of the human cervix is based on biological and sero-epidemiological data (Naib et al., 1969; Rawls et al., 1973; Nahmias et al., 1970; Aurelian et al., 1970). Exfoliated cervical tumour cells contain HSV-2 antigens (Aurelian et al., 1972; Gall & Haines, 1974). Virus was isolated from tumour cells in culture (Aurelian et al., 1971), and complete virus particles were observed in two of 14 tumours (Herrera et al., 1974). Nucleotide sequences corresponding to 40% of the viral genome were reported in one tumour (Frenkel et al., 1972). *In vitro* transformation of hamster cells with HSV-2 has been described (Rapp & Duff, 1973). However, direct evidence in support of a causative role for HSV-2 in cervical cancer is not yet available. Two questions pertaining to this problem constitute the focus of this presentation. Specifically: (*a*) do cervical tumours contain a virus-induced antigen that is cervical tumour-specific; and (*b*) what is the antibody to this antigen and is it different from antibody to HSV-2?

PRESENCE OF A VIRUS-INDUCED TUMOUR-SPECIFIC ANTIGEN IN CERVICAL CANCER

The molecular basis for understanding the possible oncogenic role of HSV-2 in humans is based on knowledge accumulated from work with animal models. Cells transformed by DNA tumour viruses: (i) acquire permanent new properties and lose normal growth control; (ii) contain viral gene copies, complete or partial, in a non-infectious state; (iii) transcribe specific viral gene sequences other than those associated with the inhibition of host macromolecular synthesis; and (iv) contain viral protein(s) that regulate the growth of the transformed cells and are most probably coded by virus-specific sequences. Secondary effects on the course of differentiation and evolution of the new (transformed) cell type would explain tumour formation. Accordingly,

it has been predicted (Aurelian, 1972) that cervical tumour cells contain the HSV-2 genome in a non-infectious state, transcribe specific gene functions and contain virus-coded protein(s) that regulate the growth of the transformed cells (Ca-Protein). The exact nature of Ca-Protein, however, could not be predicted with accuracy. In DNA tumour viruses, two specific antigens, synthesized early in the replicative cycle and non-structural in nature, are the T- and transplantation antigens; antibody to these antigens is present in tumour-bearing hosts. It should be stressed, however, that at present there is no evidence that these antigens regulate the growth of the transformed cell (Green et al., 1971). Furthermore, evidence is accumulating that T-antigen may not even be necessary for transformation (Robb et al., 1972).

The rationale for studying the presence in human sera of antibody to "early" HSV-2 induced antigens, different from those antigens involved in neutralization, is two-fold. Firstly, if cervical tumour cells contain Ca-Protein(s), cancer patients should have Ca-Protein(s) specific antibody. Ca-Protein(s) could be virion or non-virion in nature, unique to the tumour cells or also made in the productive infection; they should, however, be "early" protein(s), synthesized before the virus expresses those functions responsible for the inhibition of host macromolecular synthesis (Aurelian & Roizman, 1965; Roizman, 1971; Aurelian, 1974). Secondly, the antigens reacting in neutralization tests are indicative of previous infection with the virus and are not tumour-specific. The relatively high percentage of women without cancer, but with antibody to HSV-2, raises further doubts about the validity of the association of HSV-2 with cervical cancer.

The early HSV-2 induced antigen studied in this series (AG-4) is a crude extract of HEp-2 cells infected with HSV-2 for 4 hours. The control antigen (AG-H) is a similarly prepared extract of uninfected HEp-2 cells. The patients were seven women with atypia, 20 with carcinoma *in situ*, 34 with untreated invasive cervical cancer, 26 with invasive cervical cancer successfully treated 2 months to 19 years prior to blood collection, and seven with histological evidence of recurrent neoplastic disease. Control subjects matched for age, race, and socio-economic class according to economic deciles of the resident census tracts for the city of Baltimore were hospitalized for unrelated illness and had a negative history of cancer. The micro-quantitative complement-fixation test of Wasserman & Levine (1961) adapted to the herpesvirus system has previously been described (Aurelian et al., 1973). Sera reacting with both AG-H and AG-4 were considered negative for AG-4.

Two features emerge from the results summarized in Table 1. The first is that the prevalence of antibody to AG-4 is significantly higher in patients with invasive carcinoma (85%) than in a matched control group (10%), and correlates with the gradation (Koss, 1969) expected of cervical cancer (atypia: 35%; *in situ*: 72%). The second feature is the absence of AG-4 antibody in patients with cervical cancer successfully treated prior to blood collection and its presence in those with recurrent neoplastic disease. Neutralizing antibody to HSV-2, however, is highly prevalent in controls and is not affected by therapy.

Finally, AG-4 activity is observed in extracts of five out of six cervical tumours prepared by freezing and thawing and sonication and reacted with a serum (No. 64) containing antibody to AG-4 and obtained from a case of invasive

Table 1. Presence of antibody to AG-4 in cancer patients and controls

Group	No. tested	Positive for AG-4		Positive for AG-H		Positive for HSV-2	
		No.	%	No.	%	No.	%
Atypia	7	3	43	0	0	7	100
Matched controls	7	0	0	0	0	4	57
Carcinoma *in situ*	20	13	65	0	0	19	95
Matched controls	20	1	5	0	0	10	50
Invasive	34	29	85	0	0	33	100
Matched controls	34	4	12	5	15	24	71
Treated invasive cancer	26	0	0	0	0	26	100
Recurrent cancer	7	6	86	0	0	6	100

carcinoma, but not observed in two out of two biopsies from women without cancer and in one from adenocarcinoma of the cervix. It is also present in extracts of three cell cultures established from cervical tumours reacted with serum No. 64. AG-4 activity is not observed in all cases with a second serum (No. 28) obtained from a control and negative for AG-4 activity.

Is AG-4 the predicted Ca-Protein(s) and what do we know about it? In favour of the interpretation that AG-4 is a possible candidate are the following observations: (i) unlike the "non-virion" antigen (Tarro & Sabin, 1970; Hollinshead et al., 1973), AG-4 is specific to squamous carcinoma of the human cervix. In a recently completed blind study on 38 sera supplied by Dr R. Manaker of the National Cancer Institute, AG-4 antibody was not observed in sera from patients with carcinoma of the vulva, bladder, prostate, kidney, tonsils, salivary gland, breast, stomach, lung and bronchi. Antibody to AG-4 is also absent in carcinoma of the pancreas, vagina, and endometrium, and in adenocarcinoma of the cervix (Aurelian et al., 1973); (ii) AG-4 antibody appears to be associated with active tumour growth, being present in recurrent neoplasia and absent in successfully treated patients; and (iii) in favour of its viral nature are its absence from uninfected cells and its synthesis *in vitro*, four hours after infection of HEp-2 cells with HSV-2. However, this evidence does not prove that AG-4 is virus-coded nor that it plays a regulatory role in tumour growth. Purification and characterization studies will determine whether AG-4 is one or more proteins, virus- or cell-coded, and its location inside the cells. The question of its role in control of tumour growth must, however, await the results of the purification studies.

WHAT IS THE ANTIBODY TO AG-4?

The specificity of the AG-4 antibody to cancer and the observation that AG-4 does not block the neutralizing ability of human sera (Aurelian et al., 1974) are of particular significance in view of the presence of structural (at least HSV-1) viral antigens in cells infected between 0-4 hours (Honess & Roizman, 1973), and the presence of neutralizing antibody in patients' sera (Table 1). It is probable that, during the first four hours after infection, not enough viral structural proteins are made to be detectable. Possibly also, neutralizing antibody recognizes those antigens after they undergo some modification (individual or due to interaction) occurring during the later intervals of the replicative cycle. Finally, it is possible that antibodies specific to the various viral antigens (and AG-4) reside in different immunoglobulin fractions and preferentially react in some, but not other, serological procedures.

To inquire into this possibility, we studied 11 human sera obtained from cancer and control subjects, and fractionated by zone centrifugation in 10-40% (w/v) sucrose gradients at 35 000 rpm for 18 hours in an SW50 rotor. Protein distribution in fractions collected from the bottom of the tube was monitored by absorbance measurements at 280 nm in an ISCO model UA-4 absorbance monitor. IgG and IgM quantities in the peak fractions were determined by immunodiffusion assays using Hyland immuno-plates (Hyland, California). They were tested for their ability: (*a*) to fix complement with AG-4, control antigen, AG-H and HSV-2; (*b*) to neutralize HSV-2 in an artificial mixture with HSV-MP by the multiplicity analysis previously described (Aurelian et al., 1970); and (*c*) to stain HEp-2 cells infected with HSV-2 for four and 24 hours by the indirect immunofluorescence procedure, using Evans Blue as a counterstain. The anticomplement immunofluorescence test (Reedman & Klein, 1973) was also done on HEp-2 cells infected with HSV-2 for four hours.

The results of these studies (Table 2) may be summarized as follows. Antibody to AG-4 is present only in cancer but not in control sera, and resides in the IgM fraction; IgG does not fix complement with AG-4. In contrast, complement-fixing activity to HSV-2 resides in the IgG fractions; IgM does not fix complement with HSV-2 in nine out of 11 sera. The IgM fractions also do not stain HSV-2 infected cells. All IgG fractions stain 24 hours-infected cells and nine out of 11 stain the four hours-infected cells. Neutralizing antibody to HSV-2, however, resides in both IgG and IgM fractions of most sera, and addition of complement does not substantially improve the neutralizing potential of either fraction in seven of these sera. Following absorption with HSV-2, anticomplement immunofluorescent staining was observed only in the four hours-infected cells stained with IgM from AG-4 positive sera.

CONCLUSIONS AND PERSPECTIVES

The data presented provide evidence for the presence in cervical tumours of a virus-induced antigen, designated AG-4. This antigen is specific to squamous cervical cancer, consistent with the infectious epidemiological pattern of the disease (Kessler, 1974). The conclusion that AG-4 is associated with tumour growth rests on the following observations: (i) antibody to AG-4 shows the gradation expected of the development of cervical cancer (Koss, 1969); it is present in 35% of women

Table 2. Immunological reactivity to HSV-2 and AG-4 of immunoglobulin fractions from human sera

Serum No.	Diagnosis[a]	Positive for AG-4		Positive for HSV-2							
		CF[b]		CF[b]		NT[c]		IF[d]			
		IgM	IgG	IgM	IgG	IgM	IgG	IgM		IgG	
								4h	24h	4h	24h
3	CaCx	+(1/2)	-	ND[e]	ND[e]	+	+	-	-	+	+
8	CaCx	-	-	+(1/2)	+(1/4)	+	+	-	-	-	+
12	CaCx	+(1/2)	-	-	+(1/8)	+	+	-	-	+	+
13	CaCx	+(1/2)	-	-	+(1/4)	+	+	-	-	+	+
15	Control	-	-	-	+(1/16)	-	+	-	-	+	+
17	CaCx	+(1/2)	-	-	+(1/16)	-	+	-	-	+	+
10	Control	-	-	-	+(1/4)	+	+	-	-	-	+
25	Control	+(1/2)	-	+(1/8)	+(1/8)	+	+	-	-	+	+
63	CaCx	+(1/8)	-	-	-	-	+	-	-	+	+
160	Atypia	+(1/4)	-	-	+(1/8)	-	+	-	-	+	+
183	CaCx	-	-	-	+(1/32)	+	+	-	-	+	+

[a] CaCx: invasive cervical cancer.

[b] Complement fixation. The antibody titre, defined as the highest serum dilution fixing more than 10% of complement, is given in parentheses.

[c] Neutralization.

[d] Immunofluorescence.

[e] Not done.

with atypia, 65% of those with carcinoma *in situ* and 85% of those with invasive cancer; (ii) antibody to AG-4 is not present in sera of control subjects even with recurrent HSV-2 disease; (iii) antibody is absent in sera from cancer patients who have undergone successful therapy but present in the sera of those with recurrent neoplastic disease; and (iv) AG-4 is present in squamous cervical tumours biopsied or grown in culture but not in normal cervical tissue or in adenocarcinoma of the cervix. Viral antigens detected by other tests (Table 2) cannot be correlated with cancer.

It is significant that IgM and IgG immunoglobulins differ in their ability to recognize HSV-2 and AG-4 in different serological assays. AG-4 antibody is an IgM immunoglobulin in all sera positive for AG-4 studied in this series. Antibody to HSV-2, in contrast, is in the IgG fraction if assayed by complement fixation or immunofluorescence, or in both the IgG and IgM immunoglobulins, if assayed by neutralization.

The significance of this observation in terms of the possible confusion in interpreting the results of various serological assays for HSV-2 and "non-virion" antibody in cancer patients should be given special consideration. Unfractionated serum possesses the sum of activities of the various immunoglobulin fractions; however, their different avidity, the proportion and nature of the various antigens in the specific preparation used as antigen, and the sensitivity of the specific modification of the test used, will clearly affect the final interpretation of the data.

Of particular interest is the observation that antibody to AG-4 is an IgM immunoglobulin. The ratio of different Ig classes is not the same in all general responses. Thus, IgM is the preferential response to a sarcoma-specific antigen (Eilber & Morton, 1971). Antibody to antigens having a large number of identical epitopes, like bacterial polysaccharide, are mainly IgM, and antiflagellin responses are richer in IgG than antiflagella responses (Makela et al., 1971). The IgM nature of anti-AG-4 antibody further suggests that immunity to AG-4 may be T-cell independent (Mitchell et al., 1971). The exact significance of this observation in terms of tumour growth and/or control must await further investigation. It should be pointed out, however, that a few observations indicate the beneficial nature of IgM responses. Firstly, 19 S antibody appears to be cytotoxic in presence of complement, whereas 7 S of the same specificity can inhibit cytotoxicity (Makela et al., 1971). Secondly, IgM is not as effective as, or less effective than, IgG in the feedback-type inhibition of antibody synthesis and immune response (Moller & Wigzell, 1965). Finally, IgM cannot inhibit cell-mediated immune responses *in vitro* (Shin[1]).

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[1]Personal communication.

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T-LYMPHOBLASTOID CELL LINES FROM MAREK'S DISEASE LYMPHOMAS

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Marek's disease (MD) is a contagious lymphoproliferative disease of the domestic fowl associated with infection with a herpesvirus (MDV). Interest in the disease has been a consequence of its importance as a major cause of loss to the poultry industries of many countries (Biggs, 1971). With the development of successful vaccination techniques against MD, attention has been focused upon its relationship to other neoplastic diseases, especially those putatively associated with herpesviruses. Investigation of the nature of MD has been hampered by the lack of any MD lymphoma-derived cell lines (Klein, 1972); this contrasts with Burkitt's lymphoma (BL), where cell lines were readily produced soon after the description of the clinical condition (Epstein, 1970). Recently, MD lymphoblastoid cell lines have been established *in vitro* (Akiyama et al., 1973; Powell et al., 1974), and *in vivo* (Theis et al., 1974). We now describe the establishment and characterization of two cell lines, HPRS Line 1 and HPRS Line 2.

ESTABLISHMENT AND CHARACTERIZATION OF MD CELL LINES

The approach used was that found to be successful in the long-term culture of lymphocytes from BL biopsies (Epstein & Barr, 1964). Ovarian lymphomas were collected from experimentally infected birds, and dispersed into single-cell suspensions by agitation in 0.05% trypsin. The cells were adjusted to a concentration of 5×10^6 per ml and cultures of volume 5 ml were incubated at 40°C in screw-cap glass universal bottles, 20 mm in diameter. The medium used was RPMI 1640 supplemented with 20% fetal calf serum (FCS), 10% tryptose phosphate broth, and 1% non-essential amino-acids. Cultures of peripheral blood lymphocytes were initiated in a similar manner. The cultures were re-fed by carefully replacing half the supernatant medium, taking care not to disturb the settled cells, thrice weekly for the first week, and thereafter weekly, until signs of growth of non-adherent cells were apparent. Many cultures gave rise to

a profuse growth of adherent fibroblast-like cells. Of 120 cultures initiated from 49 tumours and 11 blood samples, only two continuous cell cultures have been established; a third culture, which started to grow as non-adherent lymphoblastoid cells after a latent period of 90 days, failed to survive subculture. A further 32 lymphocyte preparations from normal blood failed to grow in long-term culture. More recent attempts to produce additional cell lines have so far been unsuccessful. This contrasts with the relative ease with which lymphoblastoid cell lines may be established from human blood and BL biopsies, and suggests that the method described is not optimal, though it may reflect the notorious difficulty of establishing avian cell lines (Klein, 1972).

All cultures were re-fed weekly for a period of three months before being rejected. One culture was found to be growing as non-adherent lymphoblasts after 31 days of incubation. This was designated "HPRS Line 1" and has now been growing for 12 months. HPRS Line 2 had a latent period of 92 days, and has now been growing for 9 months. Subculturing by division of the cultures into two was done every seven days.

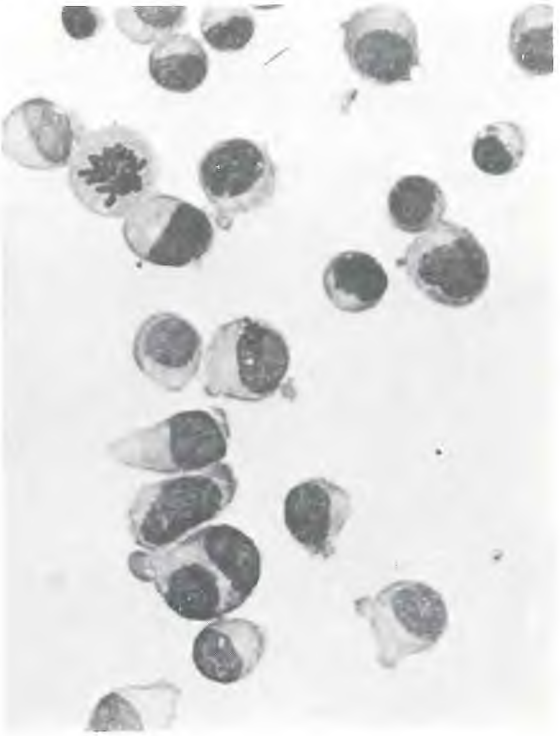
In the growth of both cell lines, a high proportion of dead cells has been consistently observed. Medium supplemented with 30% FCS or with 15% FCS and 15% chick serum supported growth but did not provide the solution to this problem. The cells of both cell lines were fastidious about their culture conditions. Growth was better at 40°C than at 37°C. Line 1 cells failed to divide at the lower temperature; Line 2 cells divided more slowly. The percentage of FCS in the medium was critical. Medium containing less than 10% or more than 30% did not support growth. Normal calf serum could not be used to replace FCS in the medium. Normal chick serum could be used (at 20%), but some sera were toxic.

Cultures grew to a cell concentration of about 1.2×10^6 per ml, which remained static on refeeding. Division of such a culture into two containing 0.6×10^6 cells per ml was followed by rapid cell death, presumably owing to the fragility of the cells, producing cultures containing 0.3×10^6 cells per ml after 24 hours. Cell numbers then increased with a doubling time of 3-4 days. The cells were never attached to glass. Line 1 cells appeared singly or in pairs, but Line 2 cells consistently formed clumps of large numbers of cells. Most cells were round, but some cells had one or many pseudopodium-like projections. The living cells ranged in size from 5 μm to 12 μm, with a mean of 8 μm. In smears, and in suspensions stained with Natt and Herrick's stain (Natt & Herrick, 1952), the cells showed a degree of heterogeneity, perhaps related to their stage in mitosis. Smears showed cells to have large nuclei and a rim of intensely basophilic cytoplasm containing vacuoles (Fig. 1). Some large cells contained two nuclei. Nucleoli were not seen.

Ultrastructurally, the cells were similar to those seen in MD lymphomas (Fig. 2). The nucleus was round or oval and sometimes slightly indented, with marginated chromatin. Nucleoli were occasionally seen. The cytoplasm contained several mitochondria and osmiophilic (lipid) bodies, but sparse endoplasmic reticulum. Single ribosomes and polyribosomes were frequent. The cells were remarkably similar to cultured BL lymphoblasts (Frazier & Powell, 1975); about 3% of the cells showed projections of the nuclear envelope enclosing portions of cytoplasm, and parallel

FIG. 1. PHOTOMICROGRAPH OF A STAINED FILM OF LYMPHOBLASTS OF HPRS LINE 1

The regular rounded cells, which vary considerably in size, have large nuclei and basophilic cytoplasm. One large binucleate cell is present. (May-Grünwald Giemsa) (x 1 300)



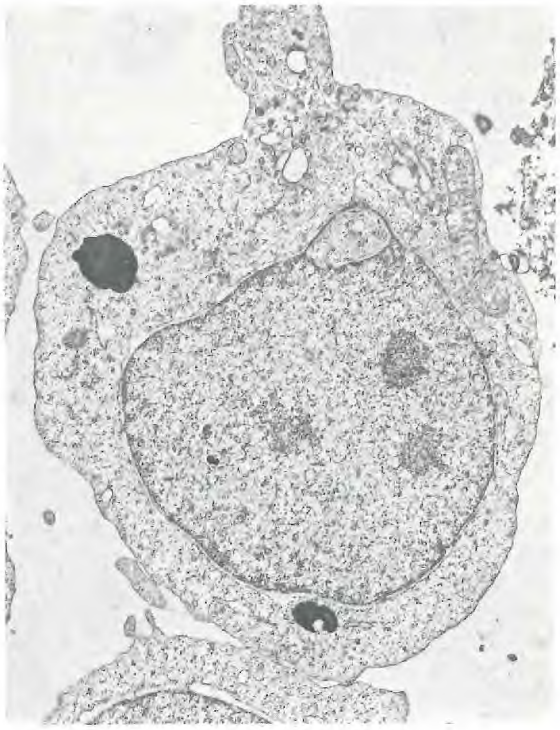
arrays of annulate lamellae were present in the cytoplasm of a few cells. A very small proportion of Line 1 cells (<0.5%) contained immature intranuclear herpesvirus capsids (Fig. 3). The particles measured about 90-95 nm in diameter. No enveloped or cytoplasmic particles were observed. No herpesvirus particles have yet been seen in Line 2 cells.

Both cell lines acted as plaque-forming infectious units (PFU) to chick kidney-cell monolayers, as well as being infectious *in vivo*. The numbers of PFU varied considerably with time. Mean estimations were 660 and 145 PFU per million viable cells for Line 1 and Line 2 respectively. Neither cell line produced any cell-free virus.

Suspensions of living cells were examined for membrane antigens, using the indirect fluorescent antibody technique (Hudson & Payne, 1973). The specific antisera were raised in chickens against HPRS-16 infected chick-kidney cells (anti-MDV) and in rabbits against suspensions of MD lymphoma cells (antitumour), normal thymus cells (anti-T), and normal bursa cells (anti-B). All antisera

FIG. 2. ELECTRON MICROGRAPH OF A TYPICAL LYMPHOBLAST OF HPRS LINE 1

The large nucleus contains a nucleolus, and has a projection of the nuclear envelope. The cytoplasm is rich in ribosomes, and also contains two large lipid bodies, vacuoles and mitochondria. (x 10 000)

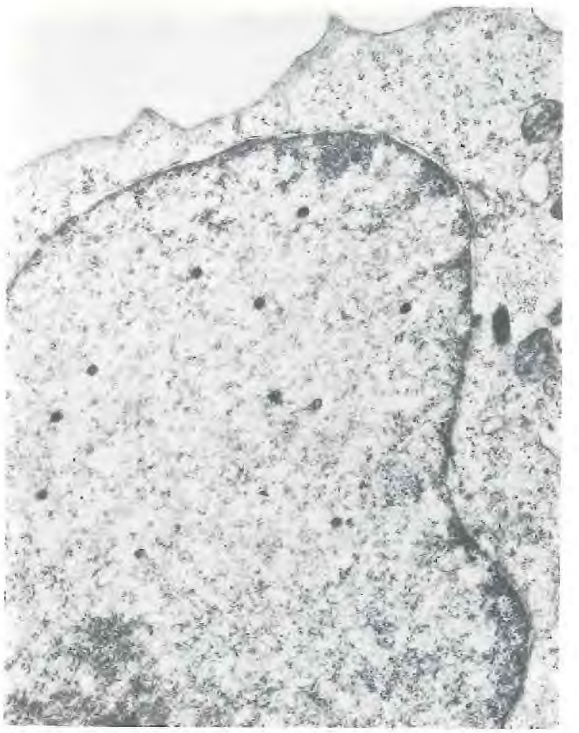


had been extensively cross-absorbed (Payne et al., 1974). The results are shown in Table 1. The anti-B and anti-T antisera were specific, as assessed by the staining of suspensions of bursa and thymus cells. These sera stained B and T lymphocytes, respectively, in the blood. The anti-MDV serum was specific for virus plaques in chick-kidney monolayers. The staining of a high proportion of cell-line cells and of lymphoma cells with antitumour antiserum suggests the presence of a tumour-specific antigen. The results indicate that such an antigen is not identical with the known virus-associated antigens. Line 1 cells stained for T-cell determinants, showing smooth staining with frequent capping, typical of T cells (Fig. 4). Line 2 cells also stained for T-cell determinants.

The immunocompetence of the cells as T cells has been measured by their response to stimulation with phytohaemagglutinin (PHA), and by

FIG. 3. PORTION OF A CELL CONTAINING IMMATURE INTRANUCLEAR HERPESVIRUS PARTICLES

(x 16 000)



their capacity to mount a graft-versus-host (g.v.h.) reaction. Fig. 5 shows that the background level of incorporation by the cells of tritiated thymidine was high, and higher than that of freshly explanted lymphoma cells. There was little or no response to increasing PHA levels, in contrast to the effect of PHA on the incorporation of tritiated thymidine by blood lymphocytes. The specificity of the action of PHA on T cells was demonstrated by the response of the bursa- and thymus-cell suspensions to PHA.

The results of the g.v.h. reactions are presented in Fig. 6. Line 2 differed from Line 1 in possessing no detectable g.v.h. capacity. Line 1 was intermediate between Line 2 and normal blood. Histologically, the splenic enlargement produced by Line 1 cells was typical of a g.v.h. reaction (Biggs & Payne, 1961). This difference may reflect the variable effect of malignant transformation upon cells. Further work is necessary

Table 1. Percentage of cells showing specific membrane fluorescence[a]

Antiserum	HPRS Line 1	Thymus suspension	Bursa suspension	Blood lymphocytes	MD lymphoma	MD virus plaques in chicken-kidney cells
Anti-T	99.5 ± 0.3	93.9 ± 2.3	4.0 ± 0.4	73.5 ± 2.8	76.6 ± 3.9	-
Anti-B	1.1 ± 0.4	4.3 ± 0.3	94.0 ± 0.9	21.8 ± 3.3	20.4 ± 2.4	-
Antitumour	96.3 ± 1.2	0	0	1.3 ± 0.2	35.1 ± 9.6	-
Anti-MDV	1.0 ± 1.0	0	0	0	2.8 ± 1.8	+++

[a] Mean of five determinations ± standard error.

to establish the immunological nature of this reaction, and to eliminate the possibility of its being due to the differential ability of the two cell lines to multiply *in vivo*. The properties of the two cell lines are summarized in Table 2.

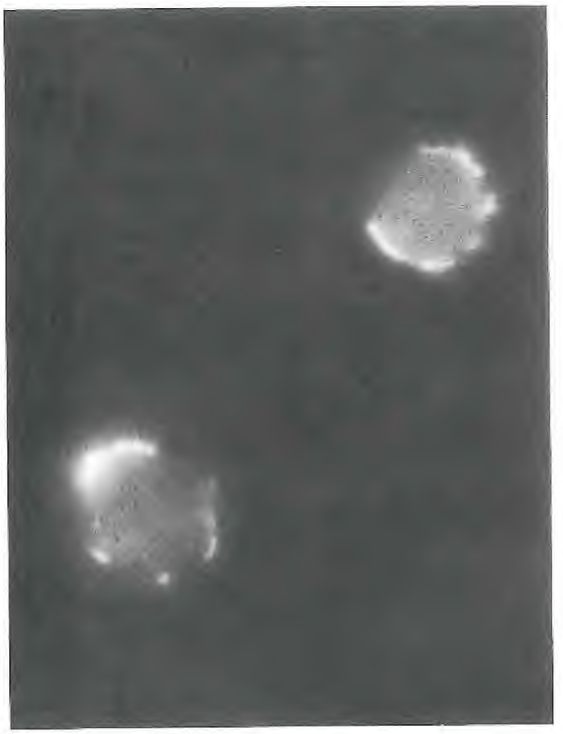
DISCUSSION

The majority of cells in MD lymphomas are of thymus origin (Hudson & Payne, 1973; Rouse et al., 1973; Payne et al., 1974). The role of these cells in the histopathogenesis of the disease is not clear.

Payne (1972) suggested either that they represent foci of malignantly transformed lymphoid cells (intrinsic mechanism), or that these lymphocytes are participating in an excessive proliferative response to new viral antigen (extrinsic mechanism). Since MD usually has a multifocal origin, an extrinsic mechanism is obviously a possibility. There is, however, a poor correlation between the presence of viral antigen and the site of tumour development (Payne & Rennie, 1973). Cell-surface antigens have been reported in MD (Ishikawa et al., 1972); the results of our fluorescent-antibody studies suggest that the cell lines carry membrane tumour-specific antigens. Preliminary results of ^{51}Cr-release cytotoxicity tests suggest that immune lymphocytes are capable of killing the cells of HPRS Line 1 and Line 2. This and other evidence supports the role of MDV as an agent capable of transforming lymphoid cells, and associated with the continuous growth of such cells *in vitro*. DNA hybridization studies have revealed the presence of 60-90 MDV genome equivalents per diploid cell in the cell line MSB-1 (Nazerian & Lee, 1974); after cloning of the same line, all ten clones produced had a small proportion of MDV antigen-expressing cells (Akiyama & Kato, 1974), and induction with IUDR and BUDR caused the increased expression of virus-specific antigens. The failure of the cell lines to respond to PHA stimulation, and their reduced capacity to mount a g.v.h. reaction, suggest that transformation of the cells has altered their functional capabilities. The striking similarity between the cell lines and cultured human lymphoblasts is surprising in view of the B-cell nature of the latter.

FIG. 4. CELLS OF HPRS LINE 2 SHOWING T-CELL SURFACE DETERMINANTS

Two cells of HPRS Line 2 showing T-cell surface determinants stained by the indirect fluorescent antibody technique. (x 2 000)



Herpesviruses are, however, involved in the transformation of T lymphocytes in other systems (herpesvirus saimiri). We conclude that the development of acute Marek's disease involves the malignant transformation of thymus-dependent lymphocytes by Marek's disease virus.

SUMMARY

The establishment and continuous culture of two lymphoblastoid cell lines derived from Marek's disease lymphomas is described. Although the cells carried T-lymphocyte surface antigens, they had many features in common with cultured Burkitt's lymphoma lymphoblasts, which carry B-cell determinants. A small proportion acted as infectious units in tissue culture, and a similarly small proportion contained intranuclear immature herpesvirus particles. The cells did not respond to phytohaemagglutinin. One cell line possessed some graft-versus-host capacity, as measured by the induction of splenomegaly. It is concluded that the development of acute Marek's disease involves the malignant transformation of thymus-dependent lymphocytes by Marek's disease virus.

FIG. 5. EFFECT OF STIMULATION BY PHYTOHAEMAGGLUTININ

Effect of stimulation of suspensions of various cells by phytohaemagglutinin, as assessed by incorporation of tritiated thymidine. Each point is the mean of eight cultures; vertical lines show standard errors. Culture period: 48 hours; pulse labelling for 16 hours; cell concentration 2 x 10^6 per ml; culture medium RPMI 1640 + 10% FCS.

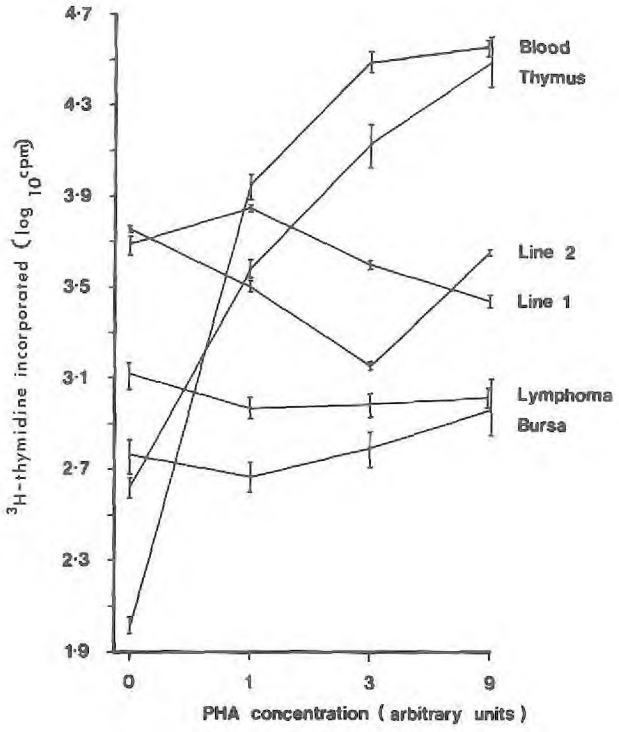


FIG. 6. INDUCTION OF SPLENOMEGALY

Splenomegaly-inducing capacity of cells of HPRS Line 1 and Line 2 and of allogeneic peripheral blood in R.P.L. Line 6 chick embryos. Dashed lines indicate ± 2 standard errors of the mean of uninoculated control embryos.

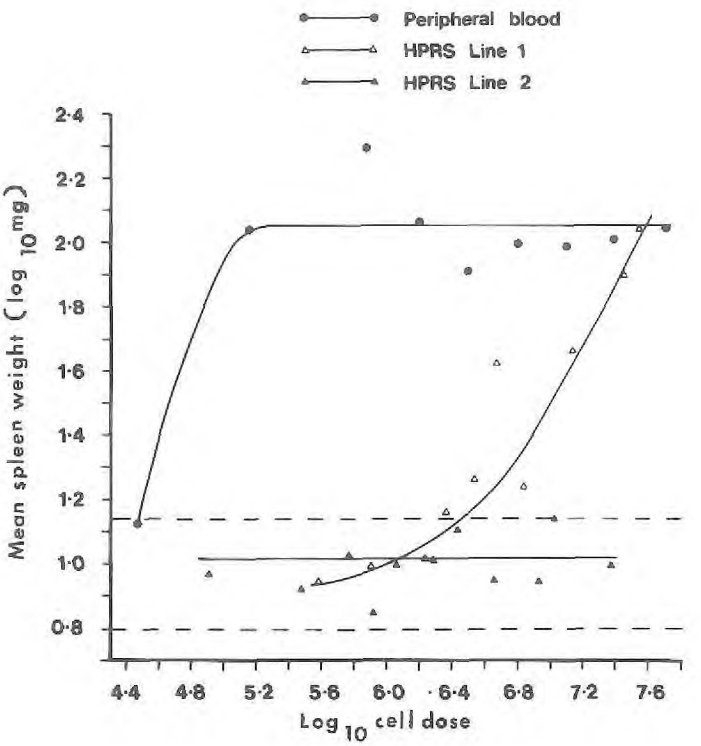


Table 2. Properties of lymphoblastoid cell lines

Cell line	Source	Latent period (days)	Growth *in vitro*	Infective *in vitro*	Infective *in vivo*	Anti-MDV fluorescence	Herpesvirus particles	Response to PHA	g.v.h. capacity	gs-antigen
HPRS Line 1	Ovarian lymphoma	31	Singly	0.066%	Yes	<0.5%	Yes	No	Yes	No
HPRS Line 2	Ovarian lymphoma	92	Clumps	0.015%	Yes	<0.5%	No	No	No	No

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LYMPHOID CELL LINES FROM LYMPHOMAS OF MAREK'S DISEASE

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Among oncogenic herpespviruses, Marek's disease virus (MDV) has been studied extensively, especially from the standpoint of host-virus interactions *in vivo*. However, lack of a lymphoid cell line derived from MD lymphoma has prevented studies on virus tumour-cell interactions *in vitro* such as those pursued extensively with Epstein-Barr virus. Two cell lines, the MOB-1 line and the MSB-1 line, derived from MD lymphomas, were established in 1973 (Akiyama et al., 1973; Akiyama & Kato, 1974a), and recently we have established a third line, the MOB-2 line, from another MD lymphoma.

The present paper reports further studies on the characterization of these three cell lines. The cell lines were also compared with a cell line, 1104B, derived from a bursal tumour induced by avian leukosis virus (ALV) (Hihara et al., 1974).

CONTINUOUS CELL CULTURES FROM LYMPHOMAS OF MAREK'S DISEASE

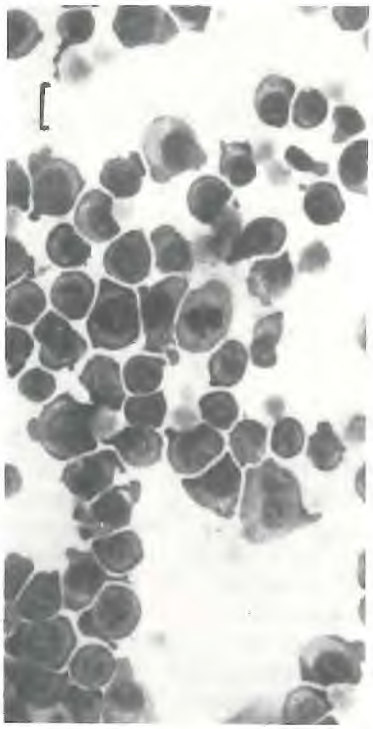
Three cell lines were obtained from two ovarian lymphomas and one splenic lymphoma, of chicks with Marek's disease. They were designated as lines MOB-1, MOB-2 and MSB-1, respectively. They were grown in RPMI 1640 medium supplemented with 10-20% fetal calf serum and incubated at 41°C in a humidified atmosphere of 5% carbon dioxide in air. These lines consisted of lymphoblastoid cells (Fig. 1) and grew singly without becoming attached to the surface of the culture vessel. They grow better at 41°C than at 37°C. MSB-1 cells have now grown well for over 420 days. The MOB-1 cells degenerated after 320 days, but recultivation of the cells from an early culture that had been kept in a deep freeze is under way. MOB-2 cells have grown for 60 days. The biological and virological characteristics of these cell lines are summarized in Table 1.

CLOSE ASSOCIATION OF THE CELLS WITH MDV

A small proportion of the MOB-1, MSB-1 and MOB-2 cells were consistently MD viral antigen (VA)-positive (Fig. 2), but the percentage of VA-positive cells varied (0.1-10%) from passage to passage.

FIG. 1. SMEAR PREPARATION OF MSB-1 LINE CELLS AFTER 385 DAYS' CULTIVATION

Giemsa stain. Scale: 10 μm.



These lines also consistently contained a small proportion of cells with MDV-induced cell-surface antigen (CSA). Electron microscope examination showed that a similar number of nuclei contained herpesvirus nucleocapsids (Fig. 3). Attempts to recover cell-free virus from cells of these lines failed. However, MDV could be easily isolated by co-cultivation of cells of these lines with susceptible cells, such as chick kidney cells or chick embryo fibroblasts (CEF). All 10 cloned cultures derived from the MSB-1 line contained some MDV antigen-positive cells. It is unlikely that cell-free virus was transmitted to all these cloned cells, since cell-free virus was rarely obtained from the line. Since the proportion of cells in this line showing replication of MDV is very small, it is probable that the genome is closely associated with most of the cells.

ONCOGENICITY OF CELLS

When day-old chicks were inoculated with MOB-1 cells or MSB-1 cells, MD lesions appeared as frequently as after inoculation of virulent MDV. A dose of over 10^3 cells of MSB-1 line per chick was enough to produce MD lesions, and these cells were as virulent as the original MDV BC-1 strain (Okaniwa

Table 1. Characteristics of MD cell lines[a]

Characteristics	MOB-1	MSB-1	MOB-2	1104B[b]
Origin of cells	MD ovarian tumour	MD splenic tumour	MD ovarian tumour	ALV-induced bursal tumour
Origin of virus	MDV BC-1	MOB-1 line-associated MDV	MSB-1 line-associated MDV	ALV, subgroup A
Length of cultivation	320 days (22 Jan 1973) >126 days (a subline)	>420 days (10 Aug 1973)	>60 days (10 Aug 1974)	>4 years
Latent period to obtain actively growing cells	Short (4-5 days)	Short (4-5 days)	Long (30 days)	NT
Manner of growth	Singly, transient clump	Singly, transient clump	Singly, transient clump	Singly
Morphology	Lymphoblast	Lymphoblast	Lymphoblast	Lymphoblast
Peroxidase reaction	-	-	NT	NT
Optimal temperature for growth	41°C	41°C	41°C	38-41°C
Doubling time	Ca. 20 hours	Ca. 10 hours	Ca. 48 hours	Ca. 16 hours
Surface character with scanning electron microscopy[c]	With scattered microvilli	With scattered microvilli	NT	With microvilli
Immunoglobulin with cytotoxic test	-	-	NT	-
Karyotype	Chicken female	Chicken female	Chicken female	NT
Chromosomal aberration	-	+	+	NT
Cells with MDV viral antigen	0.1-5%	0.3-12%	11%	-
Cells with MDV CSA	0.1% or less	3% or less	0.9%	-
Herpes-type capsids	+	+	NT	-
Cell-free MDV with CEF	-	-	NT	NT
Isolation of MDV by co-cultivation with CEF	+	+	+	NT
Cloning efficiency	Poor	High	Poor	High
Clones free from MDV antigen	NT	-	NT	NT
MDV viral genome	NT	+[d]	NT	NT
Agglutinability with Concanavalin A	++	+++	++	NT
Oncogenicity in chicks	+	+	NT	+
Oncogenicity in chick embryos	+	NT	NT	NT
Oncogenicity of virus from cells	NT	+	NT	NT
Transplantability in chicken wing web	-	± ~ +	NT	NT
Transplantability on chorioallantoic membrane	+	+	NT	NT
C particles	-	-	NT	+
Complement-fixation test for avian leukosis	-	-	NT	+
Resistance-inducing factor	-	-	NT	+
gs-Antigen	±	-	NT	+[e]
Susceptibility to ALV, subgroup A	NT	+	NT	NT
Susceptibility to ALV, subgroup B	NT	-	NT	NT

[a] NT: not tested.

[b] Hihara et al. (1974).

[c] Akiyama & Kato (1974b).

[d] Nazerian & Lee (1974).

[e] ALV, subgroup A.

FIG. 2. MDV VIRAL ANTIGEN-POSITIVE CELLS OF THE MSB-1 LINE DEMONSTRATED BY IMMUNOFLUORESCENCE

et al.[1]). The distribution of MD lesions in the tissues of chickens inoculated with the cells of this line is similar to that found with the MDV BC-1 strain. Chicks that had been raised in contact with chicks inoculated with the cells, also developed MD lesions, due to contact infection. This indicates that MSB cells behave like the original MDV BC-1 strain when inoculated into chicks. No difference was found between the original MDV BC-1 strain and MDV isolated from MSB-1 cells in plaque morphology or antigenicity, as demonstrated by gel diffusion (Ishikawa & Kato[1]). MDV isolated from the cells (210 days cultivation level) was inoculated into eight day-old chicks intraperitoneally. Six chicks developed MD lesions in various visceral organs and nervous tissues within 10 weeks after inoculation.

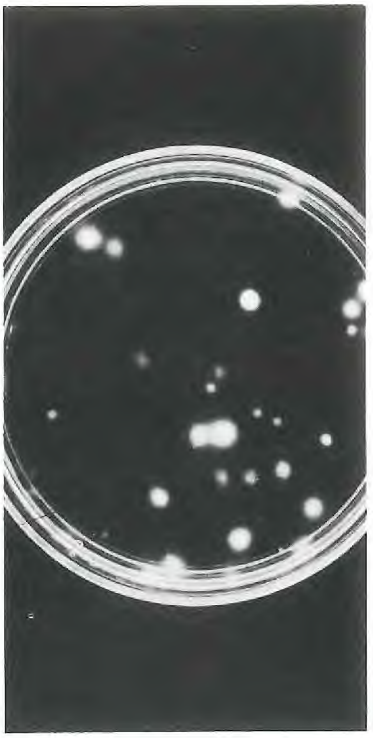
We have not obtained any evidence of continuity of growth of the inoculated cells. When a male chick was inoculated with MSB-1 cells, which have the female karyotype, gonad lymphoma lesions appeared. Twenty mitotic lymphoid cells from these lesions were examined and all were found to have the male karyotype (Akiyama & Kato[2]).

Transplantability of the cells in day-old chicks was also examined by

[1] Unpublished data.

[2] Unpublished data.

FIG. 3. COLONIES OF MSB-1 LINE CELLS 10 DAYS AFTER PLATING



inoculating 10^7 cells of the MSB-1 line or the MOB-1 line per chick into the wing webs of 10 chicks. Transient tumour growth was observed in the wing webs after inoculation of 10^7 MSB-1 cells, and one chick died due to progressive growth of the local tumour. In contrast, chicks inoculated with 10^7 MOB-1 cells did not show any tumour growth. However, most of the chicks developed MD lesions in various visceral and nervous tissues after the transplants had regressed (Akiyama & Kato[1]).

These results indicate that MDV associated with the cell lines remains oncogenic, even after long-term cultivation, and that most if not all of the lymphoma cells were derived from cells newly transformed by MDV from the cultured cells.

DISCUSSION AND CONCLUSIONS

Three chicken lymphoid cell lines derived from Marek's disease lymphomas have been obtained. One of the essential conditions for successful cultivation of chicken cells seems to be the incubation temperature, since the optimal temperature for growth of these lines is 41°C, rather than 37°C, and this is supported by the similar successful establishment of other chicken lymphoid cell lines (Powell et al., 1974; Hihara et al., 1974).

[1] Unpublished data.

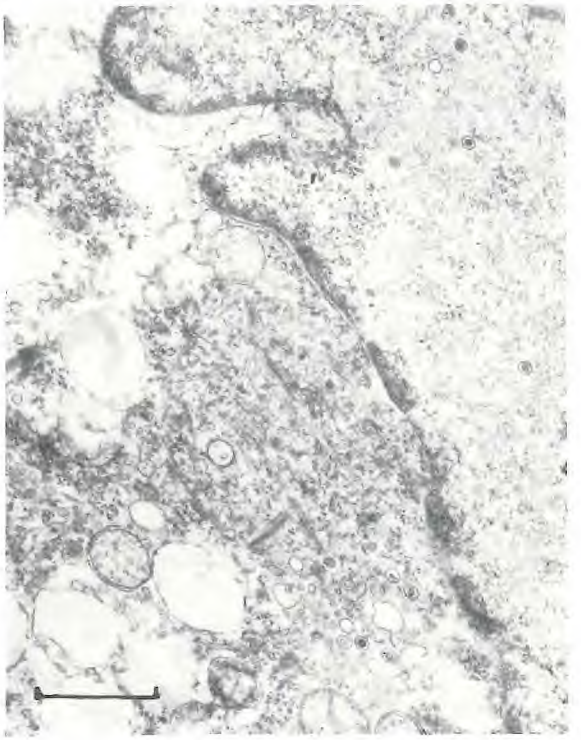
The former authors established two chicken lymphoblastoid cell lines from MD lymphoma, which also grew better at 40°C than 37°C. The latter authors developed lymphoid cell lines associated with avian leukosis virus, which again grew well at 38-41°C. However, other factors also seem to be involved in the establishment of chicken cell lines, since the rate of success was rather low (three out of 53 trials in our work and two out of 120 in that of Powell et al. [1974]). The close association of these cell lines with MDV has been demonstrated by the consistent presence of MDV antigen, herpesvirus capsids and infectious virus in these lines during long-term cultivation. This was further supported by the presence of MDV antigen-positive cells in all of 10 sublines cloned from the MSB-1 line; since the proportion of cells showing replication of MDV is very small, a large proportion of the cells in the line must therefore have MDV genome in a repressed form. This possibility is supported by the presence of 60-90 genome equivalents per diploid cell in cells of the MSB-1 line, as demonstrated by DNA-cRNA hybridization experiments (Nazerian & Lee, 1974).

MDV associated with these cell lines is as oncogenic as the original MDV BC-1 strain, in spite of the long-term *in vitro* cultivation. This contrasts with studies on MDV grown in normal avian cells, where attenuation was easily achieved by repeated passages through chick or duck cells (Churchill et al., 1969; Kato et al., 1970). It is most likely that the oncogenicity of these cell lines in chicks is largely due to the oncogenicity of the MDV they release. We have not obtained any evidence of growth of the cultured cells inoculated in chicks.

The characteristics of the lines vary considerably. In the case of MSB-1 cells, the characteristics that may be related to transformation are the lymphoblastoid appearance of the cells, long-term cultivation, unusually high growth rate, high cloning efficiency in soft agar (Fig. 4), agglutinability with Concanavalin A, and transplantability in chicks. Further studies are in progress to clarify the characteristics of these lines as regards their transformed state.

FIG. 4. THIN-SECTION ELECTRON MICROGRAPH OF A LYMPHOID CELL OF THE MSB-1 LINE

Several herpes-type capsid structures are observed. Scale: 1 μm.



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CYTOMEGALOVIRUS INFECTION OF POLYMORPHONUCLEAR AND MONONUCLEAR LEUKOCYTES IN IMMUNOSUPPRESSED TRANSPLANT PATIENTS, PATIENTS WITH CMV MONONUCLEOSIS AND A PATIENT WITH LEUKAEMIA

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We have found that cytomegalovirus (CMV) viraemia associated with the leukocytic fraction of blood ("buffy-coat" cells) was an early diagnostic finding in nine (35%) of 26 renal transplant patients with disseminated CMV infections, in a patient with mononucleosis and thrombocytopaenia and in another patient with mononucleosis and hepatitis. All transplant patients with CMV viraemia showed a rise in complement-fixing antibody, viruria or both and manifested compatible clinical findings (Fiala et al., 1975).

CMV viraemia appears to be important in the pathogenesis of virus dissemination for the following reasons: (1) it was noted early in the natural history of CMV disease; (2) the onset of CMV viraemia preceded or was concurrent with viral pneumonitis in four transplant patients, whereas chronic viraemia (lasting 1 and $2\frac{1}{2}$ years) was complicated by retinitis in two patients; (3) antibody response to CMV did not interrupt viraemia as the virus is protected inside leukocytes.

CMV titres in the blood cells were determined using a microplaque assay (Fiala et al., 1973) of blood fractions separated on the Ficoll-Hypaque (F-H) gradient (Böyum, 1968) (Table 1). With the exception of the patient with lymphoblastic leukaemia, the polymorphonuclear (PMN) fraction had the highest titre of virus. This was approximately ten-fold higher than the mononuclear fraction. Electron microscopy of about 300 leukocytes from the buffy-coat preparations of three viraemic patients were negative for virus particles.

CASE HISTORIES

An adult male without a significant past medical history developed fever, arthralgias, rash, hepatomegaly and elevation of serum glutamic pyruvic transaminase to 700 units per ml. CMV was first isolated from both PMN and mononuclear leukocytes, then from PMN leukocytes and the pharynx and finally, from the urine.

However, we have recently seen a seven-year-old female with a four-year history of acute lymphoblastic leukaemia in haematological remission with a two-week history of fever and cough. Chest X-ray revealed bilateral interstitial infiltrates. Lung tissue obtained by open biopsy revealed intranuclear inclusions compatible with CMV, and CMV was isolated from the tissue. After the surgery, the patient developed a haemorrhagic pleural effusion, a sample of which was separated on the F-H gradient. CMV grew, at a high titre, from the mononuclear leukocytes, whereas the PMN leukocytes were virus-negative.

DISCUSSION

The relationship of CMV to leukocytes may be highly complex and dynamic. CMV viraemia in PMN leukocytes may represent phagocytosis of virus or virus-antibody complexes. In immunologically competent patients, phagocytized virus may be rapidly killed, whereas in immunologically deficient patients, it may persist. In leukaemic patients, however, mononuclear cells may, perhaps because of co-infection with another helper virus, such as Epstein-Barr virus, be permissive for CMV.

Table 1. Cytomegalovirus titres in blood fractions (separated on Ficoll-Hypaque gradient) from six patients with CMV infections

Patient	Immunological status and diagnosis	Interval after transplantation or onset of illness (months)	CMV titre[a]		
			Mononuclear cells	Polymorpho-nuclear cells	Red cells
F.M.	Immunosuppressed renal transplant recipient	12.2	1.2	+[b]	0.003
		12.6	4.3	ND[c]	ND
		14.4	2.4	9.6	ND
		14.8	0.4	9.6	0
C.R.	Immunosuppressed renal transplant recipient	1.2	0.13	ND	ND
		2.1	0.33	+[b]	0
		2.2	0.5	ND	ND
		2.4	0.22	3.1	0
		2.7	0	0	0
L.M.	Immunosuppressed renal transplant patient	14.0	+	+	0
J.L.	Chronic uraemia; haemodialysis	14.0	+	+	0
W.C.	Normal; CMV mononucleosis and hepatitis	0.5	0.6	3.0	ND
		1.0	0	5.0	ND
A.S.	Acute lymphoblastic leukaemia	48	>20.0	0	0

[a] Virus titre is expressed as cytomegalovirus focus-forming units per 10^5 inoculated leukocytes or red cells separated from peripheral blood, except for the last patient (A.S.), in whom haemorrhagic pleural effusion was used for isolation of blood cells.

[b] Virus was found in the blood fraction but the titre was not determined.

[c] Not done.

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ULTRASTRUCTURAL AND IMMUNOFLUORESCENT STUDIES OF THE REPLICATION OF HERPESVIRUS SAIMIRI IN CULTURED LYMPHOCYTES OF INFECTED OWL MONKEYS

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Herpesvirus saimiri (HVS), first isolated in 1968 (Meléndez et al., 1968), is an indigenous virus of squirrel monkeys that causes malignant lymphoma and/or lymphocytic leukaemia when injected into owl monkeys, marmosets and several other species of New World monkeys (Meléndez et al., 1972; Deinhardt, 1973). Virions of HVS cannot be detected in normal or neoplastic tissues of infected monkeys by electron microscopic (EM) examination, nor can HVS nucleocapsid antigens be detected in these tissues by fluorescent antibody examination (Ablashi et al., 1971; Falk et al., 1972; King & Meléndez, 1972; Wolf et al., 1971). HVS can be isolated from intact tumour cells, however, by establishing cell cultures of them or by co-cultivating them with permissive cells. Falk et al. (1972) reported that peripheral lymphocytes from HVS-infected monkeys latently carry the HVS genome, because viral antigens can be demonstrated by immunofluorescent (IF) microscopy of lymphocytes within 24-72 hours after *in vitro* incubation. Infectious virus was not produced by lymphocyte cultures alone, but when lymphocytes were co-cultivated with permissive cells, viral antigens and infectious virus particles were produced. The cellular events underlying the replication of HVS in latently-infected lymphocytes are not known. Preliminary EM examination failed to reveal HVS virions in cultured lymphocytes, and it was speculated that transmission of the genome from lymphocytes to permissive cells might occur through some other medium, such as cell fusion or cell-to-cell transfer of viral genome in some form other than the HVS virion (Falk et al., 1972; Deinhardt, 1973).

I conducted an ultrastructural study of short-term cultured lymphocytes from control and HVS-infected monkeys in order to determine whether virions were produced, whether defective or complete particles were formed, the percentage of cells in which

replication occurred, and the extent to which virion production observed by EM was related to the presence of viral antigens determined by IF.

Five male and female adult owl monkeys (*Aotus trivirgatus*) weighing 600-800 g were inoculated with 10^5 tissue culture infective doses of herpesvirus saimiri, stock E940F, strain S295C (kindly supplied by Dr L.V. Meléndez and associates). They, and three uninfected control monkeys, were part of an experiment aimed at providing an animal model for nasopharyngeal carcinoma (Giddens, 1974). Monkeys were bled at 91-210 days post infection and lymphocytes were separated on Ficoll-Hypaque gradients, cultured in RPMI 1640 medium with 20% fetal calf serum, and examined by IF and EM at 24, 48 and 72 hours after the beginning of culture (AC). Virus isolation was also attempted by co-cultivation of lymphocytes with monolayers of Vero cells (Giddens, 1975).

At the time of lymphocyte culture, two of the five infected monkeys were leukaemic: pre-injection white blood cell counts had doubled and there were large numbers of atypical lymphocytes. Within 50 days AC, four of the five infected monkeys had died with malignant lymphoma and lymphocytic leukaemia. Lesions did not differ significantly from those described previously (Ablashi et al., 1971; Hunt et al., 1970; Wolfe et al., 1971). The fifth monkey is still alive and has a normal white cell count at 380 days post infection.

In each specimen of cultured lymphocytes or buffy coats studied by EM, I examined 50-100 lymphocytes. No virus particles were observed in control monkeys or in buffy coats of infected monkeys. Herpesvirus nucleocapsids were evident in two of five lymphocyte cultures from infected monkeys 24 hours AC, four of five at 48 hours AC, and all of five at 72 hours AC (Table 1). Virus particles were seen only in

Table 1. Percentage of lymphocytes containing HVS particles by electron microscopy and isolation of HVS by co-cultivation with Vero cells

Animal		Buffy coat	Time after beginning of culture			HVS isolated by co-cultivation
			24 h	48 h	72 h	
62	Control	0	0	NE[a]	0	0
63	Control	0	0	0	0	0
61	Control	NE[a]	NE[a]	NE[a]	0	NE[a]
74	Infected	0	0	5-10%[b]	10-20%[b]	+
73	Infected	0	1-5%	5-10%	5-10%	+
56	Infected	0	0	1-5%	1-5%	+
52	Infected	0	5-10%	5-10%[b]	5-10%	+
70	Infected	0	0	0	1-5%	+

[a] Not examined.

[b] Enveloped virions were observed.

lymphocytes, which had always undergone degenerative changes. The capsids were 95-105 nm in diameter and had a hollow core, an inner ring, or a dense core (Fig. 1). Nucleocapsids were often seen in the cytoplasm of cells that had advanced degenerative changes in their nuclei (Fig. 2). Enveloped virions were 160-180 nm in diameter and were extremely rare, having been observed in only three cells. Both naked and enveloped virions were similar in size and morphological features to those described by King et al. (1972) and to the pleomorphic virions described by Heine & Ablashi (1974) and Heine et al. (1971). Viruses were isolated from all five infected monkeys by co-cultivating their lymphocytes with Vero cells (Table 1). No virus was isolated from control monkey lymphocytes and no cytopathic effect was produced in them.

Indirect IF studies using anti-HVS serum from guinea-pigs and owl monkeys were conducted on cultured lymphocytes and on Vero cells that had been infected with the viruses isolated from the co-cultivation experiment. Of the cultured lymphocyte preparations from infected monkeys, 5-20% stained brilliantly with anti-HVS serum (Table 2). Staining was in a variety of patterns, and occurred in both cytoplasm and nucleus. No significant difference was observed in the percentage of stained cells at 24, 48 and 72 hours. There was relatively good agreement between the EM (Table 1) and IF (Table 2) data. Generally, lymphocytes with positive IF staining were about 1-2 times as frequent as

FIG. 1. LYMPHOCYTE FROM HVS-INFECTED MONKEY 48 HOURS AC

Capsids have empty centres (A), inner rings (B), or dense cores (C). An enveloped virion is present in the cytoplasm (D). (x 48 000)

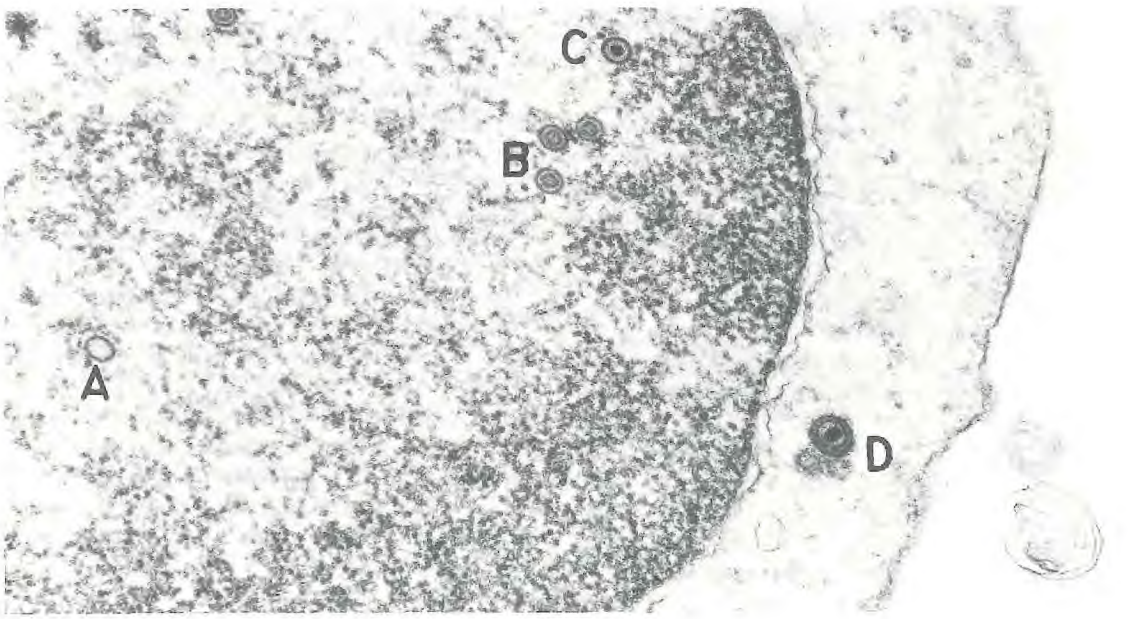
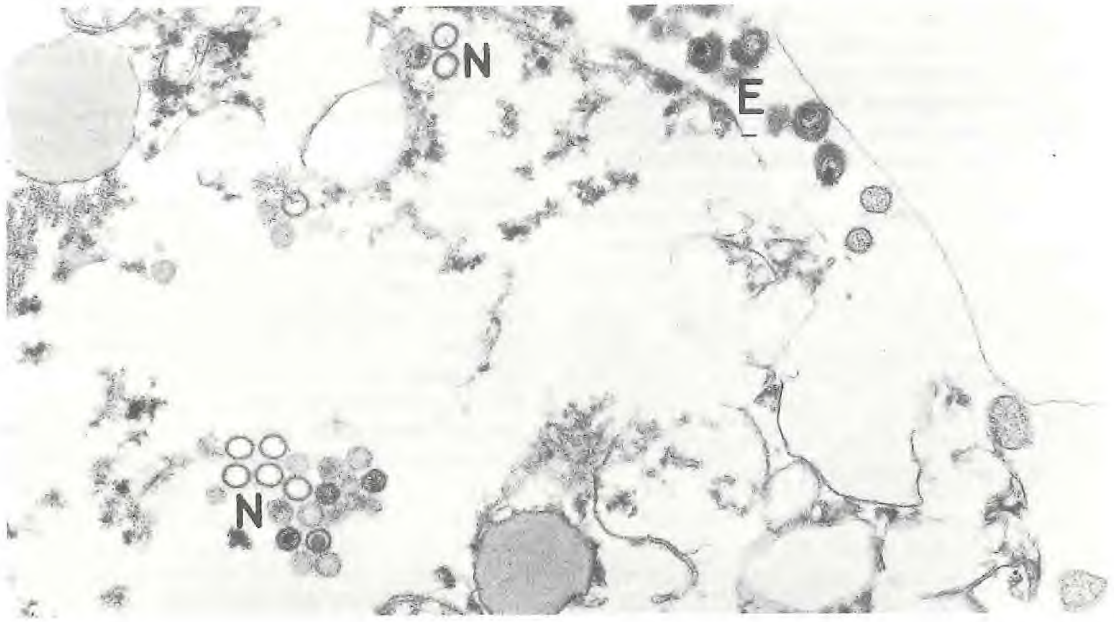


FIG. 2. DEGENERATED LYMPHOCYTE FROM HVS-INFECTED MONKEY 48 HOURS AC
There are nucleocapsids (N) in the centre and enveloped virions (E) in the periphery of the cell. (x 28 100)



lymphocytes with virus particles on EM examination.

The virus in the cultured lymphocytes of infected monkeys was identified as HVS on the basis of its ultrastructural features, its presence in HVS-infected monkeys but not controls, the characteristic cytopathic effect produced in Vero cells by cocultivation with lymphocytes, and specific IF staining of cultured lymphocytes and infected Vero cells with anti-HVS guinea-pig and owl monkey sera, but not with preinoculation sera or anti-herpes simplex serum.

The results of these studies confirm those of Falk et al. (1972) on the latency of HVS infection of circulating lymphocytes. They provide EM evidence that nucleocapsids are formed within 24 hours AC and that enveloped virions are produced as early as 48 hours AC. Production of enveloped virions is rare, however, and this is probably why studies by others have failed to isolate HVS from the supernatant of lymphocytes when they have been cultured alone. A sensitive, permissive cell system, such as the Vero cell, appears to be needed to pick up the occasional complete virions and become infected.

There seemed to be some prognostic value in the incidence of lymphocytes containing virus particles. Monkey 70, which had the smallest percentage of virus-containing lymphocytes of any of the infected monkeys studied,

Table 2. Percentage of positive lymphocytes with HVS antigens by immunofluorescent examination

Animal		Time after beginning of culture		
		24 h	48 h	72 h
62	Control	0	NE[a]	0
63	Control	0	0	0
61	Control	NE[a]	NE[a]	0
74	Infected	10-20%	10-20%	10-20%
73	Infected	5-10%	5-10%	10-20%
56	Infected	5-10%	5-10%	5-10%
52	Infected	10-20%	10-20%	10-20%
70	Infected	5-10%	5-10%	5-10%

[a] Not examined.

is still alive, and HVS can still be isolated from its lymphocytes by cocultivation.

Preliminary studies in my laboratory of lymphocytes from healthy squirrel monkeys with naturally occurring HVS infection indicate that, although HVS can be isolated by cocultivation with Vero cells, it has not been detected by ultrastructural and IF examination similar to that described herein. This suggests that only a very small percentage of lymphocytes in squirrel monkeys is latently infected with HVS. This may be the result of some mechanism, possibly immunological or genetic in nature, that has evolved in the squirrel monkey to allow it to live symbiotically with HVS. Klein et al. (1973) have shown that squirrel monkeys have an earlier antibody response than owl monkeys to experimental HVS infection. There may also be a difference in the cells that are infected and in their susceptibility to malignant transformation. Wallen et al. (1974) and Falk et al. (1974) have shown that T lymphocytes are infected with HVS in owl monkeys and marmosets that have HVS-induced lymphoma. Perhaps in the squirrel monkey, as in man with Epstein-Barr virus (EBV) infection, B lymphocytes are infected, and malignant disease is apparently a rare event.

SUMMARY

The lymphocytes of five owl monkeys infected with herpesvirus saimiri (HVS) and of three control owl monkeys were studied by electron microscopic (EM) and immunofluorescent (IF) techniques. Buffy coats of whole blood immediately after bleeding were also prepared for study. At the time of the study, two of the five infected monkeys were leukaemic; within 50 days after the study, four of the five had died with malignant lymphoma and lymphocytic leukaemia. HVS virions were demonstrated by EM and HVS antigens by IF in 1-20% of the

lymphocytes from infected monkeys in 2/5 cultures at 24 hours AC, 4/5 at 48 hours AC, and 5/5 at 72 hours AC. There was quite good agreement between the EM and IF data. None of the control monkey cultures and none of the buffy coat preparations contained HVS virions or antigens. By EM, the great majority of the virus particles were nucleocapsids within the nuclei of lymphocytes. Enveloped virions were rare. There was some evidence to suggest that the higher the percentage of lymphocytes containing HVS, the poorer the prognosis for the monkey. HVS was isolated from all five infected monkeys by co-cultivation of lymphocytes with Vero cells. Control lymphocyte co-cultivations were negative.

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TUMOUR INDUCTION IN VIVO: MAJOR EVENTS LEADING TO TUMORIGENESIS IN EMBRYOS INOCULATED WITH ONCOGENIC HERPESVIRUSES

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Inoculation of *Rana pipiens* embryos with zonal centrifuge-purified fractions of Lucké tumour herpesvirus (LTHV) results in a high percentage of renal adenocarcinomas (Mizell, 1969; Mizell et al., 1969b). The study of tumorigenesis in these inoculated embryos has revealed characteristics and conditions that must be fulfilled for tumours to develop. Paramount among these are the following two requisites: (1) the injected animal must be sufficiently young to be immunologically tolerant; and (2) the virus "target" tissue must contain dividing and differentiating cells. Frog embryos and young tadpoles fulfill these conditions.

These frog embryo-LTHV inoculation studies have provided us with an approach to an understanding of the *mechanism* of tumour induction by herpesvirus in amphibia. This is of obvious value to the study of the putative oncogenic herpesviruses of man. Nevertheless, it has become increasingly evident that, in order to understand the underlying mechanism of such tumour induction in mammals, a comparable mammalian model system is needed.

The newborn opossum, *Didelphys marsupialis virginiana* (Kerr), a marsupial common in North America, has promise of providing this mammalian model system. After an extremely short gestation period ($12\frac{3}{4}$ days), the young opossums leave the birth canal, migrate to the marsupial pouch, attach to the nipples and complete their extra-uterine development and differentiation within this readily accessible maternal environment. Another unique feature of newborn opossums - also related to their immaturity at birth - is the absence of the usual mammalian immune mechanism. In fact, lymphocytes are absent at birth and the thymus is merely an epithelial sheet or anlage. Because of the above attributes, these unique mammals have been inoculated with Epstein-Barr virus (EBV).

In this paper I shall discuss and compare some of the results of these dual herpesvirus inoculation studies.

FROG EMBRYOS AND THE LUCKÉ TUMOUR HERPESVIRUS

Injection experiments have

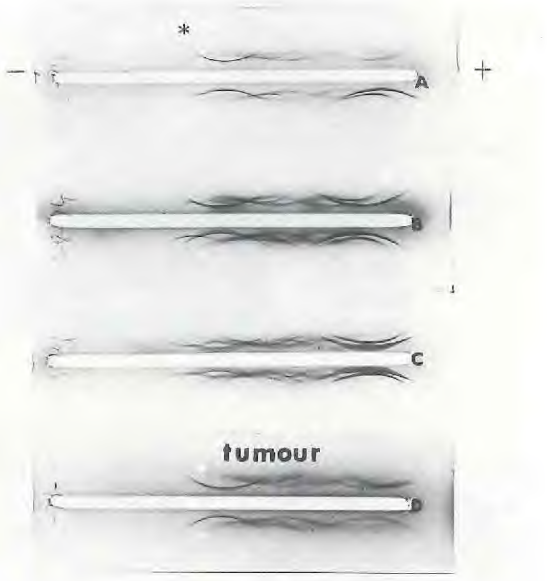
indicated that the LTHV has a preferential affinity for differentiating kidney tissue. Only if the pronephric kidney was differentiating at the time of injection did an unusually high incidence of pronephric tumours develop during subsequent metamorphosis (see Fig. 1); if injection was delayed until after this early larval kidney was fully formed and the late larval kidney or mesonephros was differentiating, then mesonephric tumours resulted. Thus these studies have indicated that the target cell of the Lucké tumour herpesvirus is the *differentiating* kidney cell - *not* a differentiated cell, but a cell in the process of differentiating. It is during this period of ontogeny that immunological competence first emerges in these larval forms of the leopard frog (Marchalonis, 1971). Although proteins similar to the IgM and IgG antibodies of higher vertebrates are found in adult leopard frogs, IgG does not make an appearance until late metamorphic or young adult stages (see Fig. 2). Thus injection of LTHV into embryos or tadpoles results in a high incidence of renal tumours if the inoculation was performed before the emergence of immunological competence. Even when these inoculated larvae enter the later stages of metamorphosis or complete metamorphosis and emerge as young froglets, the tumour-bearing animals often retain the immunoglobulin pattern of a *young* tadpole (see Fig. 2D). Frequently, many of these tumour-bearing

FIG. 1. LATERAL VIEW OF STAGE 19 FROG EMBRYO

Lateral view of Stage 19 frog embryo removed from egg membranes. The longitudinal bulge (arrow) indicates the region of the developing pronephric kidney into which LTHV fractions were injected. (From Mizell, 1969)

FIG. 2. IMMUNOELECTROPHORESIS PATTERNS OF METAMORPHIC TADPOLE SERUM

Immunoelectrophoresis patterns of tadpole serum samples of increasing age during metamorphosis into frogs. Anode is at right. A: upper well - Metamorphic Stage XIV; lower well - Stage XVIII. B: upper well - Stage XIX; lower well - Stage XX. C: upper well - Stage XXI; lower well - Stage XXII. Note that immediately below the asterisk an IgG precipitin arc is lacking in the Stage XIV animal's serum. However, in the Stage XVIII tadpole (lower portion of A), IgG is detectable as a faint line. With increasing age (B and C) the IgG line becomes increasingly prominent. D: upper well, serum from a tumour-containing tadpole. This animal, a full two months older than the Stage XXII animal illustrated in C, merely has a faintly detectable IgG arc. (The lower well of C was prepared with serum from the Stage XIV tadpole in A for comparison). The antiserum used in these studies was produced in rabbits using adult frog serum.

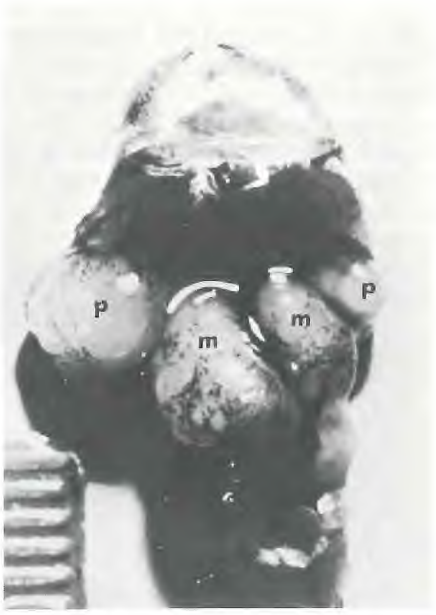


larvae are delayed in metamorphic development for appreciable periods of time (see Fig. 3).

Unlike the characteristic constant temperature of mammals and birds, northern *Rana pipiens* undergo seasonal changes in body temperature. Minimal temperatures are attained during the winter and spring (hibernation) only to rise once again with the onset of summer. Concomitantly, the spontaneous renal carcinoma of northern *R. pipiens* exists in two seasonal forms within the same populations of northern United States frogs. Neoplasms of hibernating frogs or frogs that have been maintained in the cold for several months ("winter" tumours) contain LTHV, whereas "summer" tumours from animals maintained at or near room temperature are devoid of

FIG. 3. METAMORPHIC TADPOLE WITH PRONEPHRIC AND MESONEPHRIC TUMOURS

Ventral internal view of a metamorphic tadpole with bilateral pronephric (p) and mesonephric (m) tumours. The animal was injected with 0.2 µl of LTHV at Embryo Stage 19 (see Fig. 1), seventeen weeks before this photo was taken. (From Mizell, 1969)

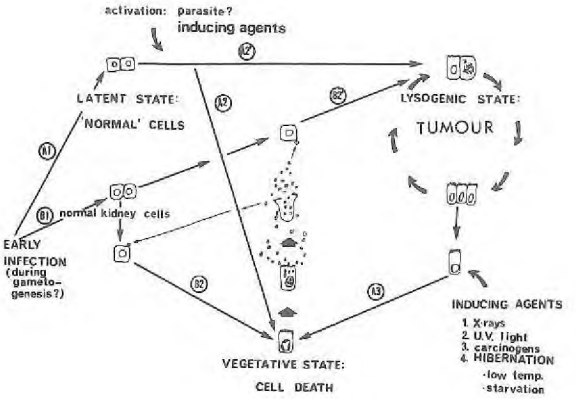


demonstrable virus. Because of these observations, we suggested, at the Frog Kidney Adenocarcinoma Conference of 1961, that the Lucké tumour agent existed in two recognizably different forms and that the Lucké agent might represent a metazoan counterpart of temperate bacteriophage (Mizell, 1961).

In 1965, we published a possible life cycle for the Lucké agent that was consistent with the then available evidence and postulated the existence of an integrated provirus state (Mizell & Zambernard, 1965) (see Fig. 4). The main feature of this scheme is that the virus can exist in three states within the frog kidney: (1) latent state (within "normal" cells); (2) lysogenic state (actively growing tumour); and (3) vegetative state (degenerating "tumour" with visible virus particles). The first two states represent integrated states: (1) the latent state is a highly integrated state, in which the presence of provirus is virtually unexpressed; (2) the lysogenic state is a less integrated state, in which the provirus gains expression within the genome of the host cell. Its presence in the host cell is reflected by features usually associated with typical, growing neoplasms - mitotic activity, cytoplasmic basophilia, etc. Seldom, if ever, can visible viral particles be seen. The non-integrated state is represented by (3) the vegetative

FIG. 4. POSTULATED LIFE CYCLE OF THE LUCKÉ TUMOUR HERPESVIRUS

Postulated life cycle of integrated and non-integrated states of the Lucké tumour herpesvirus. An essential feature of the scheme is that the virus can exist in three states within the frog kidney: (1) latent state, (2) lysogenic state and (3) vegetative state. The various pathways of the life cycle are indicated by arrows, and the two major routes are marked by letters that facilitate recognition of the sequence of events. (For further details, see text) (From Mizell & Zambernard, 1965)



state. It is during this state that visible viral particles can be demonstrated. Host cells contain intranuclear inclusions, lack mitotic activity, and manifest margination of chromatin material. Viral particles can be demonstrated by electron microscopy during this stage.

The various pathways of the life cycle are indicated by arrows in Fig. 4. The two major routes are marked by letters that facilitate recognition of the sequence of events (e.g., A1→ A2'→A3). Note that the latent state, upon activation, can follow one of two pathways: (i) A2' leading to the lysogenic state; or (ii) A2 leading to the vegetative state. A similar choice may be available to normal kidney cells via B2' or B2. In this same paper (Mizell & Zambernard, 1965), we pointed out that: "the definitive relationship of the suspected viral etiology would have to await the fulfillment of conditions similar to Koch's Postulates - requirements which had been used to establish proof of a causal relationship in bacterial diseases. In fact, Koch's first tenet, that the investigator should find the agent in every case of the disease, was obviously not fulfilled: some adenocarcinomas contained an appreciable number of virus particles, whereas other tumours were 'virus free'".

During the ensuing years, low

temperature was used as a probe to test this postulated life cycle and to gain insight into the tumour and the suspected provirus state. Virus production was induced by low-temperature treatment of the "virus-free" form of the frog adenocarcinoma. Virus recovered after this low-temperature treatment fulfilled the morphological requirements of herpesvirus (Mizell et al., 1968). Herpesvirus development was also induced in tumour explants of these "virus-free" primary tumours; in homologous host eye chambers (Mizell et al., 1968; Skinner & Mizell, 1972) and in heterologous host eye chambers (Mizell et al., 1969a). *In vitro* herpesvirus rescue from cultured explants of "summer-phase" kidney was attained in 1971 (Breidenbach et al.). All results indicated that the two naturally occurring seasonal forms of the Lucké tumour were temperature-related states of the same tumour and suggested that the "virus-free" tumour cells contained the LTHV genome in masked or latent form (Mizell, 1972). Direct evidence demonstrating the presence of LTHV genetic information in summer-phase tumour cells was recently obtained. By means of molecular hybridization, we were able to demonstrate that the so-called "virus-free" carcinoma transcribes Lucké tumour herpesvirus-specific RNA (Collard et al., 1973). Koch's first postulate was thereby fulfilled.

Although the Lucké tumour herpesvirus has not been cultivated in "pure" culture, we had previously succeeded in preparing zonal centrifuge-purified fractions of enveloped LTHV from productively infected winter tumours. When the enveloped LTHV zonal cut was injected into frog embryos, typical Lucké tumours developed (adjacent fractions lacked this particle and were not oncogenic). The resulting tumours, as expected, were found to be "virus-free". "Small portions of one of these pronephric tumours were transplanted to eye chambers of 20 frogs. Ten of these frogs were transferred to a low-temperature environment (7.5°C). After 11 weeks, virus was noted in the transplants of animals maintained at low temperature. Thus, a fraction of the frog LTHV purified by zonal centrifugation, when injected into an embryo, induced a pronephric renal adenocarcinoma which was initially "virus-free"; however, after low-temperature treatment, pronephric tumour parenchymal cells contained herpes-type virus" (Mizell et al., 1969b).

This 1969 experiment was recently repeated in another laboratory. Although a crude mitochondrial fraction (which was not further purified by zonal centrifugation) was used to inoculate the embryos, similar results were reported (Naegele et al., 1974).

Thus the Lucké tumour - a spontaneous tumour of an outbred population of animals - with its herpesvirus, external fertilization, and the ability to work at egg and embryo levels, and its readily available embryo-injection carcinoma-induction attributes, provides an ideal system whereby additional insight can be gained into the *mechanism* of herpesvirus tumour induction.

OPOSSUM "EMBRYOS" AND THE EPSTEIN-BARR VIRUS

Undoubtedly it would be beneficial to have a comparable mammalian model system to study the Epstein-Barr virus. The newborn opossum is uniquely suited for such investigations; many of the desirable characteristics of the LTHV-embryo system discussed in the previous section are present in

this mammal. Opossums are born at a very early state of development (see Fig. 5). Estimates have been made that indicate that, when born after $12\frac{3}{4}$ days of gestation, the newborn has attained a stage of development corresponding only to that of a 12-day mouse embryo or a 2-month human fetus. The progressive development of immunological competence in opossum pouch "embryos" has been studied by Rowlands & Dudley (1969), and the immaturity of the neonate immune system has been used to advantage in previous investigations in our laboratory (Mizell & Isaacs, 1970). In recent experiments, we have been able to predict the time of birth and thus obtain neonates as they leave the birth canal and before they reach the maternal pouch (see Fig. 6). With the aid of timed matings, we have been able to extend our studies to earlier embryonic stages by removing developing young from the uterus and culturing these embryos *in vitro* (New & Mizell, 1972).

Therefore we now have a mammalian system that indeed does approach the LTHV-amphibian embryo system.

The preliminary results obtained this year, after inoculation of pouch young with live human EBV-containing cells (kindly provided by M.A. Epstein) are briefly described below. Approximately nine months after inoculation, a female opossum (in whose pouch the litter of young were injected and reared) was sacrificed and autopsied. The autopsied animal

FIG. 5. LITTER OF NEWBORN OPOSSUM "EMBRYOS"

Litter of 10 newborn opossum "embryos" (less than 24 hours old) nursing in pouch of the anaesthetized mother. Scale in centimetres. (From Mizell & Isaacs, 1970)

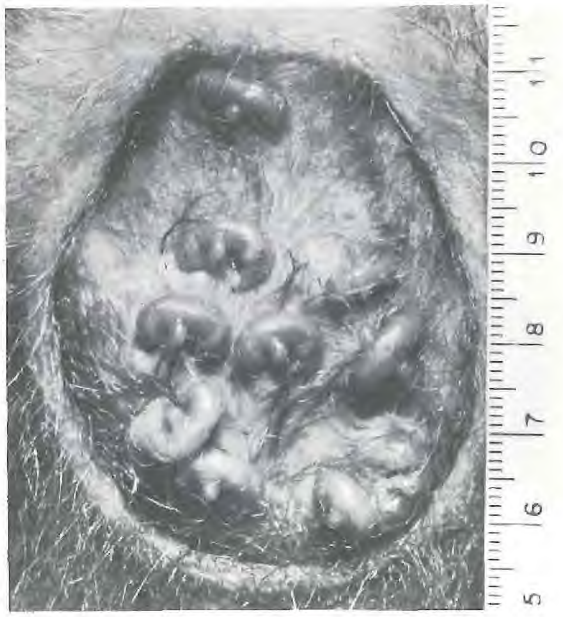
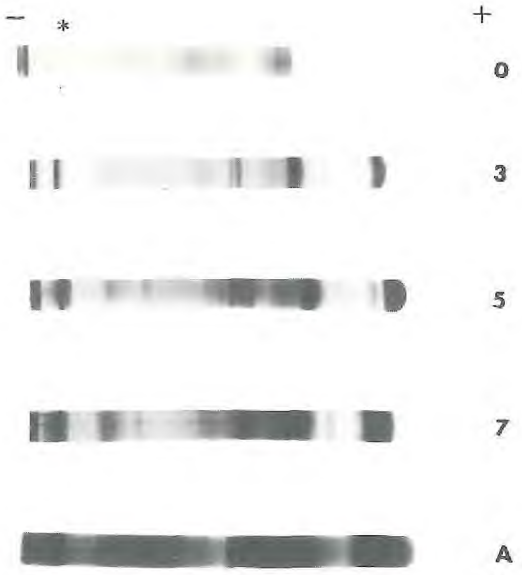


FIG. 6. ELECTROPHORETOGRAMS OF SERUM PROTEINS OF DEVELOPING OPOSSUM "EMBRYOS"

Acrylamide gel electrophoresis of the serum proteins of developing opossum "embryos". Numbers at the right of each gel indicate the age of the serum donor in days. The anode is at right; the gels were stained with Coomassie Brillant Blue. The top "0"-day gel was obtained by pooling the serum gathered from three neonates immediately after birth and before they entered the maternal pouch. Six µl of serum was used for the run. The 3-, 5- and 7-day pouch-young gels were prepared by using a similar amount of serum from each known aged "embryo". The adult serum pattern ("A") is shown in the bottom gel and was prepared in an identical manner as the other gels using one-third the volume of serum (2 µl). Since all gels were treated identically (including staining, destaining, etc.), the relative concentrations (or absence) of the various proteins are meaningful. Note the lack of banding in the newborn immunoglobulin area (under the asterisk). Also note that, during the first week of pouch life, the banding in this area, although much lighter than the immunoglobulin area of the adult, becomes increasingly evident.



contained a large tumour located on one of the lobes of its liver (see Fig. 7), and histological preparations revealed that the lesion was a malignant carcinoma (see Fig. 8). Surprisingly, although located on the liver, the lesions resembled nasopharyngeal carcinoma in morphology. These studies are still in progress, and injected young from this experiment are still being monitored for evidence of EBV antigens.

To date, no new lesions have occurred in this experiment and no EBV antigens have been detected in the young of this series. However, Drs Harald zur Hausen and Hans Wolf have tested the liver carcinoma for hybridization with EBV cRNA. Positive hybridization was obtained (the tests with appropriate controls were repeated three times), and it appears that the opossum tumour contains DNA sequences that are at least in part homologous to EBV DNA. (The opossum carcinoma cells contain approximately 10 EBV genome equivalents).[1] (See Table 1).

Obviously these experiments must be completed and additional experiments must be undertaken before any definite conclusions can be drawn. Nevertheless, a mammalian experimental system may now be available for use in closely studying the relationship between EBV and carcinoma induction.

[1] Unpublished data.

FIG. 7. OPOSSUM LIVER AND CARCINOMA

Macrophotograph of opossum liver and carcinoma. Scale in centimetres.

FIG. 8. HISTOLOGY OF OPOSSUM LIVER CARCINOMA

Histological section of opossum tumour seen in Fig. 7 showing the typical pattern of an undifferentiated carcinoma. (Haematoxylin and eosin) (x 200)

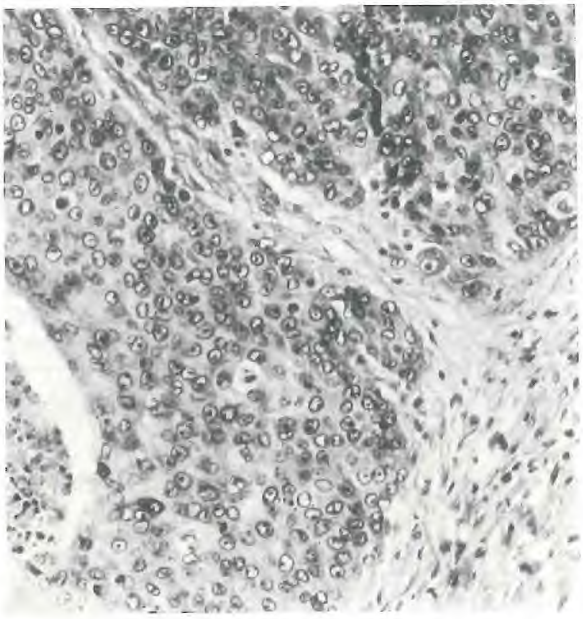


Table 1. Opossum liver carcinoma hybridization with EBV cRNA

Specimen	DNA hybridized (cpm)	Number of genome equivalents (approx.)
Opossum No. 1 (normal tissue)	39	0
Opossum No. 2 (normal tissue)	19	0
Opossum tumour	258	10
Raji	1 203	50
P3HR-1	3 303	139

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IMMUNOLOGICAL ASPECTS OF HERPESVIRUS INFECTIONS IN PRIMATES

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Knowledge concerning herpesvirus infections is important because these viruses are exceedingly ubiquitous, produce acute lytic infections, are involved in latency, and have been implicated in the development of neoplasia in man and other animals. Non-human primates, like man, respond to herpesviruses with a variety of clinical manifestations ranging from latent or subclinical infections to primary localized infections, generalized infections including involvement of the central nervous system, and finally to a highly fatal tumour. The mechanisms underlying these widely divergent clinical responses are essentially unknown. It is assumed that many biological and physiological factors are involved, one of which is undoubtedly immunological. Following the observation that four non-human primate species, namely the baboon (*Papio cynocephalus*), squirrel monkey (*Saimiri sciureus*), cebus monkey (*Cebus albifrons*) and marmoset (*Saguinus oedipus*), demonstrated graded responses to infection via the genital tract with herpes simplex virus type 2 (HSV-2) (Kalter et al., 1972), it was decided to determine the immune response of

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these simian species to herpesviruses and other antigens.

The bursal- and thymic-dependent immune systems, and the phagocytic system of these animals were evaluated by a variety of clinical immunological techniques (Harvey et al., 1974). It was found that none of the non-human primates involved in this study demonstrated an immune competence of the same magnitude as that observed in normal humans.

Baboons inoculated intravaginally with HSV-2 exhibited no overt evidence of infection or induction of *in vitro* specific sensitization to the virus, as exhibited by lymphocyte transformation. However, upon intravenous inoculation with the herpesvirus, there was depressed responsiveness to mitogens one week later, with recovery at two weeks, associated with a brisk specific blastogenic response to HSV antigens. In contrast, previously immunized cebus monkeys, after corneal inoculation with the same virus, showed depression of lymphocyte responsiveness to mitogens and specific herpesvirus antigens in subsequent weeks. Blood lymphocyte background counts increased in all animals, suggesting that the lymphocytes had been stimulated by systemic spread of viral antigens or perhaps had even been infected.

In herpesvirus saimiri (HVS) infections of its natural host (*S. sciureus*), no obvious disease occurs. Some animals display an elevation of (background) lymphocyte thymidine incorporation that may reflect lymphocyte infection. In the cebus monkey, a depressed response to mitogen stimulation coincident with markedly elevated background counts occasionally follows HVS infection. In contrast to this is the response to such infection seen in the marmoset. Lymphocytes from the leukaemic animals *in vitro* demonstrate an extremely high rate of thymidine incorporation as the lymphomatous disease progresses. As compared to this background stimulation, thymidine incorporation is actually less upon stimulation with phytohaemagglutinin (PHA), pokeweed mitogen (PWM) and HVS antigen, suggesting that the disseminated virus infection is stimulating lymphocytes to their maximum potential.

Additional studies were then carried out to ascertain, if possible, the immunological factors in the baboon, cebus and marmoset that are responsible for the defence of each animal species against infectious and oncogenic agents. As seen in Table 1, the blastogenic response of the three simian species to inactivated HSV-1 antigen demonstrates that the baboon was most responsive whereas little difference was noted between the cebus monkey and the marmoset. A comparison of the responsiveness of these three simians (and man) to a variety of immunological parameters (Table 2) emphasizes the differences between the species. As compared to man, these simians appear to be less competent; the baboon, however, manifested immune responsiveness most closely resembling that of man. This species displayed no abnormalities in circulating leukocytes or ability to form antibodies to certain antigens (*Salmonella* H and influenza A2). Lymphocyte stimulation *in vitro* by five antigens (HSV-1, mumps, *Candida*, tetanus and dinitrochlorobenzene (DNCB)) and Rebuck skin window (RSW) most closely approximated to the degree of response observed in humans. Cebus monkeys showed several abnormalities, including significant leukopenia, very low serum alpha-1 globulin levels, cutaneous anergy to mitogens and poor skin window response. The most marked abnormalities were seen in the marmoset: persistent leukocytosis, absent to poor antibody and T-lymphocyte responses to immunogens, weak responses to

phytohaemagglutinin *in vivo* and *in vitro*, with a uniquely greater response to pokeweed mitogen *in vitro*.

In conclusion, studies are under way in our laboratory aimed at elucidating the immunological mechanisms underlying the differences in susceptibility among primates to herpesvirus infections. Our results to date demonstrate that the baboon, which is relatively resistant to a variety of infectious and oncogenic agents, is reasonably competent immunologically. The cebus monkey and especially the marmoset appear to be deficient in several immunological parameters. The baboon appears to be similar to man from birth to maturation, whereas the marmoset resembles the newborn human in many immunological parameters and perhaps remains at that level. The cebus monkey is more or less intermediate in character.

Table 1. Blastogenic response to inactivated HSV-1

Species	Time (days)							
	0		14[a]		30		70	
	Absolute counts	BI[b]	Absolute counts	BI[b]	Absolute counts	BI[b]	Absolute counts	BI[b]
Baboon	9 971	4.1	4 823	4.9	10 775	18.6	20 309	24.5
Cebus	9 391	3.1	1 350	3.5	15 899	5.9	5 379	3.8
Marmoset	1 063	2.2	1 556	1.3	1 656	7.1	5 428	5.1

[a] Time of first immunization with HSV; second immunization on day 30, and third immunization on day 60.

[b] $$\text{Blastogenic index} = \frac{\text{absolute counts per minute}}{\text{resting counts per minute.}}$$

Table 2. Comparative immunological parameters in primates

Species	T system			B system				Phagocytic-complement system		
	E rosettes[a] (%)	BI (PHA)	Skin delayed hypersensitivity[b]	EAC rosettes[c] (%)	Antibody response (titres)		BI[d] (PWM)	Skin window		CH_{50}[e] (units/ml)
					Salmonella (H antigen)	Influenza (A2)		3 hours	22 hours	
Baboon	32	23	10	26	300	160	4	± PMNs[f]	Clumps with 15-40% macrophages	17
Cebus	53	14	20	41	200	160	7	± PMNs	5-15% macrophages	69
Marmoset	32	8	3	40	80	40	12	± PMNs	5-15% macrophages	15
Man[g]	40-70	60-250	50-95	15-35	ND[h]	ND	25-75	2-3 + PMNs	Clumps with 50-80% macrophages	43

[a] Rosette formation of lymphocytes with sheep red blood cells (SRBC).

[b] Percentage of animals responding with >5 mm of induration at 48 hours to battery of five immunogens.

[c] Rosette formation of lymphocytes with SRBC and amboceptor.

[d] Reflects both B and T responses.

[e] One 50% haemolytic unit (CH_{50}) of serum complement was defined as the volume of serum in ml that lysed 50% of 5×10^8 EA in 7.5 ml of reaction medium after incubation at 37°C for 30 minutes.

[f] Polymorphonuclear leukocytes.

[g] Normal range of values.

[h] Not determined.

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DISCUSSION SUMMARY

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Experiments related to reactivation of latent herpesviruses and to the mechanism of latency were considered to be of great interest. It was pointed out that IgG antibodies may play a role in the prevention of herpesvirus reactivation though it seemed unlikely that their presence was the only prerequisite for virus repression. The herpesvirus of Marek's disease, for example, can grow perfectly well in the presence of IgG antibodies. In addition, it has been reported that reactivation of herpes simplex virus (HSV) in explanted ganglia takes place in the presence of IgG antibodies.

Concerning the latency of HSV DNA in mice, as demonstrated by *in situ* hybridization, it was stated that viral DNA could be detected in neurons of trigeminal ganglia of practically every mouse infected by herpes simplex virus. Apart from a few positive neurons, a majority that appears to be negative for viral DNA is always present. DNA replication after explantation would seem to explain why positive cells are found in these experiments; this is indicated by the heavy labelling observed in the nuclei of the positive neurons.

This led to the question whether it is possible to demonstrate the presence of the DNA of a latent herpesvirus before its reactivation. This is indeed the case: it was reported that, in frozen sections of trigeminal ganglia of a rabbit latently infected by HSV-1, viral DNA was demonstrated by *in situ* hybridization. The label was observed exclusively in the nuclei of a small number of ganglion cells.

A small percentage of cells of a lymphoblastoid cell line, established from a chicken with Marek's disease, contain immature herpesvirus particles; these are mainly in the nucleus, but some are also present in the cytoplasm. The question was raised whether some of the cytoplasmic

particles might not be Type-C viruses and whether it has been possible to show by other methods that the cell line concerned is free from avian leukosis viruses. Exclusion of this possibility was based on the observation that the cells concerned at least do not show the presence of gs-antigens.

The successful establishment of cell lines from chickens with Marek's disease was related to the increase in incubation temperature from 37°C to 41°C.

Finally, the possible induction of tumours by EBV in opossums aroused some interest. Tumours have not yet been induced in these animals by injecting viral preparations. The only tumour obtained thus far originated in an animal that had been in contact with infected newborns. The question whether the tumours observed after injecting EB-3 cells consist of human or of opossum cells has still not been answered.

TRANSFORMATION IN VIVO

TRANSFORMATION IN VIVO – A REVIEW

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As recently as a decade ago, there was almost no evidence to suggest that herpesviruses might be involved in the causation of tumours. It had been known at that time for some years that the cells of the seemingly infectious Lucké carcinoma of the leopard frog kidney (Lucké, 1934, 1938) contained a herpesvirus (Fawcett, 1956) but there was considerable confusion as to its significance when other, quite different, cytoplasmic, DNA viruses were isolated from the tumour material. Ten years ago was also the time at which Epstein-Barr virus (EBV) was first detected in cultured lymphoblasts from Burkitt's lymphomas (Epstein et al., 1964) but, apart from the circumstance that the cultured material came from tumour biopsies, there was little to connect this virus, either, with oncogenesis; indeed, the absence of oncogenic herpesviruses at that time was a significant factor in limiting the importance attributed to EBV when it was first discovered (Epstein et al., 1964).

The mere fact that it is now hardly necessary to stress how important herpesviruses have become in relation to oncogenesis speaks volumes for the immense progress that has been made in the last decade. The scale of this progress was reflected in the outstandingly successful first symposium on Oncogenesis and Herpesviruses held in Cambridge in June 1971, and there is little doubt that the publication resulting from that meeting (Biggs et al., 1972) has proved a most important and valuable contribution to the literature of viral oncology. The many new developments since the Cambridge meeting clearly justify the holding of a further International Symposium and the complexity of these developments rapidly becomes evident when the present programme is examined.

MAJOR DEVELOPMENTS IN HERPESVIRUS ONCOGENESIS

In recent years, the confusion over the role of the Lucké herpesvirus has been resolved and it has been clearly shown to be the etiological agent of this naturally occurring tumour (Mizell et al., 1969; Naegele et al., 1974). Marek's disease, a long recognized highly contagious malignant lymphoid tumour of chickens (Marek, 1907) has finally been shown

to be due to a herpesvirus (Churchill & Biggs, 1967; Solomon et al., 1968; Epstein et al., 1968) and is now readily controlled by a viral vaccine (Churchill et al., 1969; Okazaki et al., 1970). In addition, a new herpesvirus isolated from cultured kidney cells of normal squirrel monkeys, although non-pathogenic in its natural host has been found to induce rapidly fatal malignant lymphomas on inoculation into owl monkeys, cotton-topped marmosets, and rabbits (Meléndez et al., 1969; Daniel et al., 1970). Further studies have shown that this virus, herpesvirus saimiri, both causes leukaemia and is carcinogenic in several additional species of subhuman primates (Meléndez et al., 1971; Hunt & Meléndez, 1972). It is thus not only an oncogenic herpesvirus affecting animals phylogenetically related to man, but also the first DNA virus known to be leukaemogenic. Since the discovery of herpesvirus saimiri, a further oncogenic primate herpesvirus has been found (Meléndez et al., 1972). And contemporaneously with these very important discoveries of oncogenic animal herpesviruses, an immense body of information has been accumulated on EBV strongly suggesting that it is a possible human tumour virus (Epstein & Achong, 1973a; Klein, 1973; Henle & Henle, 1973), whilst herpes simplex virus type 2 has likewise been implicated, though much less strikingly, in human neoplasia (Rawls et al., 1968; Nahmias et al., 1969).

FORMS OF HERPESVIRUS INFECTION IN GENERAL

It has, of course, long been recognized that herpesviruses can and do initiate straight forward productive infections with replication of viral progeny (Table 1, item 1) and it is evident that this type of infection always leads to the death of the affected cells (Roizman, 1972). It is this cell death that underlies the pathological lesions caused by herpesviruses *in vivo* and cell lysis in tissue culture. In addition, it has also become clear over the years that herpesviruses can also initiate non-productive, non-cytocidal infections in which the viral genetic information remains latent and quite unexpressed within the cell, but liable to subsequent activation to a productive cycle with the inevitable cell lysis this entails (Table 1, item 2a). This process is found with herpes simplex virus in experimental animal models and in natural infection in man (Stevens et al., 1972; Stevens & Cook, 1973; Cook et al., 1974; Bastian et al., 1972) and there is reason to believe that varicella-zoster virus behaves in exactly the same way (Bastian et al., 1974). Although herpes simplex virus and varicella virus apparently adopt this non-productive, latent type of infection exclusively in nerve cells, it would seem that cytomegalovirus, for example, may exhibit a similar type of infection in human lymphoid cells (Doisi et al., 1969; Fiala et al.[1]).

With oncogenic herpesviruses, there is yet another type of infection; this too, is a non-productive infection, but unlike that just described it is a non-productive infection in which the viral genome is expressed not only by transforming cells into malignant tumour cells, but also by inducing at the same time the formation of virus-determined neo-antigens (Table 1, item 2b). Two important points require to be emphasized: (i) non-productive infections with expression of the viral genome to bring about malignant transformation can, like non-productive infections without expression of the viral genome, be activated to a produc-

[1] See p.109.

Table 1. Forms of infection of cells by herpesviruses

1. Productive - Virus replication leading to cell death
2. Non-productive - Virus genome present
(a) *Unexpressed* but often activated to a productive cycle
(b) *Expressed* in malignant transformation but can be activated to a productive cycle

tive cycle; and (ii) a combination of two or all of these various forms of infection can be, and frequently is, present in the same individual at the same time. In this connection, the herpesviruses of Marek's disease and the Lucké frog carcinoma deserve special attention because they provide important examples of these cell-virus interactions. In the Marek's disease-infected bird, productive infection with release of virus particles takes place in the feather follicle, and this plays a large part in the horizontal transmission of infection, whilst at the same time, the cells of tumours caused by the virus show a non-productive infection with the viral genome expressed in malignant transformation. It is interesting to note that, when such tumour cells are put into tissue culture, a productive infection is induced in some of them and virus structural antigens and virus particles appear (Ahmed et al., 1970; Kato & Akiyama, 1973; Powell et al., 1974).

A somewhat different example of these various types of herpesvirus infection is provided by the Lucké virus in its relationship to the tumour cells. Here we get a dramatic illustration of the way in which infection can switch from productive to non-productive cycles under natural conditions. Thus, during warm weather, there is a non-productive infection of the tumour cells with the virus genome expressed to give malignant transformation: summer tumours are free of virus particles, grow rapidly, metastasize, and often lead to death of the host. But in those frogs that survive into the autumn and winter, the low ambient temperature activates the virus to a productive cycle with virus replication leading to cell death and consequent regression of tumour growth (Rafferty, 1964; McKinnel & Ellis, 1972). With the return of warm weather in the spring and summer, the productive lytic cycle is again switched off and the virus genome expresses itself, as before, in summer tumours, by malignant transformation with the promotion of rapid tumour growth (McKinnell & Ellis, 1972). It is of great interest that the type of virus infection can be changed experimentally at will in either direction merely by manipulation

of the temperature at which tumour-bearing frogs are kept in the laboratory (Zambernard & Vatter, 1966; McKinnell & Ellis, 1972).

FORMS OF INFECTION BY PUTATIVE HUMAN ONCOGENIC HERPESVIRUSES

As a result of extensive sero-epidemiological surveys, it has been recognized for some years that there is a striking association between infection with herpes simplex virus type 2 and cervical neoplasia in women (using this term to include dysplasia, carcinoma *in situ*, and frank invasive carcinoma). For some time there was doubt as to whether the association reflected a causal relationship or merely resulted from the coincidental liability to a high incidence of infection with herpes simplex virus type 2 and to cervical neoplasia in those women who followed a particular sexually permissive life-style; recent studies appear to have eliminated the latter alternative (Adam et al., 1974), leaving the possible causal relationship between the virus and the tumour urgently in need of confirmation by evidence of a more direct kind.

As to the form of infection occurring with herpes simplex virus type 2, it is quite clear that productive infections are very frequent in the cervix and manifest themselves not only in those relatively rare cases where overt herpetic genital lesions are found, but also in the vastly greater number of seropositive individuals in whom evidence for virus production in the cervix is obtained in the course of cervical cytology screening programmes (Naib et al., 1966). In addition, it has recently been shown that herpes simplex virus type 2 causes non-productive, unexpressed infections (Table 1, item 2a) in sacral ganglia 3 and 4 (Baringer[1]); however, if the virus is indeed causally related to cervical cancer it must, by analogy with all we know of DNA tumour viruses in general and oncogenic herpesviruses in particular, surely be capable of causing a non-productive infection in the tumour cells with expression of the viral genome to bring about the malignant transformation responsible for the tumour (Table 1, item 2b). Investigations aimed at determining whether virus-determined neo-antigens are present in such tumours have resulted so far only in somewhat equivocal findings (Royston & Aurelian, 1970). But much more telling biochemical evidence that the virus is not causally related to the tumour has recently been obtained, since nucleic acid hybridization tests have failed to reveal the presence of viral genetic information in the tumour cells (zur Hausen et al., 1974), thus suggesting that a non-productive infection with expression of the viral genome to bring about malignant transformation is not present.

Understanding the various forms of infection possible with herpesviruses (Table 1) is of even greater significance in relation to the problem of a causative role for EBV in Burkitt's lymphoma and nasopharyngeal carcinoma. A steadily growing body of solid evidence links this virus ever closer with these two tumours (Epstein & Achong, 1973a; Klein, 1973; Henle & Henle, 1973), but certain difficulties remain. Thus, the virus is widespread in human populations and infects huge numbers of individuals who show no evidence of Burkitt's lymphoma or nasopharyngeal carcinoma, and it has sometimes been regarded as an objection to a carcinogenic role for the virus that transformed lymphoblastoid

[1] See p.74.

lines carrying EBV can be readily established *in vitro*, not only from biopsies of Burkitt's lymphomas, but also from the peripheral lymphocytes of such seropositive normal individuals. However, there are now reasons for believing that this objection is apparent rather than real, and that the conceptual difficulty involved rests only on a failure to understand the various possible forms of herpesvirus infection. It is clear that infection by EBV results from horizontal transmission and is widespread throughout the world (see Epstein & Achong, 1973b). Very young infants frequently have transient maternally-derived antibodies that disappear. Thereafter, the proportion of antibody-positive individuals increases with age as more and more become infected, so that by late adolescence the incidence has reached the region of 80% and persists close to this level in subsequent age-groups, although the incidence level is raised or lowered by standards of living. Thus, most primary infections with EBV occur in childhood and early adolescence, and are accompanied by permanent seroconversion, total immunity to infectious mononucleosis, and lifelong harbouring of the virus. This harbouring is manifested in two ways: (i) by productive infection (Table 1, item 1) of cells somewhere in the oropharynx with release of infectious virus particles into the buccal fluid (Gerber et al., 1972; Miller et al., 1973) (such infected fluids play a most important part in horizontal transmission of the virus); and (ii) by the non-productive infection of peripheral lymphoid cells, which therefore contain the unexpressed viral genome (Table 1, item 2a). In this young age-group, the vast majority of people undergoing primary infection show no recognizable illness but an occasional individual may react with the clinical manifestations of infectious mononucleosis.

In late adolescence and young adult life, primary infection also leads to seroconversion and harbouring of the virus but, in contrast, one-half to two-thirds of those who become infected in this age-group develop infectious mononucleosis and only a substantial minority remains without clinical manifestations. The possible reasons why primary infection in young adults is so often accompanied by infectious mononucleosis are not relevant to the problem of malignant transformation, but a recent discussion of them has been presented elsewhere (Epstein & Achong, 1973b). What is relevant is the state of the virus in normal seropositive individuals or in individuals who are seropositive after an attack of infectious mononucleosis, in contrast to the state of the virus in an individual with Burkitt's lymphoma.

All seropositive people, irrespective of whether they are completely normal or have a history of infectious mononucleosis, must clearly have in common a low-grade productive infection somewhere in the oropharynx with liberation of infectious virus particles; in addition, they would all also appear to harbour the virus as a non-productive infection in their peripheral lymphocytes. It is this virus genetic material that allows continuous lymphoid lines to be established in culture from the peripheral lymphocytes of seropositive people, irrespective of whether their primary infections were clinically manifest or not. But the fundamental question concerns the form of non-productive infection shown by these genome-containing peripheral lymphocytes of seropositive people. Are these cells malignantly transformed *in vivo* (Table 1, item 2b) and prevented from forming tumours by some type of immunological surveillance, in which case cell lines would be established from the peri-

pheral lymphocytes of seropositive people by the direct outgrowth of these already transformed malignant cells, or are these cells infected in the unexpressed form of non-productive infection (Table 1, item 2a) in an analogous manner to ganglia cells infected by herpes simplex or varicella? Recent observations (Rickinson et al., 1974; Jarvis et al., 1974) strongly indicate that the second alternative is the correct one, that the genome-containing peripheral lymphocytes are indeed infected by EBV in the non-productive, unexpressed form (Table 1, item 2a), and that cell lines are established from such cells, not by direct outgrowth, but by a different and more complicated two-step process occurring in the culture vessels; these experiments do not require elaboration here since they are fully described later[1].

With the Burkitt's tumour cell, the situation is different. Here, too, the cell contains EBV DNA but immunofluorescence studies on membrane antigens and EBV-determined nuclear antigen (EBNA) (Klein, 1972; Reedman & Klein, 1973) indicate that the viral genome is expressed to give viral-determined neo-antigens and thus presumably, by analogy with animal systems, is present in the form of non-productive infection in which it is also expressed to transform cells into tumour cells *in vivo*. This concept of transformation by the virus *in vivo* is supported by the fact that, when biopsies of Burkitt's lymphomas are cultured *in vitro*, cell lines are established by the outgrowth of the tumour cells themselves (Nadkarni et al., 1969; Fialkow et al., 1970; Manolov & Manolova, 1972), a mechanism of origin clearly different from that seen where lines are obtained from EBV genome-containing peripheral lymphoid cells of seropositive normal individuals (Rickinson et al., 1974). The demonstration that there are these two separate mechanisms for establishing cell lines in culture, one relating to tumour cells and the other to viral genome-containing cells from individuals free of malignant disease, has removed an apparent difficulty in accepting that EBV might be a carcinogenic agent responsible for the malignant transformation of Burkitt's lymphoma, since it shows that EBV can cause non-reproductive infections without expression of the viral genome (Table 1, item 2a) in non-malignant lymphoid cells analogous to those seen with herpes simplex virus, varicella virus, and cytomegalovirus, as well as the non-productive infection with expression of the genome (Table 1, item 2b) that appears to occur in the malignant cells of Burkitt's tumour.

As to the situation with EBV and nasopharyngeal carcinoma, although close links between the virus and this human malignancy have been established, the nature of the association is less easy to understand. Biochemical studies (Wolf et al., 1973) supplemented recently by immunological evidence (Klein et al., 1974), clearly show that the viral DNA is present in the malignant epithelial cells of this tumour, apparently with expression of the viral genome (Table 1, item 2b), suggesting that here, too, EBV may have a causative role. But how this unique example of EBV infecting a non-lymphoid cell comes about, and how it relates to the many peculiarities of nasopharyngeal carcinoma, remain to be explained.

EBV AND THE INDUCTION OF TUMOURS IN PRIMATES

In addition to all the indirect circumstantial evidence suggesting that EBV is involved in the malignant

[1] See p.175.

transformation causing Burkitt's lymphoma and nasopharyngeal carcinoma, experimental studies with EBV in South American primates have demonstrated that the virus can certainly be carcinogenic *in vivo* and have thus strengthened the likelihood that it is so in man. Lymphoid tumours have recently been observed in both cotton-topped marmosets (Shope et al., 1973 and owl monkeys (Epstein et al., 1973a) following inoculation of EBV in two separate laboratories, and confirmation of the marmoset results has already been forthcoming (Falk et al., 1974). With each of these hosts, continuous lymphoblastoid cell lines have grown out in culture from tumour material with production of both viral antigens and EBV particles (Shope et al., 1973; Epstein et al., 1973b), just as happens with cell lines from Burkitt's lymphomas, and the viral genome itself has been detected in the owl monkey cells (Epstein et al., 1975).

It would seem that, in the marmoset and owl monkey, both herpesvirus saimiri and EBV can initiate non-productive infections with expression of the viral genome to cause the cellular changes of malignant transformation (Table 1, item 2b); the evidence for this appears good, but final proof requires further confirmatory experiments. Since the two species of South American primates involved are just those most susceptible to tumour induction by herpesvirus saimiri, it is not surprising therefore that they should appear to react to EBV in a similar way with the production of tumours.

CONCLUSIONS

All in all, an understanding of the different forms of infection possible with herpesviruses provides a clear explanation of the various cell-virus interactions that occur with these agents and that, if not clearly distinguished, cause conceptual confusion. With each year that goes by, understanding of the biological behaviour of oncogenic herpesviruses increases and it becomes more and more likely that some of these viruses may have a causative role in at least some human tumours.

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PREVENTION OF EPSTEIN-BARR VIRUS (EBV)-INDUCED INFECTION IN COTTON-TOPPED MARMOSETS BY HUMAN SERUM CONTAINING EBV NEUTRALIZING ACTIVITY

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Epstein-Barr virus (EBV) is intimately associated with three human lymphoproliferative diseases - infectious mononucleosis, Burkitt's lymphoma and nasopharyngeal carcinoma. Although there is extensive virological, molecular, and immuno-epidemiological evidence in favour of EBV as the causative agent (for review see Miller, 1974), the lymphoproliferative disease in man has not been experimentally reproduced.

Because the direct experimental EBV induction of lymphoproliferative diseases in man is impossible for ethical reasons, attempts have been made to produce disease in animals. Two general approaches have been taken. In the first, living cells carrying EBV have been experimentally inoculated. Hetero-transplanted human cells will produce a fatal lymphoproliferative disease in the immunodeficient rodent but are rejected by the immunointact rodent (Adams et al., 1973). In contrast, autochthonous EBV-converted cells have produced malignant lymphoma in one of four inoculated cotton-topped marmosets (Shope et al., 1973), but similar experiments in five gibbons (Werner et al., 1972) and four squirrel monkeys (Shope & Miller, 1973) have not resulted in tumours. In the second approach, cell-free EBV has been experimentally inoculated. Although the material has failed to produce disease in rhesus monkeys, gibbons and squirrel monkeys (Miller et al., 1974; Werner et al., 1972), it has induced lymphoproliferative change (including lymphoma) in cotton-topped marmosets (Shope et al., 1973; Miller et al., 1974) and owl monkeys (Epstein et al., 1973).

Of 17 cotton-topped marmosets given between 10^4 and 10^5 transforming units of EBV, five developed malignant lymphoma, three lymphoid hyperplasia, seven inapparent infection (antibody response only), and two failed to develop antibodies (Miller et al., 1974). In this report, neutralization experiments, carried out to establish that EBV is essential to the induction of the

experimental lymphoproliferative disease in cotton-topped marmosets, will be described.

MATERIALS AND METHODS

Marmosets

Wild-reared cotton-topped marmosets (*Saguinus oedipus*), of mixed age and sex, were purchased from a commercial dealer who obtained the animals in Colombia, South America. The blood of all the animals contained parasites (trypanosomes and microfilariae).

Virus

A single stock of cell-free EBV was prepared in marmoset cells (line B-95-8) transformed by an infectious mononucleosis strain of EBV (designated 883L) (Miller et al., 1972). Aliquots of the stock virus were stored frozen at -70°C until used. The infectivity of virus in the stock and in various inocula was measured by an end-point dilution assay utilizing long-term transformation of leukocytes from human neonatal cord blood cocultivated with human placental fibroblasts (HPF) (Grogan et al., 1970). All cultures were grown in RPMI 1640 medium with 20% heat-inactivated fetal bovine serum, plus penicillin, streptomycin and fungisone. Leukocyte cultures were fed twice weekly.

Serum

Reference sera from two healthy adult males, one (designated WP) with EBV antibody, the other (designated RB) without, were obtained by plasmapheresis. Serum was separated aseptically from the clot and stored frozen at -20°C until use. Sera were examined for antibody to EBV capsids (VCA), using P3HR-1 cells as antigen (Henle & Henle, 1966), nuclear antigen (EBNA), using Raji cells (Reedman & Klein, 1973), crude complement-fixing antigens (CF) derived from P3HR-1 cell lysates (Miller et al., 1972), and neutralization of infectious virus (NT), using B95-8 cell-free virus (Miller et al., 1971). Sera from the experimental animals obtained before inoculation and serially afterwards were examined for VCA antibody by indirect immunofluorescence using P3HR-1 cells as the test antigen.

Neutralization

Undiluted stock virus was combined with human serum (heat-inactivated at 60°C for 20 minutes) in equal (1 ml) volumes, mixed and held at 37°C (water) for 1 hour. Following incubation, the virus-serum mixture was held on ice until inoculated (not more than 60 minutes). Aliquots of the mixture, as well as stock virus simultaneously incubated without serum, were frozen at -90°C for subsequent infectivity titrations.

Inoculation of animals

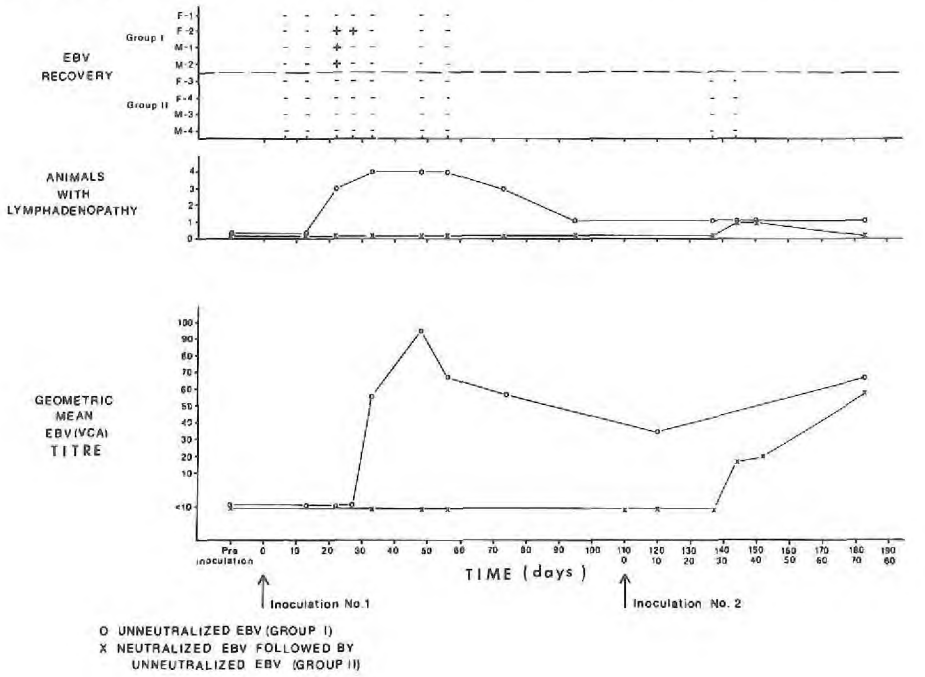
During the experiment, two sets of inoculations were performed. In the first set, four animals received stock virus mixed with EBV antibody-deficient serum (group I in Fig. 1), and four animals received stock virus mixed with EBV antibody-containing serum (group II in Fig. 1). Each animal received the simultaneous inoculation of 1 ml of mixture by the intravenous, 0.5 ml by the subcutaneous, and 0.5 ml by the intraperitoneal route. In the second set, performed sixteen weeks after the initial inoculation, animals in group II, which had received "neutralized" material, were challenged with the EBV serum antibody-deficient inoculum. Inoculations were given by the same routes.

EBV recovery

Peripheral blood, collected in

FIG. 1. SEQUENCE OF EVENTS IN COTTON-TOPPED MARMOSETS FOLLOWING INOCULATION OF EBV

The first inoculation consisted of unneutralized EBV in group I (indicated by "O") and neutralized EBV in group II (indicated by "X"). The second inoculation consisted of unneutralized EBV and was given to group II animals only. Successful recovery of EBV from peripheral blood cultures is indicated by "+", failures by "-".



heparin, was serially obtained following inoculation. Lymphocytes were separated on Ficoll-Hypaque gradients (Böyum, 1968), washed three times, adjusted to 5 x 10^5 cells/ml, placed into culture tubes with "feeder" cells (HPF) and incubated at 35°C in a 5% carbon dioxide and air atmosphere. When transformation of cultures became evident, cell stocks were grown in the absence of HPF until tested for the presence of EBV-specific antigens. Harvests of supernatant fluids at seven days were frozen and thawed three times, passed through a 0.8-µm Millipore filter, and then tested for the presence of infectious virus by long-term transformation of leukocytes from human neonatal cord blood. Cells were examined for the presence of EBNA using the reference sera described.

RESULTS

The four animals that received virus mixed with human EBV antibody-deficient serum (group I in Fig. 1)

developed disease characterized by intra-abdominal lymphadenopathy (by palpation), first detected in three animals 22 days following inoculation and in the fourth animal by 33 days. Nodules, sometimes discrete and solitary, but more often five or six in chains, increased in size to no larger than 1 x 0.5 cm, then decreased until they disappeared in three of four animals by 95 days after inoculation. Small nodules have persisted in one animal without change for the last three months. No intra-abdominal nodules were palpated in the four animals that received virus mixed with human serum containing EBV antibody (group II).

EBV was recovered from the peripheral lymphocytes of three of four marmosets (in group I) 22 days following inoculation and one of four on the 27th day. Thereafter no virus was found in peripheral lymphocytes. Lymphocytes obtained from animals in group II failed to yield virus.

Antibody to viral capsids first appeared in all animals in group I on day 33 and has persisted for 4.5 months. Antibody titres peaked at 48 days (3 at 1:80, 1 at 1:160). No antibody was detected in the sera of group II animals.

Serum WP contains VCA, EBNA, and CF antibody (see Table 1). After one hour of incubation at 37°C with the virus stock that contains approximately 500 000 leukocyte transforming units (TU), WP serum completely and repeatedly prevented transformation. Incubation at room temperature for 5 minutes also resulted in complete inhibition of transformation. In contrast, virus titration after incubation for 1 hour with antibody-negative RB serum was virtually the same as the titration of virus incubated for one hour without human serum ($10^{4 \cdot 3}TU_{50}$/ml and $10^{4 \cdot 25}TU_{50}$/ml respectively).

Thus, following inoculation of $10^{4 \cdot 3}TU_{50}$ of unneutralized EBV, animals simultaneously developed lymphadenitis (intra-abdominal) and viraemia. Within two weeks of the viraemia, all animals had EBV-VCA antibody and virus could no longer be isolated from cultures of peripheral lymphocytes. The animals given neutralized EBV failed to display adenopathy, did not have viraemia and did not produce antibody.

The animals given neutralized EBV were challenged with unneutralized

Table 1. EBV antibody findings in the human reference sera used in the neutralization experiments[a]

Serum	VCA	EBNA	CF	Nt
WP	1:640	Present	1:640	$\geq 10^{4 \cdot 3}TU_{50}$
RB	<1:2.5	<1:2.5	<1:4	No virus inhibition

[a] Antibody titre to viral capsid antigen (VCA), nuclear antigen (EBNA), and crude complement-fixing antigen (CF), and inhibition of virus by undiluted serum (Nt) (TU = transforming units).

virus 16 weeks later to determine if they could be infected. The same parameters were measured. Only one animal displayed intra-abdominal adenopathy (chain of small nodes), none had detectable circulating virus but all had developed EBV-VCA antibody. It is interesting that the time of appearance of antibody was not indicative of an anamnestic response.

DISCUSSION

The results of these experiments support the previous observations that EBV causes the lymphoproliferative disease that occurs in cotton-topped marmosets following inoculation with an infectious mononucleosis strain of virus (Shope et al., 1973). The evidence can be summarized as follows. First, only animals given EBV, unneutralized by human serum antibodies, developed signs of disease. All animals developed lymphadenopathy, all developed EBV-VCA antibody and three of four yielded EBV in cultures of peripheral lymphocytes. Second, disease developed in a progressive fashion, initially with the simultaneous appearance of viraemia and lymphadenopathy, followed by the appearance of EBV-specific antibody, disappearance of viraemia and gradual subsidence of adenopathy. Finally, animals that received neutralized EBV failed to develop lymphadenopathy, EBV-VCA antibody or viraemia.

The cotton-topped marmoset model shares some characteristics in common with infectious mononucleosis (IM) in man, with the following notable exceptions. Viraemia in IM occurs during the acute illness and well into convalescence, persisting in some for life (Diehl et al., 1968). In contrast, the marmoset viraemia disappeared with the appearance of EBV-VCA antibody. The distribution of lymphoid tissue hypertrophy in man during IM includes peripheral lymph-nodes as well as the spleen; marmosets appear to develop central lymphadenopathy without involvement of the spleen. Most EBV-induced IM in patients over 10 years old is accompanied by characteristic sheep cell heterophile agglutinin responses; the 17 marmosets previously inoculated by Miller and Shope (Miller et al., 1974) plus the eight in the present experiment have not developed heterophile agglutinin responses. Falk et al. (1974) have reported heterophile agglutinins in one cotton-topped marmoset inoculated with autochthonous EBV-infected lymphoblasts; the event is, at least, rare. Finally, IM is associated with the appearance of atypical lymphocytes. The marmosets in previous experiments have not shown remarkable changes in peripheral blood leukocyte morphology or concentration (Shope et al., 1973). In the present experiment, animals inoculated with unneutralized virus developed an absolute lymphocytosis with the appearance of large, immature lymphocytic cells. However, characterization is incomplete at present and awaits further evaluation.

Challenge of marmosets with unneutralized EBV four months following the initial inoculation of neutralized virus was not followed by viraemia and significant adenopathy, although all animals developed EBV-VCA antibody responses. The design of the experiment does not permit conclusive explanation. Perhaps the previous inoculation with inactivated antigen primed the host immune responsiveness, preventing replication of the subsequently inoculated EBV. If so, why did antibody fail to appear until 34 days following the second inoculation? Analysis of serum IgM antibody responses will perhaps be revealing.

The absence of progression of the lymphoproliferative disease to malignant lymphoma is also puzzling. Factors

such as dose and route of inoculation, immune competence of the recipient animal, and changes in the strain of virus are currently under investigation.

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RESPONSE OF MARMOSETS TO EXPERIMENTAL INFECTION WITH EPSTEIN-BARR VIRUS

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Epstein-Barr virus (EBV) has been proved to be the etiological agent of heterophil-positive infectious mononucleosis, is most probably the main cause of most Burkitt's lymphomas and may also be responsible for nasopharyngeal carcinoma (for review, see Klein, 1973). The association of EBV with neoplastic disease is based on sero-epidemiological studies, the demonstration of the EBV genome in tumour cells and the ability of EBV to transform human and simian lymphocytes *in vitro* (Werner et al., 1972; Miller et al., 1972; Falk et al., 1974b). The recent induction of lymphoproliferative diseases with EBV in non-human primates (Shope et al., 1973; Epstein et al., 1973; Falk et al., 1974a; zur Hausen[1]) further proves the oncogenic potential of EBV. This report describes the regular induction of lymphomas in some species of marmoset monkeys with the B95-8 strain of EBV and the development of lymphoblastoid cell lines from the inoculated animals.

[1] Personal communication.

MATERIALS AND METHODS

Virus and lymphoblastoid cell lines

Concentrated EBV from P3HR-1 cell cultures was supplied by Charles Pfizer Co. (Maywood, New Jersey). These preparations had been evaluated electron microscopically to determine the number of viral particles (usually about 10^9/ml) but no infectivity studies were performed. Two lymphoblastoid cell lines (EB-2 and EB-3) derived from Burkitt's lymphoma and one (Kaplan) derived from an acute case of infectious mononucleosis were obtained from Dr Werner Henle. An additional EBV-positive lymphoblastoid cell strain isolated from the peripheral blood of a case of myeloblastic leukaemia (PBL) was supplied by Dr James Grace, and the EBV-producing marmoset

lymphoblastoid cell strain, B95-8, by Dr George Miller. The establishment and maintainance of simian lymphoblastoid cell lines transformed by EBV derived from the Kaplan cell line has been reported in detail (Falk et al., 1974b) and cells were grown in Eagle's medium or medium RPMI 1640 with 10-15% fetal calf serum and antibiotics. HR-1 virus was assayed by superinfection of Raji cells and determination of the number of cells positive for early EBV antigen (EA) 72 hours after superinfection. The titre of B95-8 virus was determined as transforming units (TFU) by transformation of human umbilical cord blood lymphocytes *in vitro*.

Lymphoblastoid cell lines were established from spleens or lymph-nodes, and virus isolation from tissues of infected animals was performed as previously described for herpesvirus saimiri-infected animals (Deinhardt et al., 1974).

Animal inoculation

Colony-reared or imported cotton-topped (CT) or white-lipped (WL) marmoset monkeys (*Saguinus* spp.) were inoculated with various materials containing EBV (Table 1). Animals were examined clinically at intervals post inoculation and blood samples were collected for haematological and serological studies. Complete necropsies were performed on all dead animals or animals sacrificed when moribund.

Serology

Sera were evaluated for antibodies against EBV-specified antigens (viral capsid [VCA], early [EA], membrane [MA] or EBV-determined nuclear [EBNA] antigens) by indirect immunofluorescence tests using acetone-fixed smears of HR-1 (VCA), Raji (EBNA) or superinfected Raji cells (EA); viable HR-1 cells were used for detecting antibodies to MA.

RESULTS

Tumour induction in marmosets

Table 1 summarizes our studies with EBV in marmosets over the past nine years. Twenty-five marmosets were inoculated with human or simian EBV-carrying lymphoblastoid cells and none of these animals developed overt clinical or haematological disease other than short-lived and possibly non-specific local lymph-node swellings. Marmosets inoculated with P3 or HR-1 virus likewise remained healthy. In contrast, the majority of marmosets inoculated with B95-8 virus developed a lymphoproliferative disease. Two groups of CT marmosets were inoculated with two separate preparations of B95-8 virus (Table 2). In the first group, one animal died 19 days post inoculation (PI) from an undetermined cause, one developed a lymphoma and died 31 days PI, and three are still surviving and healthy. A separately prepared stock of EBV-B95-8 was titrated in marmosets of the second group to establish the minimum virus dose needed for tumour induction. All three marmosets inoculated with 10^4 TFU of EBV died with lymphomas 35-110 days PI, and one of three marmosets inoculated with 10^3 TFU likewise developed a lymphoma. One animal of the latter group died accidentally 191 days PI and one is still surviving. None of the animals inoculated with 10^2 TFU developed tumours but one died 50 days PI from unrelated causes.

Macroscopic lesions observed in inoculated marmosets included generalized and visceral lymphadenopathy, splenomegaly and enlarged thymuses. Microscopically, malignant lymphoproliferative lesions, consisting of

Table 1. Inoculation of marmosets with EBV-carrying lymphoblastoid cell cultures or with cell-free preparations of EBV

Inocula	Number of marmosets inoculated	Species[a]	Results
Human EBV-carrying lymphoblastoid cell cultures[b]			
PBL strain (6×10^7 - 7×10^7 cells)	6	WL	No disease
EB-2 (3×10^6 cells)	4	WL	No disease
EB-3 (4×10^7 cells)	5	WL	No disease
Kaplan (2×10^7 cells)	1	WL	No disease
Simian EBV-carrying lymphoblastoid cell cultures[c]			
Autochthonous (5×10^8 cells)	1	WL	No disease
Allogeneic (0.3 - 5×10^8 cells)	2	CT	No disease
	4	WL	No disease
Xenogeneic (0.3 - 5×10^8 cells)	2	WL	No disease
Cell-free EBV			
P3 (10^9 particles as determined by electron microscopy)	5	CT	No disease
	33	WL	No disease
HR-1 (10^5 Raji infectious units)	4	CT	No disease
	3	WL	No disease
B95-8 (10^2 - 10^4 transforming units)	14	CT	Lymphoma

[a] CT: cotton-topped marmoset; WL: white-lipped marmoset.

[b] See Materials and methods for origin of cell cultures.

[c] Marmoset or squirrel monkey cells transformed by EBV derived from Kaplan cells.

Table 2. Inoculation of cotton-topped marmosets with EBV (strain B95-8)

Group	Number inoculated	Titre of inoculum[a]	Survival (days PI)		Lymphoma	EBV antibodies
			Alive	Dead		
I	5	10^4	3 (399)	2 (19, 31)	1	4/5
II	3	10^4	-	3 (35, 57, 110)	3	3/3
	3	10^3	1 (211)	2 (48, 191)	1	2/3
	3	10^2	2 (211)	1 (50)	-	0/3
	3[b]	-	3 (211)	-	-	0/3

[a] Assayed by transformation of human umbilical cord blood lymphocytes; values expressed as transforming units/ml.

[b] Cagemate controls.

extensive diffuse infiltrations of immature lymphocytic cells with frequent mitotic figures, were present in lymph-nodes, spleen, thymus, adrenal glands, liver, kidneys and lungs. None of the inoculated or control marmosets developed any haematological abnormalities detectable in the peripheral circulation.

Development of serum antibodies to EBV

None of the marmosets inoculated with human EBV-carrying cells (Table 1) developed antibodies to EBV-specified antigens. One marmoset inoculated with autochthonous EBV-transformed cells developed antibodies to VCA that became detectable 16 days PI; two other marmosets inoculated with allogeneic cells also developed VCA antibodies. Maximal antibody titres in these marmosets were 1:16-1:32.

In contrast, nine of 11 CT marmosets inoculated with 10^3-10^4 TFU of B95-8 EBV (Table 2) developed VCA antibodies, whereas marmosets inoculated with 10^2 TFU or uninoculated marmosets caged with inoculated animals did not develop VCA antibodies. Antibodies to EA, EBNA and MA were also demonstrated by screening some sera of the inoculated animals. For example, two animals of group I that did not develop disease, still had VCA and EBNA antibodies one year after inoculation. The exact anti-EA, anti-EBNA and anti-MA titres of these sera are still under study.

Establishment of lymphoblastoid cell lines

Attempts to establish cell lines from circulating leukocytes were unsuccessful but three lymphoblastoid cell lines were established from lymph-nodes and spleens of two group I marmosets that died 19 (animal No. 5308) and 31 (animal No. 72-FQ-2) days PI; the characteristics of these cells are presented in Table 3. All three cell lines expressed EBV antigens, contained herpesvirus particles by electron microscopy, and produced

Table 3. Properties of lymphoblastoid cell cultures established from spleen or lymph-nodes of cotton-topped marmosets inoculated with EBV (strain B95-8)

Property	Cell culture		
	5308	72-FQ-2	72-FQ-2
Tissue of origin	Lymph-node	Lymph-node	Spleen
EBV-specified antigens			
EA	6-8%[a]	4-9%	1-5%
VCA	7-11%	1-4%	1-6%
MA	+	+	+
EBNA	+	+	+
Herpesvirus particles	+	+	+
Transforming virus in culture fluids	+	+	+
Membrane immunoglobulins			
IgA	12%	-	1%
IgG	1%	-	10%
IgM	4%	1%	5%
Rosettes with sheep red blood corpuscles	-	-	-
Interferon production	-	-	-

[a] Range of values obtained in four separate tests; 400-500 cells counted in each test.

infectious, transforming virus; the cells had B-cell characteristics.

DISCUSSION

Studies in several laboratories have proved without question that some strains of EBV can cause lymphomas in some species of non-human primates. Lymphomas were induced in one of three owl monkeys inoculated with extracts prepared from EB-3 cells (Epstein et al., 1973), in one of three cotton-topped marmosets inoculated with virus derived from the

Kaplan cell line (zur Hausen[1]) and regularly in cotton-topped marmosets inoculated experimentally with $\geq 10^4$ TFU of EBV strain B95-8 (Shope et al., 1973; Falk et al., 1974a). Common marmosets (*Callithrix jacchus*) appear to be as susceptible as cotton-topped marmosets, as shown in a recent collaborative study with Drs J. Hilgers and G. de-Thé in which seven of eight common marmosets, inoculated with the same inoculum (10^4 TFU EBV-B95-8) as used for group II animals of this report, developed lymphomas in 51-90 days. The susceptibility of white-lipped marmosets (subspecies *S. fuscicollis* and *S. nigricollis*) is still under study. Although the B95-8 strain has not induced disease in three inoculated white-lipped marmosets (Miller[2]), it is questionable whether the difference in the ability to induce tumours in non-human primates, as between the various Epstein-Barr viruses, can be explained solely by a specifically higher susceptibility of cotton-topped marmosets because lymphocytes from white-lipped marmosets are as susceptible to transformation *in vitro* as cells from cotton-topped marmosets. However, the difference in responses of white-lipped as compared with cotton-topped marmosets may be caused by a difference in immune response toward EBV antigens between the two animal species. In addition, squirrel monkey lymphocytes transformed by B95-8 EBV *in vitro* induced tumours when inoculated intraperitoneally into the autochthonous lymphocyte donor animal (Liebold[1]).

As in man, the neoplastic lymphoid cells in marmosets had B-cell characteristics but, in contrast to man, the disease was probably multiclonal, as suggested by the presence of tumour cells that produce different classes of immunoglobulins. The clonality of the experimentally-induced lymphomas is being further studied by evaluation of sex chromosome markers of tumour cells from chimeric male-female twins.

The difference in oncogenic potential between the B95-8 strain of EBV and other strains of EBV cannot be explained. Marmosets were inoculated with the HR-1 virus when the very low transforming ability of this virus was not yet known. However, even EBV derived from the Kaplan cell strain, which has a good transforming capacity *in vitro*, appears to be only weakly oncogenic in non-human primates. It is possible that the B95-8 virus was altered by passage through marmoset cells, but no antigenic or nucleic acid differences (at least 90% homology between HR-1 and B95-8 virus DNA; Nonoyama[3]) have been detected; the results obtained so far, however, do not rule out minor genome changes and this needs further evaluation. Although the pathogenesis of the disease induced by EBV in non-human primates under experimental conditions is not identical with Burkitt's lymphoma in man, the regular induction of lymphomas in the experimental animals and re-isolation of EBV from the tumours nevertheless provides not only further evidence for the oncogenicity of EBV but also a good experimental model for studies of the prevention and therapy of EBV-induced diseases.

SUMMARY

The response of cotton-topped (CT) or white-lipped (WL) marmosets, inoculated with material containing Epstein-Barr virus (EBV), was studied.

[1] Personal communication.

[2] Unpublished data.

[3] Unpublished data.

Twenty-five marmosets inoculated with 10^6-10^8 cells from human or simian EBV-carrying lymphoblastoid cell cultures developed no detectable clinical disease. Transitory antibody titres to VCA were detected in sera from several of these marmosets. Thirty-eight marmosets inoculated with P3 virus (10^9 particles) and seven marmosets inoculated with 10^5 infectious units of HR-1 virus likewise developed no recognizable clinical or haematological disease over several years of observation. Fourteen marmosets were inoculated with 10^2-10^4 transforming units (TFU) of EBV strain B95-8; five of 11 animals inoculated with 10^3-10^4 TFU of virus developed malignant lymphoma and died 31-110 days PI. Antibodies to one or more EBV-specified antigens were detected in sera from nine of the inoculated marmosets. EBV-carrying lymphoblastoid cell cultures were established from the spleen and lymph-nodes of one animal that died with malignant lymphoma and from another animal that died 19 days PI from undetermined causes.

ACKNOWLEDGEMENTS

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OBSERVATIONS ON THE NATURE OF EPSTEIN-BARR VIRUS INFECTION OF PERIPHERAL LYMPHOID CELLS IN INFECTIOUS MONONUCLEOSIS

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Continuous lymphoid cell lines can readily be established from biopsy samples of Burkitt's lymphomas (Epstein, 1970), and both the lymphoma cells and the cells of tumour-derived lines are known to contain Epstein-Barr virus (EBV) DNA (zur Hausen et al., 1970; Nonoyama & Pagano, 1971). Furthermore, it is clear from experiments using a variety of cytological markers that, in the majority of cases, it is the Burkitt's tumour cells, already malignantly transformed *in vivo*, that in culture grow out to give the lymphoid cell lines (see Epstein & Achong, 1973).

EBV DNA-containing lymphoid lines can also be obtained from cultures of peripheral lymphocytes from healthy individuals, patients with a variety of irrelevant diseases, and patients with infectious mononucleosis (IM), provided that the donors are seropositive for EBV (Nilsson et al., 1971), and this raises the important question as to the nature of the EBV infection in those lymphocytes from which these cell lines originate. If EBV really is an etiological agent in the malignant transformation underlying Burkitt's lymphoma, can it be resident in the tumour cells in the same state as in the peripheral lymphoid cells of the huge number of seropositive people who do not have lymphomas? Alternatively, might not the virus-cell interaction in lymphoma-free individuals be of a different nature to that seen in the tumour?

These questions must be considered in the light of the ability of herpesviruses in general to establish two distinct types of non-productive infection *in vivo*, one in which the virus genome is expressed to cause malignant transformation, the other a latent infection in which the virus genome is seemingly unexpressed (see Epstein & Achong, 1973). If the EBV-cell interaction in lymphoma-free individuals is of the first type, then continuous cell lines would arise in peripheral lymphocyte cultures by direct outgrowth of *in vivo* transformed cells, exactly as is known to occur in cultures of Burkitt's lymphoma biopsies; in contrast, if the interaction is of the second type, other mechanisms

can be envisaged whereby cell lines could arise. The present paper describes experiments designed to investigate how lymphocytes from one category of seropositive tumour-free individuals, namely patients with IM, give rise to continuous cell lines in culture.

MATERIALS AND METHODS

Details of materials and methods are as described elsewhere (Rickinson et al., 1974; Jarvis et al., 1974), except that, in the present experiments, cultures were set up using mononuclear cells harvested after centrifugation of whole blood on Ficoll-Isopaque (Böyum, 1968). Transformation of cultures was recognized by the appearance of progressively growing clumps of lymphoblastoid cells (Figs. 1 and 2).

EXPERIMENTAL PROCEDURE

In a first set of experiments, equal numbers of IM leukocytes and fetal leukocytes from donors of opposite sex were co-cultivated in medium with 20% fetal calf serum (FCS) and, soon after the onset of trans-

FIG. 1. CULTURE OF IM LEUKOCYTES THAT HAVE NOT UNDERGONE TRANSFORMATION AFTER 28 DAYS IN VITRO

(x 65)

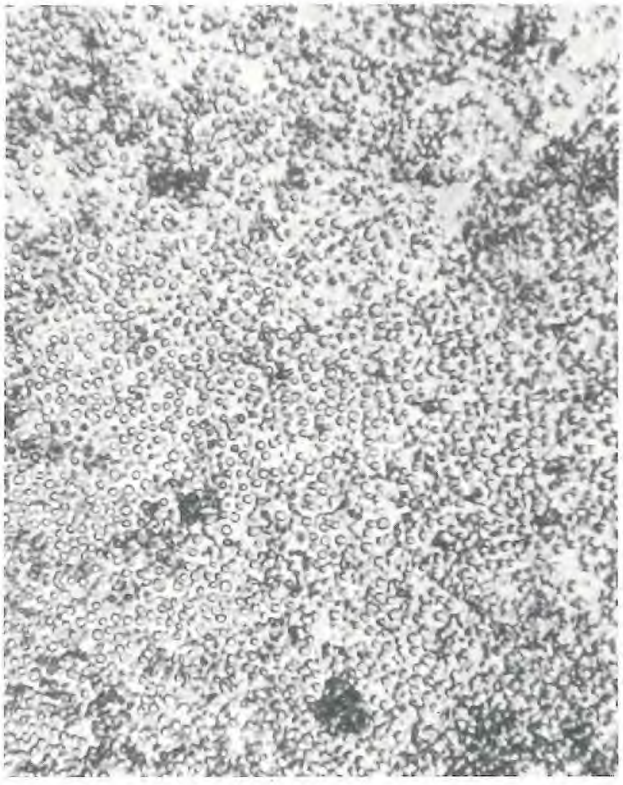
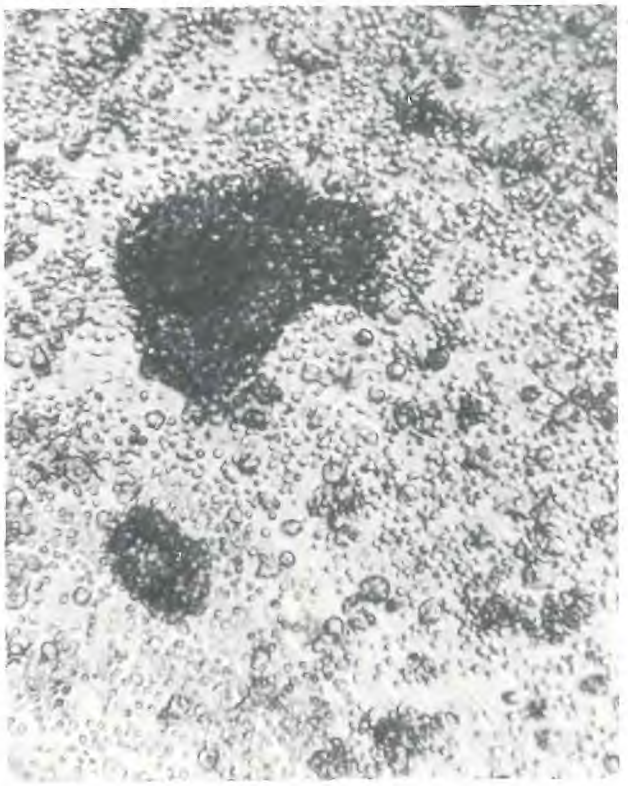


FIG. 2. CULTURE OF IM LEUKOCYTES AFTER 28 DAYS IN VITRO

Clumps of transformed cells can be seen; these were first recognizable as small aggregates of lymphoblastoid cells seven days earlier. (x 65)



formation, the emerging cell lines were harvested for cytogenetic examination.

In further experiments, the incidence of transformation in cultures of IM leukocytes alone was compared: (*a*) with the fetal leukocyte-transforming ability of parallel cultures of IM leukocytes X-irradiated after 0-2 days in preliminary culture; and (*b*) with the fetal leukocyte-transforming ability of cell-free extracts made from parallel cultures of IM leukocytes on the third day of culture, the extracts having first been treated with antiserum to EBV or with EBV-negative serum.

A further set of experiments recorded the incidence of transformation and time to transformation in cultures of IM leukocytes alone, IM leukocytes pre-exposed to EBV *in vitro*, fetal leukocytes pre-exposed to EBV *in vitro*, and fetal leukocytes to which were subsequently added X-irradiated cells of the QIMR-WIL line (Pope et al., 1968) as an external source of EBV. All cultures were grown in medium containing 15% FCS and either 5% antiserum to EBV or 5% EBV-negative serum.

Finally, known numbers of IM leukocytes and fetal leukocytes from donors of opposite sex were mixed in a proportion of roughly 2:1 and co-cultivated in the presence or absence of antiserum to EBV as described above. The transformed cells emerging from these cultures were again taken for cytogenetic examination.

RESULTS

When IM and fetal leukocytes were co-cultured in medium with 20% FCS, transformation occurred as readily as in the corresponding cultures of IM leukocytes alone, and the transformed cell populations were made up of mixtures of IM-derived and fetal-derived cells. Detailed results are presented elsewhere (Rickinson et al., 1974) and show that, in many co-cultures, the fetal cells were predominant, and that the proportions of the two cell types remained fairly constant with subsequent passage in culture.

In the second set of experiments, it was found that IM leukocytes X-irradiated immediately on collection or after one or two days of preliminary culture were able to bring about the transformation of added fetal leukocytes, the fetal origin of the transformed cells being confirmed by the sex chromosome marker. Moreover, there was good correlation between the incidence of fetal cell transformation in the co-cultures and the incidence of transformation in cultures where unirradiated IM leukocytes of similar origin were cultured alone (Rickinson et al., 1974).

When extracts were prepared from three-day cultures of IM leukocytes from different patients, it was found that, in two out of three cases, the extracts were capable of transforming fetal leukocytes and that this capacity was abolished by treatment with antiserum to EBV (Rickinson et al., 1974). In addition, those IM leukocyte populations that gave infectious extracts were also those that transformed most readily when cultured alone.

Table 1 presents data on the incidence of transformation and mean time to transformation in four different types of culture, each one set up in the presence of antiserum to EBV and of EBV-negative serum as a control. In cultures of IM leukocytes alone, antiserum both reduced the incidence of transformation and delayed its onset in the remaining successful cultures. An exactly similar effect of antiserum was seen in the co-cultures of fetal leukocytes and X-irradiated QIMR-WIL cells, in a situation where the establishment of transformation is known to depend upon the intercellular transfer of EBV. In contrast, when either fetal leukocytes or IM leukocytes themselves were exposed to active EBV preparations immediately prior to culture, transformation occurred regularly and rapidly in virtually every culture irrespective of the presence or absence of antiserum to EBV in the medium.

From the final series of experiments in which co-cultures were set up between IM leukocytes and fetal leukocytes in a proportion of 2:1, there were four cases where transformation was observed in co-cultures containing antiserum to EBV as well as in co-cultures containing EBV-negative serum. Table 2 shows the composition of the various transformed cell populations that arose in these cases, as revealed by cytogenetic analysis. Again it is clear that, in the majority of cases, transformed cells were of mixed origin, although there were instances where cells of one type or the other were entirely predominant. It is also apparent that, when cell lines did arise in co-cultures containing antiserum to EBV, their

Table 1. Incidence of transformation and mean time to transformation in four different types of culture

Cells in culture	Type of serum	Incidence of transformation	Mean time to transformation (weeks)
$2.0\text{-}4.0 \times 10^6$ IM cells alone[a]	FCS + antiserum to EBV	8/24	5.8
	FCS + EBV-negative serum	21/24	3.8
$2.0\text{-}4.0 \times 10^6$ IM cells pre-exposed to EBV[a]	FCS + antiserum to EBV	23/24	2.4
	FCS + EBV-negative serum	24/24	2.5
2.0×10^6 fetal cells pre-exposed to EBV	FCS + antiserum to EBV	12/12	2.9
	FCS + EBV-negative serum	12/12	2.8
2.0×10^6 fetal cells + 2.0×10^5 X-irradiated QIMR-WIL cells	FCS + antiserum to EBV	7/12	4.6
	FCS + EBV-negative serum	12/12	2.0

[a] Combined results from six IM donors.

composition was not consistently altered in favour either of the IM-derived or of the fetal-derived component when compared with those lines that arose in the corresponding co-cultures containing EBV-negative serum.

DISCUSSION

Patients with heterophile antibody-positive IM were used as donors in the present work since they provided peripheral lymphocytes from an EBV-infected lymphoma-free source in which the circulating lymphoid cells carrying the virus are thought to be more numerous than in ordinary seropositive donors and from which continuous cell lines can readily be established in culture (Diehl et al., 1968). In contrast, fetal lymphocytes do not transform when cultured on their own, though they can be made to do so by exposure to EBV *in vitro* (Henle et al., 1967; Pope et al., 1968; Pope et al., 1969; Nilsson et al., 1971).

The original observation that transformed cell lines arising in co-cultures of IM and fetal leukocytes were of mixed cell origin, rather than of predominantly IM origin, immediately suggested that some factor would appear to be liberated from IM leukocyte populations early in the culture period that can subsequently bring about the *in vitro* transformation of virus-free cells co-resident in the culture. Indeed, such a mechanism might well explain the consistent appearance of transformed foci in cultures of IM leukocytes alone.

The view was strengthened by the observed correlation between the capacity

Table 2. Incidence of transformation and origin of transformed cells in co-cultures of infectious mononucleosis patients' leukocytes (IM) and fetal leukocytes (F) set up in the presence or absence of antiserum to EBV

Cells in culture	FCS + antiserum to EBV			FCS + EBV-negative serum		
	Incidence of transformation	Number of cultures	Origin of transformed cells[a] (% IM : % F)	Incidence of transformation	Number of cultures	Origin of transformed cells[a] (% IM : % F)
2.0×10^6 IM1 (♂) cells + 1.1×10^6 F1 (♀) cells	4/5	1	0 : 100	5/5	1	90 : 10
		2	55 : 45		2	75 : 25
		3	100 : 0		3	100 : 0
		4	30 : 70		4	100 : 0
		Mean:	46 : 54		*Mean:*	91 : 9
2.5×10^6 IM2 (♀) cells + 1.5×10^6 F2 (♂) cells	3/6	1	100 : 0	6/6	1	100 : 0
		2	100 : 0		2	20 : 80
		3	50 : 50		3	10 : 90
		Mean:	83 : 17		*Mean:*	43 : 57
4.0×10^6 IM_3 (♀) cells + 2.0×10^6 F_3 (♂) cells	3/8	1	20 : 80	8/8	1	90 : 10
		2	0 : 100		2	0 : 100
		3	100 : 0		3	25 : 75
					4	60 : 40
		Mean:	40 : 60		5	50 : 50
					6	60 : 40
					Mean:	48 : 52
2.0×10^6 IM_4 (♂) cells + 1.1×10^6 F_4 (♀) cells	1/8	1	55 : 45	5/8	1	100 : 0
					2	100 : 0
		Mean:	55 : 45		3	100 : 0
					Mean:	100 : 0

[a] Percentage composition of transformed cell population on the basis of at least 25 spreads per culture.

of IM leukocytes to transform when cultured alone, their fetal leukocyte-transforming capacity following X-irradiation either prior to or within the first two days of culture, and the fetal leukocyte-transforming capacity of IM leukocyte extracts made on the third day of culture. The neutralization of extracts by antiserum to EBV strongly suggested that this transforming capacity resided in infectious EBV particles released by the cultured IM leukocytes.

Supporting evidence of a different kind came from the results presented in Table 1. Here, the appearance of transformed foci in IM leukocyte cultures was significantly reduced and delayed by the presence of antiserum to EBV, in a manner very like that observed in co-cultures of fetal leukocytes and X-irradiated EBV-

producing cells where the establishment of transformation is known to depend upon the intercellular transfer of EBV. This was in marked contrast to the situations where either IM leukocytes or fetal leukocytes were pre-exposed to EBV *in vitro* before culturing, for now transformation occurred regularly and rapidly both in the presence and in the absence of antiserum to the virus. The inhibitory action of antiserum upon transformation in cultures of IM leukocytes alone therefore appears to be mediated through a restriction of intercellular virus transfer rather than through a direct cytotoxic action upon virus-transformed cells. The fact that the inhibition is incomplete is not surprising in view of the known ability of herpesviruses to spread between cells in intimate contact even in the presence of antiviral serum (Lodmell et al., 1973).

All the evidence presented so far argues in favour of a two-step mechanism underlying the establishment of continuous cell lines from IM leukocyte cultures. Firstly, the EBV genome carried by a small number of peripheral lymphoid cells is activated to a productive infection when these cells are placed in culture; secondly, virus particles thus released go on to infect nearby normal lymphocytes that then undergo *in vitro* transformation to give continuous cell lines. The possibility still exists that, whilst the majority of transformed foci arise through a two-step mechanism as outlined above, there are in addition some foci arising independently through the direct outgrowth of putatively "*in vivo* transformed" cells present within the original IM leukocyte population. In order to test this possibility, co-cultures of IM and fetal leukocytes were set up in the presence and absence of antiserum to EBV, and the compositions of the transformed populations that arose in the two types of culture were compared. In such co-cultures, a process of direct outgrowth would yield transformed foci of predominantly IM, not mixed, origin, and furthermore its influence on the overall composition of the transformed population would be much greater in cultures where the contribution of the two-step mechanism has been specifically reduced by the presence of antiserum to the virus. If, therefore, direct outgrowth of "*in vivo* transformed" cells were occurring, transformed lines emerging in cultures containing antiserum should display an increased IM-derived component. The results in Table 2 clearly show that this is not the case, and so argue against the existence of any mechanism of direct outgrowth.

The present results clearly imply that EBV DNA is present in the peripheral lymphoid cells of IM patients as a different type of infection from that shown by the virus in the malignant cells of Burkitt's lymphoma, and that cell lines arise in culture from these two sources by different mechanisms (see also Jarvis et al., 1974). It is proposed that a small proportion of IM leukocytes *in vivo* carry EBV as a latent unexpressed infection, analogous to that shown by trigeminal ganglia harbouring herpes simplex virus (Stevens et al., 1972; Bastian et al., 1972); the analogy is strengthened by our recent observation[1] that IM leukocytes do not yield infectious EBV-containing extracts unless first cultured to allow infected cells to enter a virus-productive cycle.

Whilst it is possible that acute IM patients represent a special case, it seems likely that all seropositive lymphoma-free individuals likewise possess a small population of lymphoid

[1] Unpublished data.

cells harbouring EBV as a latent infection. This possibility is being tested at the moment. In any event, the present demonstration of a type of EBV infection in the lymphoid cells of at least one group of seropositive lymphoma-free individuals, distinct from that seen in Burkitt's lymphoma cells, goes some way towards resolving the problem of how a ubiquitous virus might under special circumstances be involved in the etiology of a comparatively rare tumour.

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HOMOLOGY OF EPSTEIN-BARR VIRUS DNA IN NASOPHARYNGEAL CARCINOMAS FROM KENYA, TAIWAN, SINGAPORE AND TUNISIA

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The association of the Epstein-Barr virus (EBV) and anaplastic carcinoma of the nasopharynx has added another dimension to our view of the biology of this virus. This is a consequence of the demonstration by Wolf et al. (1973) that the EBV genome is harboured in the carcinomatous - i.e., the epithelial - cells of the tumour rather than in the infiltrating lymphocytes.

Another aspect of our understanding of nasopharyngeal carcinoma (NPC), potentially even more significant, is the epidemiology of this tumour; unlike Burkitt's lymphoma (BL), it is globally distributed although there are remarkable geographical variations.

It has been shown that the EBV genome is found in NPC from Kenya (zur Hausen & Schulte-Holthausen, 1970; Nonoyama et al., 1973; Pagano, 1974). Here we show that the viral genome can be detected in malignant tissues from patients with NPC in other parts of the world outside Kenya. This is important because it confirms that the association of EBV and a human malignancy is not confined to a limited geographical area where a local etiological co-factor might be operative, as is perhaps the case with BL (Old et al., 1968; de Schryver et al., 1969).

In addition, we present evidence of variations in the viral genome found in tumour tissue taken from NPC. The data may be interpreted as indicating the existence of different strains of the Epstein-Barr virus in nature, a phenomenon long suspected but for which previously there was no substantial evidence, or the retention of defective EBV genome in tumour tissue.

MATERIALS AND METHODS

The tumour specimens were shipped frozen to Chapel Hill for analysis of viral DNA content. The histological interpretations were made in the places of origin of the specimens. Detection of viral DNA in the tumour tissue was carried out by complementary RNA-DNA hybridization on membrane filters with tritiated EBV-specific cRNA of specific activity 1×10^7 cpm/μg. The specimens were tested in duplicate with 1.2×10^5 cpm of ^{3}H-cRNA applied per filter; appropriate corrections were made for non-specific background radioactivity in the hybridization tests, as described previously (Nonoyama & Pagano , 1971; Pagano, 1975).

The viral genome content of the tissues were also analysed by DNA-DNA renaturation kinetics according to methods previously described (Nonoyama & Pagano, 1973; Pagano, 1975). For the studies of DNA homology, S1 enzyme was used to distinguish between double and single-stranded DNA (Huang & Pagano, 1974). The same methods of preparation of the enzyme and of radiolabelled prototype EBV DNA were used for all the analyses shown here.

RESULTS

Analysis of tissues by cRNA-DNA hybridization

We showed before that EBV DNA could be detected in apparently virus-free tumours by cRNA-DNA hybridization in 32 of 38 specimens obtained from Kenya (Pagano, 1974). The data in Table 1 show that EBV DNA could be detected by the same method in five of five specimens of NPC from Tunisia, four of eight specimens from Taiwan, and four of six specimens from Singapore. Other details about these tests have been published recently (Pagano, 1975), and zur Hausen et al. (1975) have obtained similar results.

The number of genome equivalents, when averaged over the total concentration of cellular DNA tested, was variable, ranging from five genome equivalents to 133 genome equivalents per cell (Table 2). Because of the small numbers, tissue sampling and differences in histology, it was impossible to decide whether the mean level of EBV DNA in specimens from one locality differed from the level of EBV DNA found in specimens from another geographical region. Also, we could not detect any correlation between the level of EBV DNA in the tissues and the antibody titre to EBV capsid antigen, nor was such a correlation found in the more extensive studies carried out with Kenyan NPC.

Negative results, i.e., specimens of NPC that did not disclose evidence of the EBV genome, may be due to different types of tumours and

Table 1. Nasopharyngeal carcinomas from Tunisia, Taiwan and Singapore

Specimens	cRNA-DNA hybridization (No. of genome equivalents per cell)	EBV viral capsid antibody titre
Tunisia[a]		
NPC 219	59	>1/2560
NPC 237	11	1/1280
NPC 238	119	>1/2560
NPC 283	48	1/640-1/1280
NPC 333	10	-
Taiwan[b]		
A390	<2	1/640
A391	<2	1/640
A404	17	1/2560
A405	<2	1/160
74M-2	32	1/640
74M-3	<2	1/640
74M-7	17	1/640
74M-8	6	1/2560
Singapore[c]		
E121074	39	NT
E121114	<2	NT
E121990	133	NT
E120187	16	NT
3585/C	30	NT
E121320	<2	NT

[a] With G. de-Thé.

[b] With C.S. Yang, S.M. Tu and W.S.J. Lin.

[c] With K. Shanmugaratnam. NT: not tested.

Table 2. Nasopharyngeal carcinoma: summary of RNA-DNA hybridization results

Origin	Specimens that contain EBV DNA	Average No. of genome equivalents per cell	
		(Range)[a]	(Mean)[b]
Kenya[c]	32/38	5-85	18
Taiwan	4/8	6-32	18
Tunisia	5/5	10-119	50
Singapore	4/6	16-133	55

[a] The proportion of tumour cells in the samples was not determined.

[b] Of positive results.

[c] Pagano (1974).

tumour-cell sampling; the histological sections and the specimens analysed were not always precisely the same because of the small amounts of tissue available. It is important to note that specimens often contain normal as well as malignant tissue in an unknown proportion.

Comparison of results from cRNA-DNA hybridization and DNA-DNA renaturation kinetics analyses

A number of specimens were tested by both hybridization techniques (Table 3). Both of the specimens that failed to disclose EBV DNA by the cRNA-DNA hybridization technique also did not contain detectable viral DNA when analysed by the more sensitive DNA renaturation kinetics procedure. The sensitivity of the former test is about two genome equivalents per cell and of the latter about 0.4 genome per cell. The estimates of the amount of EBV DNA per cell from the two different techniques were generally in good agreement. In one instance, 119 genome equivalents were indicated by cRNA-DNA hybridization whereas the DNA renaturation kinetics analysis disclosed 211 genomes per cell; the slope of the curve in this renaturation kinetics assay was rather steep for a precise plot. The data show that EBV DNA exists in substantial amounts in these apparently virus-free tumours.

Homology of the viral nucleotide sequences found in malignant tissue to prototype viral DNA

We noted recently that a portion of the viral nucleotide sequences found in the prototype EBV harvested from the virus-producing HR1K cell line originally derived from a Kenyan Burkitt's lymphoma might be missing in two specimens of nasopharyngeal carcinoma from Singapore (Pagano, 1975). The results, summarized here in tabular form (Table 4), suggested that 10-15% of the nucleotide sequences found in the HR1K DNA might be missing in the two specimens from Singapore. The DNA sequences represented in a single specimen from Tunisia appeared

Table 3. Nasopharyngeal carcinomas: Epstein-Barr viral DNA detected by cRNA-DNA hybridization and by DNA-DNA renaturation kinetics

Origin	cRNA-DNA (average No. of genome equivalents per cell)	DNA-DNA (average No. of genomes per cell)
Kenya	19	19.2
	20	48
	85	65.7
	<2	<0.4
Tunisia	119	211.2
Singapore	39	40
	30	39
	<2	<0.4

Table 4. Summary of estimates of degrees of homology of like DNA sequences in HR1K and Raji cells, Kenyan Burkitt's lymphoma and nasopharyngeal carcinomas from Kenya and Tunis: analyses conducted by hydroxyapatite chromatography[a]

Origin	DNA	No. of EBV genomes/2 mg DNA	Approximate homology to HR1K DNA (%)
Kenya	HR1K	400-800	94
Kenya	NPC	48	88
	NPC	65.7	90
Kenya	BL	44.3	86
	BL	57	92
Tunisia	NPC	211.2	92
Singapore	NPC	39	86
	NPC	40	84

[a] Results published in part in Pagano (1975)

to be comparable to the prototype viral DNA. There was also a possibility of variations in Kenyan NPC and Burkitt's lymphoma, but the differences were too small to be considered significant. All these analyses were carried out by hydroxyapatite chromatography and required confirmation.

To extend the work, we carried out the next set of DNA-DNA renaturation kinetics analyses with the use of S1 enzyme to distinguish between single-stranded and double-stranded DNA. With good preparations of this single strand-specific exonuclease, it is theoretically possible to make more precise estimates of homology. The characteristics of the enzyme preparation (gift of E.-S. Huang) used for this work are shown in Fig. 1. The enzyme digested approximately 8% of double-stranded DNA after 120 minutes and 95% of single-stranded DNA (analysis by J. Shaw). These values were constant, and therefore an appropriate correction was made in the subsequent analyses.

We first carried out several control analyses with HR1K cell DNA and Raji cell DNA. At the same time, we analysed several tumour specimens from Kenya: two specimens of NPC, neither of which contained EBV DNA, and two specimens of Burkitt's lymphoma (Fig. 2).

The Cot_{50} (time required for 50% reannealing) of the 0.02 µg of ^{3}H-EBV DNA used for the analyses was 300 hours in the presence of 2 mg of calf-thymus (CT) DNA, a value the same as that found in earlier analyses (Pagano et al., 1973; Pagano, 1975). The HR1K DNA used as the 100% reannealing control mixture was obtained from a pellet of HR1K cells; with the concentration of added DNA used,

FIG. 1. KINETICS OF S1 ENZYME DIGESTION OF ^{32}P-HEp-2 DNA

^{32}P-labelled HEp-2 DNA was mixed with calf-thymus DNA to a final concentration of 2 mg/ml. Digestion was at 37°C in 0.1 M sodium acetate, pH 4.5, 0.3 M sodium chloride, and 0.003 M zinc. A quantity of 50 µl S1 preparation in 50% glycerol was added per ml of digestion buffer.

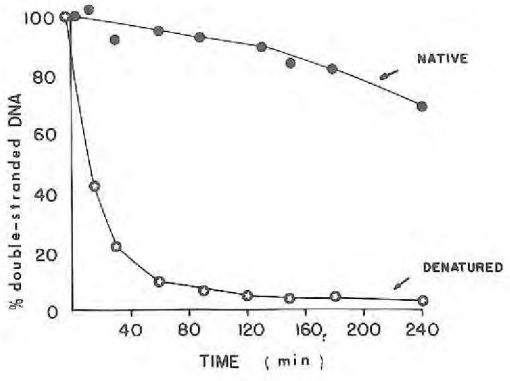
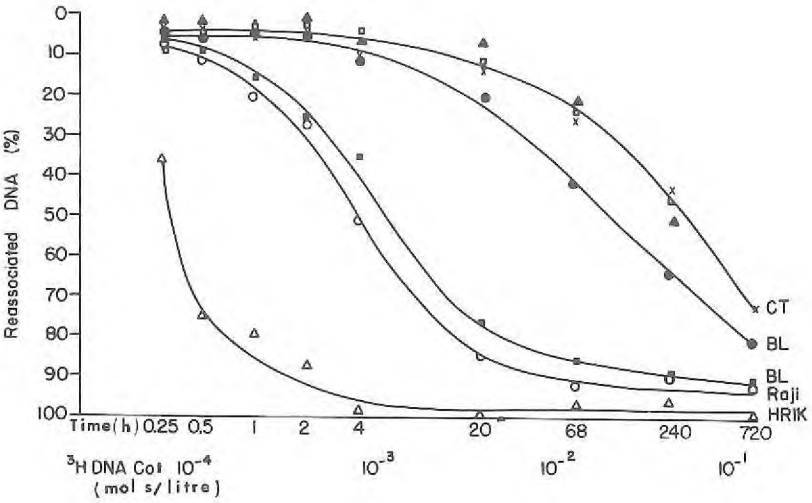


FIG. 2. HOMOLOGY OF DNA SEQUENCES IN HR1K, RAJI AND KENYAN BURKITT'S LYMPHOMA

Reannealing was carried out as described before (Nonoyama & Pagano, 1973; Pagano, 1975). Total DNA concentration was equalized to 2 mg with calf-thymus DNA in all the mixtures. The specific activity of the ^{3}H-EBV DNA was 1.8×10^{6} cpm/µg. The mixtures were as follows:

- x calf-thymus (CT) DNA (2 mg) + 0.02 µg ^{3}H-EBV DNA (in all mixtures)
- △ HR1K (cell pellet, 2 mg)
- o Raji (1 mg)
- □ NPC Kenya 48602 (1.65 mg)
- ▲ NPC Kenya 50743 (1 mg)
- ● BL Kenya 1513 (0.25 mg)
- ■ BL Kenya 59839 (2 mg)



reannealing of the index radiolabelled DNA was approximately 98% complete within four hours (Fig. 2). Raji cell DNA, previously shown to harbour 52 genomes per cell (Nonoyama & Pagano, 1973), reached 92% reannealing within 68 hours. Specimen 59839 of the Burkitt's lymphoma appeared to have at least 90% of the viral sequences found in the HR1K viral DNA and probably more, as renaturation still appeared to be proceeding at 720 hours. The relatively low level of EBV in the other specimen of Burkitt's lymphoma (1513) did not permit an inference concerning the percentage of homology, although it is obviously greater than 85%.

The problems associated with the interpretation of very small differences in completeness of reannealing will be discussed later, but these results indicate that the viral nucleotide sequences found in Raji cells and one of the specimens of Burkitt's lymphoma exhibit at least 95% homology to the prototype HR1K viral DNA.

Degree of homology of DNA sequences in Tunisian and Kenyan nasopharyngeal carcinomas

The results of the analysis of four specimens of Tunisian NPC and one specimen of Kenyan NPC are shown in Fig. 3. All four specimens of Tunisian NPC contained EBV DNA in amounts of 11.5-197.3 genomes per 2 mg of cellular DNA. However, none of the four specimens bore more than 75% homology to the prototype viral DNA. The results with specimens Nos. 396 and 393 are particularly suggestive because of the relatively large amounts of viral DNA sequences harboured in these specimens; these led to essentially complete reannealing within 20 hours of reaction time. After this, the reannealing reactions reached a plateau, as seen in Fig. 3.

The specimen of Kenyan NPC also appeared to lack some of the sequences found in prototype viral DNA.

The results shown in Figs. 2 and 3 are summarized in tabular form in Table 5. We emphasize that these are approximate estimates only and cannot be taken as firm percentages as yet.

FIG. 3. DEGREE OF HOMOLOGY OF DNA SEQUENCES IN TUNISIAN AND KENYAN NASOPHARYNGEAL CARCINOMAS

The analyses were conducted with the same radiolabelled EBV DNA (0.02 µg) and under the same conditions as those used in the experiment shown in Fig. 2. The reaction mixtures were as follows:

- x calf-thymus (CT) DNA (2 mg) + 0.02 µg ^{3}H-DNA
- △ HR1K (2 mg)
- ● Tunisian NPC 384 (0.5 mg)
- ▲ Tunisian NPC 376 (1.1 mg)
- □ Tunisian NPC 396 (1.7 mg)
- ■ Tunisian NPC 393 (1.6 mg)
- o Kenyan NPC 9574 (0.4 mg)

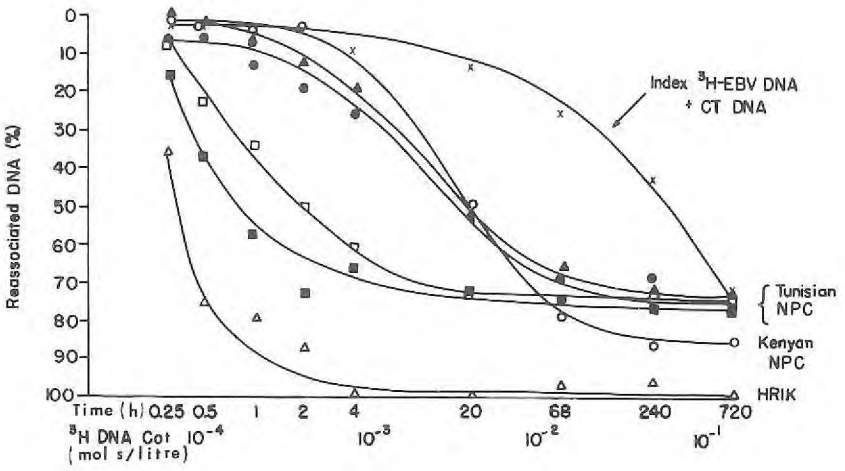


Table 5. Summary of estimates of degree of homology of like DNA sequences in HR1K and Raji cells, Kenyan Burkitt's lymphoma and nasopharyngeal carcinomas from Kenya and Tunis: analyses conducted with S1 enzyme

Origin	DNA	No. of EBV genomes/2 mg DNA	Approximate homology to HR1K DNA (%)
Kenya	HR1K	-	98-100
Kenya	Raji	60	>92
Kenya	BL	9.6	Uncertain (>85)
	BL	21.4	>92
Kenya	NPC	30.0	85
Tunisia	NPC	11.4	74
	NPC	78.7	75
	NPC	27.2	75
	NPC	197.3	76

DISCUSSION

The diversity of pathology associated with EBV and the global distribution of the virus among primates, both human and subhuman, suggest that strain variants that are biologically significant probably exist. The failure thus far to demonstrate such heterology by immunological methods means little because of the difficulty or impossibility of procuring appropriately purified structural virus antigens for the requisite analyses. Purified EBV is essentially available to date only from one source in any quantity, namely the HR1K virus-producing cell line that originates from a Ugandan Burkitt's lymphoma. Some biological evidence of possible strain differences has been proposed by Miller, based mainly on the different transforming capacity of EBV obtained from filtered throat washings from patients with infectious mononucleosis. Although quantitative assays are not yet available, it does appear that such material is generally more efficient in transforming cord-blood lymphocytes than is the prototype HR1K virus, as shown elsewhere (Miller et al.[1]); HR1K virus seems to have lost transforming ability.

At present, the only unequivocal way to demonstrate strain differences within Epstein-Barr virus is by DNA-DNA homology studies of the type that we have conducted. For definitive comparisons of two genomes, it is necessary to carry out reciprocal analyses. The two purified viruses have to be available in a quantity sufficient to allow radiolabelled viral DNA of one virus to be compared with the unlabelled viral DNA of the other, and *vice versa*. This is necessary because a one-way comparison might show complete homology whereas labelling of the other virus could disclose additional heterologous sequences in the second genome. With the limited material available, it is difficult to show that one is dealing

[1] See *Part 1*, p.398.

with viral genomes precisely equal in size.

With nasopharyngeal carcinoma especially, but also in most cases of Burkitt's lymphoma, only one-way analyses with the one available prototype viral DNA can be conducted, because it is not possible to explant the tumours and recover virus in this way. Moreover, most Burkitt's tumours do not yield enough virus to carry out a reciprocal type of analysis either.

We can, however, now at least suspect that heterologous sequences, perhaps as much as 20-25% of the EBV genome, may be present in the specimens of Tunisian NPC that we have analysed recently. Strictly speaking, we can infer only that 20-25% of the EBV genome is deleted, since we do not know whether an entire viral genome of molecular weight approximately 100 x 10^6 daltons is present in the Tunisian tumour tissue. Indeed, an attractive alternative hypothesis is that there may be a deletion of sequences in NPC and a defective genome. This situation might account for the fact that neither virus nor viral structural antigens are found in nasopharyngeal carcinoma. If such a defective genome is what is retained in some NPC, then presumably neither tumour specimens nor cell lines established in nude mice should contain unit-length viral DNA, found in Raji cells (Nonoyama & Pagano, 1972).

Finally, it is not unlikely that both situations co-exist, i.e., that parts of the genome are simply deleted and other portions are replaced with heterologous regions. Both the deleted and the heterologous regions could be quite variable, but there must also be constant regions. This may seem at first to be a complicated proposal, but in biological and molecular terms this conception offers a flexibility that may well conform to the diverse situations of the EBV genome in nature, as distinct from idealized models presented by cloned transformed cell lines derived *in vitro* in experimental tumour-virus systems.

There could be trivial explanations for the apparent differences found in the renaturation kinetics analyses. Especially with long-term renaturation reactions, hydrolytic artefacts and secondary and tertiary reannealing phenomena may occur that hinder complete reannealing. This does not seem to be the case in the study shown here because the analyses of Raji and HR1K DNA are in good agreement. With carefully conducted analyses, it does appear to be possible to achieve in excess of 90% complete reannealing, but these results must be confirmed by additional analyses. Certainly differences of 10-15% are completely ambiguous unless a consistent pattern emerges upon repeated analysis of the same specimens, and such analysis has not been possible because of the scarcity of material.

Probably the most persuasive result is shown in Fig. 3 where, already after only about four hours of reaction time, the reannealing reaction seems to have reached its limit at about the level of 75% homology to the prototype viral DNA with two of the NPC specimens from Tunisia. Results of this type can be confirmed by approaches such as addition experiments, in which a reannealing reaction that has apparently reached a plateau is driven by the addition of more of the same NPC DNA or HR1K-cell DNA. Experiments of this type and possibly also recycling experiments, in which the residual unannealed labelled EBV DNA is recovered and used for a reanalysis, should prove to be helpful in sustaining the conclusion that differences exist, but these experiments may also produce their own artefacts.

In any case, these differences have been observed in precisely conducted analyses. If confirmed, they would have great significance for the insights that they could afford into both the pathogenesis of diseases associated with the Epstein-Barr virus and the epidemiology of infection with this virus and its variants. A sort of molecular pathology and molecular epidemiology based on nucleotide sequence differences appears to be in the offing, and may provide the next insights into the association of the Epstein-Barr virus with disease and population groups.

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FURTHER STUDIES ON THE DETECTION OF THE EPSTEIN-BARR VIRUS DNA IN NASOPHARYNGEAL CARCINOMA BIOPSIES FROM DIFFERENT PARTS OF THE WORLD

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Wolf et al. (1973) showed by *in situ* nucleic acid hybridization that the epithelial cells of nasopharyngeal carcinoma (NPC) contain Epstein-Barr virus DNA (EBV DNA). These results have been confirmed by Klein et al. (1974) by passaging NPC tumour cells in nude mice. Hybridization of DNA from the original tumour material as well as from the passaged epithelial tumour cells revealed fairly constant amounts of EBV DNA. In order to establish whether EBV DNA was present or absent in the lymphocytes consistently found in NPC, nucleic acid hybridizations were performed with EBV cRNA and DNA derived from separated epithelial and lymphoid cell populations of NPC biopsies from Nairobi, Singapore and Tunisia.

While a piece of each tumour was kept for further DNA extraction, the remainder was manipulated in tissue-culture medium with mounted needles so that lymphoid and epithelial cells were liberated in the medium. The degree of dissociation was followed in phase-contrast microscopy and, if dissociation was not complete, clumped cells were separated with trypsin. When a homogeneous cell suspension was obtained, it was carefully layered on Radioselectan-Ficoll gradient, as previously described by Yata et al. (1973), and centrifuged at 400 g for 30 minutes. The epithelial cells sedimented out at the bottom, whereas the lymphocytes remained on top of the Radioselectan-Ficoll layer. The lymphocyte-containing band (more than 90% lymphocytes) and the pellet of epithelial cells were removed, washed with phosphate-buffered saline (PBS) and stored at -70°C until DNA extraction.

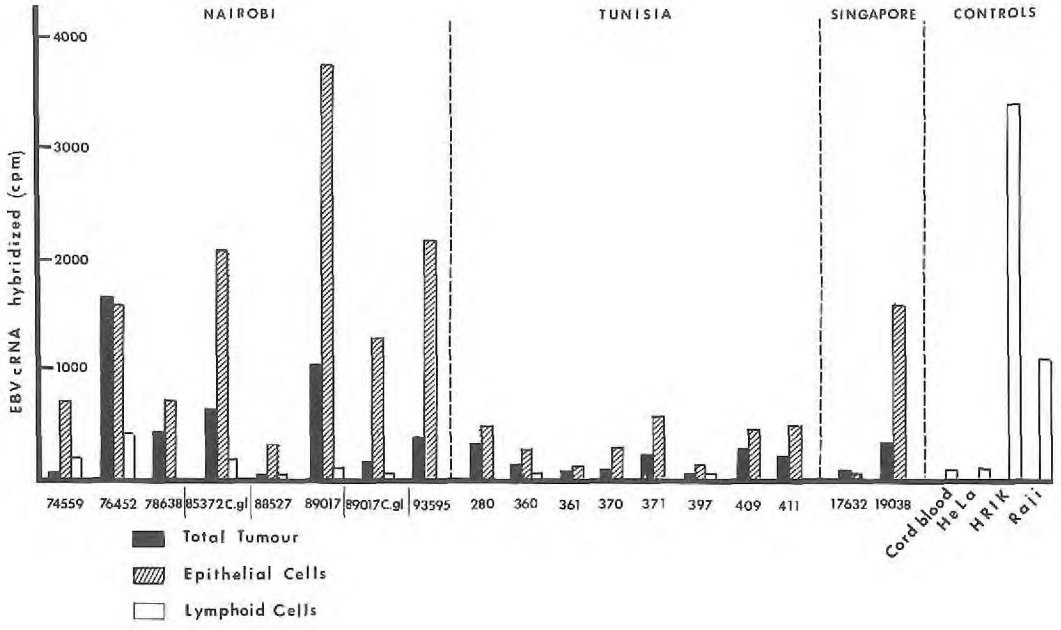
DNA extraction from non-fractionated tumour material, and from separated lymphocytes and epithelial-cell populations was done

as described previously (zur Hausen & Schulte-Holthausen, 1970). These DNAs were then bound to membrane filters and tested for the presence of EBV DNA by hybridization with labelled EBV-specific complementary RNA (57 000 cpm) under conditions described elsewhere (Wolf, 1974). The results are shown in Fig. 1, where epithelial fractions are seen to exhibit the highest hybridization counts as compared to non-fractionated material and to lymphoid cell populations. Hybridization of lymphocyte DNA with EBV cRNA was usually low and barely significant, with the exception of one highly positive tumour (K-76452), in which the lymphoid population may have been contaminated with non-lymphoid elements. There were variations between biopsies; these are being analysed with regard to pathology, serology and geographical areas.

In summary, these data confirm that, in NPC biopsies, EBV DNA is present predominantly in epithelial tumour cells. These results do not exclude the presence of EBV DNA within the infiltrating lymphocytes, as the sensitivity of the method used does not permit the detection of minute amounts of EBV DNA.

A detailed account of the findings summarized here will be published elsewhere.

FIG. 1. HYBRIDIZATION OF CELLULAR DNA FROM NPC BIOPSIES WITH EBV cRNA



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IN VITRO TRANSFORMATION BY HSV-2 FROM A HUMAN PROSTATIC CARCINOMA

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Our previous work on the incidence of herpesvirus in a random population of asymptomatic males has demonstrated by special co-cultivation techniques that the male genito-urinary tract serves as the reservoir for this virus (Centifanto et al., 1972). Further investigations on the presence of herpesvirus particles or their antigens in genito-urinary tissues led to the discovery of a herpesvirus particle in cancer cells from an untreated carcinoma of the prostate (Centifanto et al., 1973). This virus particle, which was identified as a herpesvirus on the basis of specific immunofluorescence staining, morphology and size, can transform cells in culture.

Inoculation of hamster embryo monolayers with a cell-free homogenate of the original cancer tissue resulted in the appearance of foci of transformed cells that developed into colonies of piled-up cells lacking contact-inhibition, and of an epithelial morphology different from that of the parental cell. Both the colony and cell morphology are reproducible after several passages (Fig. 1). The transformed cell cultures have a fast growth rate, release no infectious virus into the supernatant and can be grown in soft agar suspensions. The presence of the tumour-associated herpesvirus particle in the transformed cells was determined by specific immunofluorescence assays and colony inhibition test. Immunofluorescence staining with specific anti-HSV-2 serum showed an intense and distinctive nuclear and perinuclear staining in about 93% of the transformed cells (Fig. 2), while a dull non-specific fluorescence was observed in the cells of the control samples. No specific immunofluorescence staining was seen in the cytoplasm. The involvement of this tumour-associated herpesvirus in the transformation of hamster embryo cells was also investigated by the colony inhibition test (Hellstrom, 1967). A single-cell suspension of the transformed cells, which was incubated for 24 hours at 37°C, was exposed to splenic lymphocytes from either HSV-2 sensitized or non-sensitized guinea-pigs and their

FIG. 1. PHOTOMICROGRAPH OF A YOUNG COLONY OF TRANSFORMED HAMSTER EMBRYO CELLS (x 60)

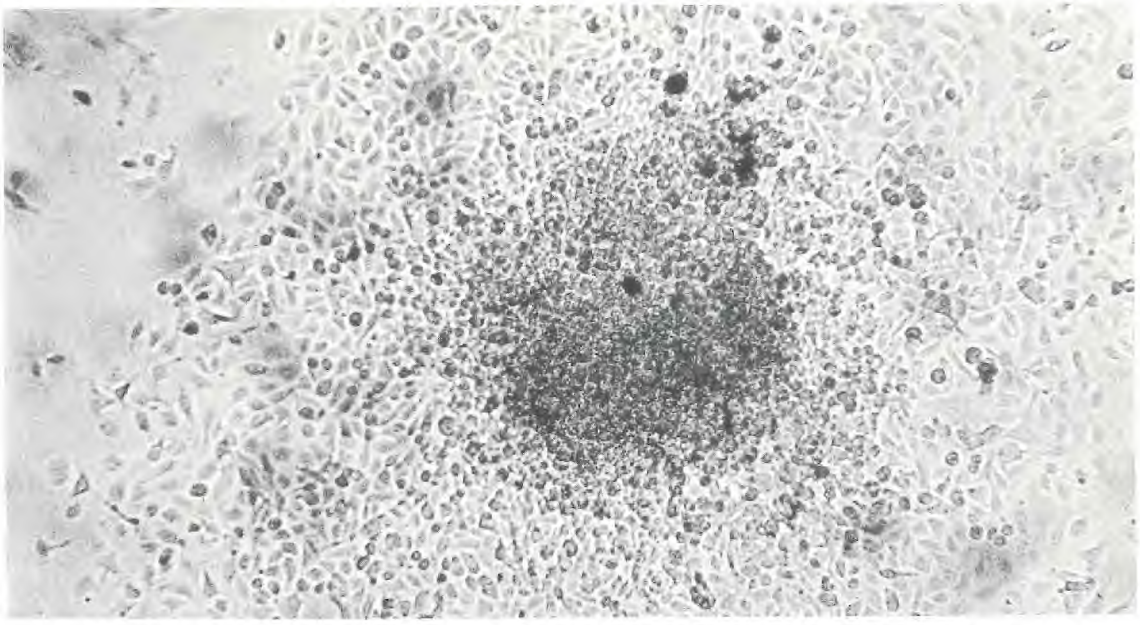
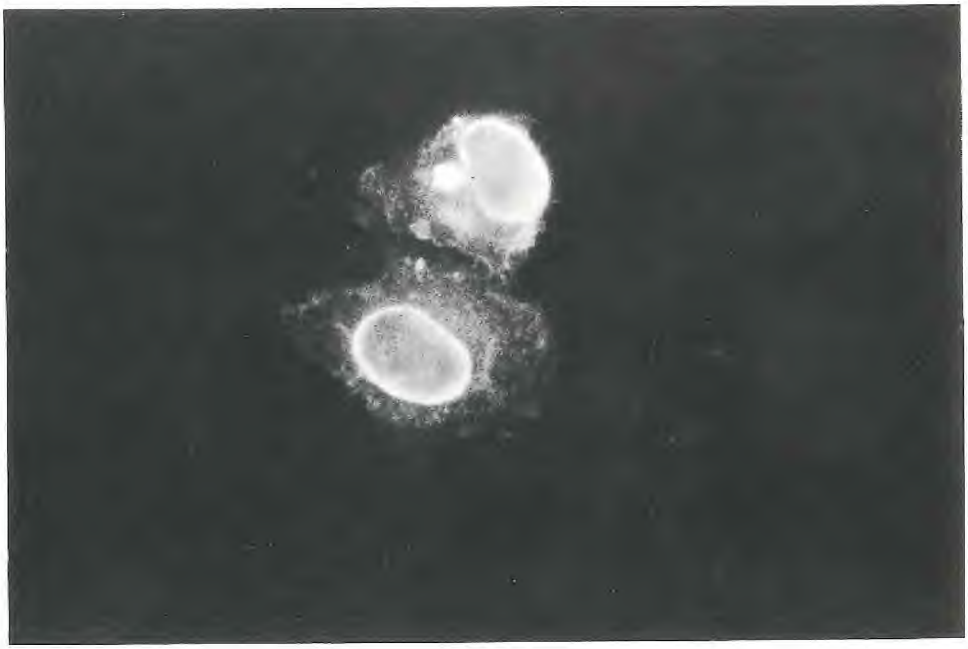


FIG. 2. FLUORESCENT STAINING OF TRANSFORMED HAMSTER EMBRYO CELLS

Specific immunofluorescent staining for herpes simplex virus type 2 (HSV-2) is seen in the nucleus and nuclear membrane of the cell.



growth measured after 72 hours incubation. At this time, control bottles that received media only and cultures with non-sensitized lymphocytes had a comparable number of healthy colonies while no growth was seen in those cultures that received the HSV-2 sensitized lymphocytes. Newborn Syrian hamsters were inoculated with 10^5 transformed cells in the intrascapular region. At the end of eight weeks, enlargement of the testes was observed and HSV-2 antigens were shown by immunofluorescence in the biopsy specimens.

In summary, we have described the transformation of hamster embryo cells by a herpesvirus particle found in human prostatic carcinoma cells. Although this virus particle has been identified as a herpesvirus, it does not fit the general characteristics of the known standard herpesvirus: it does not produce a lytic infection and is not found either in extracellular spaces or supernatant fluid of the transformed cell cultures. This virus is unique in that it is cell-associated, non-cytopathogenic, and capable of transforming cells *in vitro*, and its antigens persist in the transformed cells.

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MAREK'S DISEASE VIRUS DNA IN A CHICKEN LYMPHOBLASTOID CELL LINE (MSB-1) AND IN VIRUS-INDUCED TUMOURS

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Marek's disease (MD) is a neoplastic disease of chickens characterized by nerve lesions and lymphoid tumours. Its causative agent is a herpesvirus (MDV), but virus-specific antigens and virus particles are usually absent in the tumours. However, tumour-specific antigens have been detected on the surface of MD lymphoma cells (Ishikawa et al., 1972; Powell et al., 1974). The mechanism by which MDV induces tumours in chickens is still unclear. Early findings of significantly higher rates of DNA synthesis in lymphoid cells from MDV-inoculated, tumour-bearing chickens than in lymphoid cells from MDV-inoculated, non-tumour-bearing chickens indicated the oncogenic potential of MDV in neoplastic transformation (Lee, 1972). The recent establishment of cell lines from tumours of chickens inoculated with various strains of MDV (Akiyama et al., 1973; Powell et al., 1974) and the demonstration of MDV DNA in tumours and in a chicken lymphoblastoid cell line (Nazerian et al., 1973; Nazerian & Lee, 1974) have provided further evidence supporting the role of MDV in neoplastic transformation.

The present study deals with the presence of Marek's disease virus genome in cultured lymphoblastoid cells (MSB-1), in tumours from chickens inoculated with MSB-1 cells, in tumours from chickens inoculated with several strains of MDV, and in tumours from different organs of the same chicken.

A clone-purified pathogenic GA strain of MDV was used for the preparation of MDV DNA and complementary RNA (cRNA). The virus was propagated in duck embryo fibroblasts (DEF) in roller bottles (Lee et al., 1973). The culture fluid containing large numbers of virus particles was

harvested 96 hours after infection, concentrated and purified (Nazerian & Lee, 1974), and the virus DNA extracted and purified through two cycles of caesium chloride density gradients in an angular Spinco rotor 65. Transcription of purified MDV DNA into ^{3}H-labelled cRNA was accomplished by employing *Escherichia coli* RNA polymerase and ^{3}H-labelled ATP and unlabelled nucleoside triphosphates (Nazerian & Lee, 1974).

Chicken embryo fibroblasts (CEF) cultures and DEF cultures infected with the GA and JM strains of MDV were prepared (Nazerian et al., 1973). A chicken lymphoblastoid cell line (MSB-1), kindly provided by Dr S. Kato, was propagated as described (Nazerian & Lee, 1974).

Three groups of susceptible day-old specific pathogen-free chicks from inbred line 7_2 were separately inoculated, per chick, with 100 000 MSB-1 cells, and 500 000 cells each of DEF infected with the GA and JM strains of MDV respectively. One week after the appearance of MD clinical symptoms, the birds were sacrificed and tumours from various organs immediately removed and stored at -70°C.

Tumour DNA and cellular DNA were extracted by the procedure of Pettersson & Sambrook (1973). Heat-denatured DNA (10 µg) from various sources was fixed to 13-mm nitrocellulose membrane filters. The DNA-containing filters were baked at 80°C for 4 hours, incubated in 6 x SSC[1] containing 50% formamide and labelled MDV cRNA (30 000 cpm) for 72 hours at 43°C, and treated with ribonuclease followed by washing with 3 x SSC. The radioactivity on each filter was measured by liquid scintillation spectrometry and the DNA content on each filter colorimetrically determined by the diphenylamine reaction. For hybridization calibration, known quantities of MDV DNA were mixed with 10 µg of calf thymus DNA, heat denatured, fixed to membrane filters and incubated with MDV cRNA as above.

[1] Standard saline citrate (0.15 M sodium chloride, 0.015 M trisodium citrate).

HYBRIDIZATION OF MDV cRNA WITH MDV AND CELLULAR DNA

The results of cRNA hybridization experiments with MDV DNA, cellular DNA, and the DNA from several preparations of MSB-1 cell line, are summarized in Table 1. The specificity of the cRNA for MDV DNA was demonstrated by the binding of approximately 22.5% of cRNA to DNA from GA-MDV infected CEF. In contrast, no significant amount of MDV cRNA was bound to DNA from calf thymus, uninfected chicken embryo fibroblasts, normal chicken kidney, or normal chicken spleen. A standard curve for the estimation of the number of MDV genome equivalents per cell was constructed, based on the amount of cRNA bound to different quantities of MDV DNA (Table 1). The molecular weight of MDV DNA was 1×10^{8} daltons (Lee et al., 1971) and that of chicken DNA 1.7×10^{12} (Atkin et al., 1965). The amount of cRNA bound to MDV DNA from MSB-1 cells did not vary significantly among cell preparations from the 5th, 10th and 40th passages cultured for two days. In one preparation of MSB-1 (70th passage), in which the cells were cultured for four days, the amount of cRNA bound to MDV DNA was significantly higher. Approximately 130 genomes were found in these cells. The increase in the number of virus genomes per cell could be the result of the derepression of the resident genome for active replication of the virus, due to the aging of MSB-1 cells in culture.

Table 1. Hybridization of ^{3}H-MDV cRNA with MDV and cellular DNA

Source of DNA	^{3}H-MDV cRNA bound per 10 µg of DNA[a] (cpm)	No. of genome equivalents per diploid cell (approx.)
Calf thymus	205[b]	-
+ 27 ng MDV DNA	685	-
+ 55 ng MDV DNA	1 017	-
+ 110 ng MDV DNA	1 593	-
+ 220 ng MDV DNA	2 553	-
Chicken embryo fibroblasts	215	-
Chicken kidney	218	-
Chicken spleen	235	-
GA-MDV infected CEF[c]	6 751	-
MSB-1 (5th passage)[d]	764	54
MSB-1 (10th passage)[d]	859	68
MSB-1 (40th passage)[d]	813	61
MSB-1 (70th passage)[d]	1 250	130

[a] 30 000 cpm per 0.4-ml reaction mixture per filter.

[b] Average of two readings.

[c] GA strain of Marek's disease virus-infected chicken embryo fibroblasts.

[d] MSB-1 lymphoblastoid cell line.

HYBRIDIZATION OF MDV cRNA WITH TUMOUR DNA

Data given in Table 2 summarize the results of *in vivo* experiments on three groups of day-old MD-susceptible chicks inoculated with MSB-1 cells and the GA and JM strains of MDV. Both the GA and the MSB-1 inoculated chickens developed extensive visceral tumours, while the JM-inoculated chickens showed predominantly lymphoid lesions in the nerves, with some incidence of tumours in the ovary and testis. Tumour DNA from each of the three inoculated groups was examined for the presence of virus genome by cRNA hybridization. All tumour cells, irrespective of the source of MDV inoculum, contained MDV DNA. The amount of MDV DNA in tumours seemed to vary more between individual chickens in the same group than between groups of chickens inoculated with MSB-1 cells, and the JM and GA strains of MDV. The number of MDV genome equivalents per cell in tumours from different chickens was in the range 22-80. Since no hybridization was detected between MDV DNA and normal chicken DNA (Table 1) and no

Table 2. Marek's disease virus genome in chicken tumours induced by several strains of MDV

Bird number	Inoculum[a]	Source of tumour	^{3}H-MDV cRNA bound per 10 µg of DNA[b] (cpm)	No. of genome equivalents per diploid cell (approx.)
14033	GA-MDV	Ovary	616[c]	37
14820		Ovary	960	80
14822		Testis	770	54
14823		Testis	640	41
14828		Ovary	916	75
14793	JM-MDV	Ovary	559	32
14794		Testis	619	38
14795		Testis	750	53
14796		Ovary	629	39
14799		Ovary	453	22
15551	MSB-1	Testis	620	38
15557		Testis	471	24
15560		Ovary	821	61
15568		Ovary	583	34
15575		Ovary	858	66

[a] GA and JM strains of Marek's disease virus and MSB-1 lymphoblastoid cell line.

[b] 30 000 cpm per 0.4-ml reaction mixture per filter.

[c] Average of two readings.

DNA homology was found between duck embryo fibroblast DNA and chicken cell DNA (Lee[1]), the interaction between MDV cRNA and tumour DNA appeared to be highly specific, and only MDV DNA sequences in tumours were detected.

Table 3 presents the data on hybridization between MDV cRNA and DNA from various tumours of the same chicken. In tumours from different chickens, the amount of MDV DNA varied greatly from chicken to chicken. The significance of this variation is not known. No such variation, however, was detected between tumours from the same chicken; this agrees with similar results obtained with different tumours from the same Burkitt's lymphoma patients (zur Hausen et al., 1970; Lindahl et al., 1974). A stable virus-host interaction seemed to exist in each individual chicken.

[1] Unpublished data.

Table 3. Hybridization of ^{3}H-MDV cRNA with DNA from Marek's disease tumours

Bird number	Inoculum	Source of tumour	^{3}H-MDV cRNA bound per 10 µg of DNA[a] (cpm)	No. of genome equivalents per diploid cell (approx.)
14030	GA-MDV	Kidney	715[b]	48
		Ovary	693	46
		Spleen	701	47
14049	GA-MDV	Kidney	570	33
		Spleen	577	34
		Testis	590	36
14062	GA-MDV	Kidney	516	28
		Liver	512	28
		Ovary	533	29
14237	GA-MDV	Liver	601	37
		Ovary	619	38
		Spleen	586	35
14820	GA-MDV	Kidney	936	77
		Ovary	960	80

[a] 30 000 cpm per 0.4-ml reaction mixture per filter.

[b] Average of two readings.

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INDUCTION OF HERPESVIRUS SAIMIRI LYMPHOMA IN NEW ZEALAND WHITE RABBITS INOCULATED INTRAVENOUSLY

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The oncogenic property of herpesvirus saimiri (HVS) in various species of non-human primates has been well established by numerous investigators. These studies have been reviewed by Melendez et al. (1972a, 1972b) and by Deinhardt (1974).

In addition to non-human primate species, New Zealand white rabbits have been shown to be susceptible to HVS (Daniel et al., 1970, 1974a, 1974b).

This paper describes the induction of malignant lymphoma in adult, weaned and baby rabbits by the intravenous route, HVS being recovered from an experimentally infected rabbit.

In our previous studies (Daniel et al., 1974b), most of the rabbits were aged 3-4 months. They were inoculated by multiple routes and received various doses of virus inoculum. In the studies presented here all animals were inoculated with a single dose or occasionally two doses of virus.

MATERIALS AND METHODS

The HVS used in this study was recovered from lymphocytes obtained from a rabbit that died of malignant lymphoma (AE-270 No.2). These lymphocytes were derived from peripheral blood that was co-cultured in an owl monkey kidney cell line (210-OMK). A viral isolate was obtained after nine days in culture. This isolate was identified as HVS by serum neutralization test. A second passage of this virus in OMK was used for rabbit inoculation.

The rabbits used in this study were obtained from a local supplier and were aged four months (three rabbits), seven weeks (eight rabbits) and 10 days (seven rabbits). The four-month-old rabbits were inoculated

with 2 ml of virus intravenously. The seven-weeks-old rabbits were also inoculated with 1 ml of virus. Rabbits Nos. 1, 2 and 3 were inoculated with undiluted virus, rabbits Nos. 6, 7 and 8 with a dilution of 10^{-1}, and rabbits Nos. 4 and 5 were uninoculated controls. After 125 days, rabbits Nos. 2, 3, 6, 7 and 8 were inoculated with 1 ml of undiluted virus. The 10-day-old rabbits were inoculated by the intracardiac route with 0.5 ml of virus. After 115 days, rabbits Nos. 2, 5 and 6 received a second i.v. inoculation of 1 ml of virus. The titre of the virus inoculum was $10^{6.5}$ per ml.

RESULTS

Three four-months-old rabbits died between 17 and 84 days with malignant lymphoma.

The studies on the weaned animals (seven-weeks-old) are still in progress. Of seven animals inoculated, five have died or were sacrificed at the terminal stage between 11 and 196 days with malignant lymphoma.

Seven 10-days-old rabbits were inoculated. Of these five died with malignant lymphoma between 31 and 176 days; two are still alive after 206 days. Virus recovery was attempted from four of these rabbits from whole blood, lymphocytes, spleen and lymph gland. Virus was recovered from each of these tissues.

The histopathology of the disease seen in rabbits was similar to that described (Daniel et al., 1974b; Hunt et al., 1975). A unique feature seen in the weaned and baby rabbits was the development of leukaemia. The total white blood count ranged from 19 000 to 146 000, with lymphocytes and lymphoblasts accounting for the majority of the circulating leukocytes.

DISCUSSION

HVS is an oncogenic virus with a wide host range in monkeys. All available evidence suggests that the virus is indigenous to the squirrel monkey (*Saimiri sciureus*). In this animal host, the virus causes an inapparent persistent infection. These facts are well established.

The results of the present study confirm our previous observation (Daniel et al., 1974a, 1974b) that HVS induces malignant lymphoma in a species phylogenetically unrelated to primates. To date, no other virus has been shown to induce a neoplastic disease in New Zealand white rabbits. This is unique, both on the part of the virus, as well as on the part of the rabbit.

The disease in the rabbit is reproducible and resembles in many respects the picture seen in various species of monkeys infected with HVS. Histopathologically, the lymphoma is similar to that seen in monkeys, although the distribution of the neoplastic infiltrate is slightly different (Hunt et al., 1975). As with the monkey, no virion has been detected in infected rabbit tissues, although virus can be recovered with appropriate techniques. As in the monkey studies, recovery of virus can be accomplished through culture of circulating lymphocytes, or spleen or lymph-nodes. Virus has not been recovered from kidney cultures of infected rabbits. This is in contrast to what has been found in kidney cultures from infected monkeys. Although virus has not been isolated from rabbit kidney cultures, the presence of viral antigens in the cells has been demonstrated by fluorescent antibodies studies.

We had previously reported the absence of leukaemia in 3-4-month-old

rabbits (Daniel et al., 1974b). In the present study, we find that, in baby rabbits, leukaemia is present. The preliminary data suggest that this may be age-dependent. The presence of leukaemia is detected approximately two weeks prior to death of the animal.

The results of these studies show that:

(1) the intravenous route of inoculation produces an almost 100% incidence of disease;

(2) as in the susceptible monkey species, HVS causes a rapidly progressing lymphoproliferative disease in rabbits;

(3) animals of all ages - adults, weaned and newborn - are susceptible (animals older than four months have not been tested as yet);

(4) infected cells (*in vivo*) do not develop inclusion bodies, nor infectious virus, but proliferate as malignant cells;

(5) infectious virus is recoverable only by co-cultivation with susceptible cells.

The similarity of the rabbit-virus interrelationship with that of the monkey-virus interrelationship provides a model system that will allow further studies of the oncogenicity of HVS at considerably less expense. This is of particular importance in view of the current decreasing availability and increasing cost of non-human primates.

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DISCUSSION SUMMARY

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The papers presented were concerned with the association of herpesviruses with naturally occurring and experimentally produced tumours, and may be divided into the following categories: (1) experimental oncogenesis by Epstein-Barr virus (EBV) in New World primates; (2) the site and nature of the association of EBV with two human diseases, namely, infectious mononucleosis and nasopharyngeal carcinoma; (3) direct detection of Marek's disease virus in tumours and tumour cell lines by nucleic acid hybridization; (4) a new host, the New Zealand white rabbit, for herpesvirus saimiri; (5) establishment of cell lines from prostatic cancer biopsies.

The oncogenicity of transforming strains of EBV has now been demonstrated in four species of New World primates, namely, cotton-topped marmosets, owl monkeys, common marmosets and squirrel monkeys. Several different types of virus material have been used in these experiments, including extracellular virus from the EBV-converted marmoset cell line, B95-8, the Kaplan strain of mononucleosis-derived virus, autochthonous squirrel monkey cells transformed *in vitro* by B95-8, and an extract of the EB3 cell line. In animals given the B95-8 virus, viraemia and the development of antibody to viral capsid antigen can be prevented by prior neutralization of the virus inoculum with antiserum. As well as malignant lymphoma, two other types of infection have now been described, namely, transient lymphoid hyperplasia and inapparent infection. The entire spectrum of host responses can be seen with the same virus inoculum, and this variation may be the result of the inoculum containing a borderline oncogenic dose. It is not known whether changes in the pathogenicity of the B95-8 virus have occurred with *in vitro* passage. The problem of crossed-species oncogenicity must be considered in evaluating the animal experiments and comparing them with naturally occurring disease.

EBV has been directly demonstrated in nasopharyngeal carcinoma (NPC) in the majority of histologically confirmed

cases from several parts of the world including Kenya, Tunisia, Taiwan, and Singapore. On the basis of the rate of reassociation of cellular DNA from NPC biopsies with the ^{3}H-DNA from the P3J-HR-1 virus, it is suggested that there may be differences in the nature of the EBV sequences present in different NPC biopsies. However, the important experiment of "recycling" the unhybridized portion of viral DNA has not yet been done. In general, some doubt remains as to the ability of the nucleic acid hybridization technique to detect very small differences among EBV strains. Another important question is the significance of NPC biopsies that lack evidence of the EBV genome. At least two explanations are possible, namely, that genome-negative biopsies represent the same histological disease but with a different etiology, or that subtle differences in histology exist and that EBV is associated only with one histological type of tumour. Three types of evidence now indicate that EBV is associated with the epithelioid cells of the tumour: (1) the viral DNA has been found in epithelial cells by *in situ* hybridization; (2) when epithelial cells are physically separated from lymphoid elements of the tumour, the cell DNA from the former exhibits greater hybridization with viral DNA; and (3) epithelial tumour cells passaged in "nude" mice have been found to contain viral DNA and EBV-determined nuclear antigen (EBNA). It is apparent that this finding of the association of EBV with a carcinoma emphasizes the need to continue the search for epithelial cells in the naso- and oropharynx that are susceptible to the virus.

The nature of the association of EBV with peripheral blood lymphocytes in mononucleosis was the subject of considerable discussion. Two contrasting hypotheses were presented. The first hypothesis holds that a small number of transformed cells circulate in the peripheral blood and that immunological surveillance mechanisms continually eliminate these potentially oncogenic cells. The second hypothesis suggests that EBV is associated with non-transformed cells in the peripheral blood in a manner analogous to the association of herpes simplex virus with dorsal root ganglia cells. In favour of the first hypothesis are findings that patients with mononucleosis develop antibodies against EBNA and the unpublished findings that peripheral blood leukocytes from mononucleosis patients are capable of forming colonies in soft agar. In favour of the second hypothesis is the demonstration that a two-step process of transformation appears to occur when mononucleosis blood leukocytes are placed *in vitro*. This suggests that a latent viral genome is activated and is then transferred to uninfected cells *in vitro*. However, the exact location of the virus in the

mononucleosis cells has not been pinpointed. Among the possibilities discussed were an intracellular location in lymphocytes, a location on the surface of lymphocytes, and possibly free virus in the plasma.

A possible new model for herpesvirus saimiri (HVS) in the rabbit was described. The peculiarities of this model, in contrast to the disease in monkeys, are the involvement of conjunctiva and nose and lips, the failure of rabbits to develop antibodies to HVS, and the lack of involvement of the kidney. Further work is necessary to prove that HVS is the etiological agent solely responsible for the lesions described.

Marek's disease virus can now be demonstrated by nucleic acid hybridization in tumours and in cell lines derived from tumours. Complementary sequences to the virus are not found in normal chick embryo fibroblasts or in organs obtained from non-infected chickens.

Cell lines have been established from prostatic carcinoma and the cell lines are transplantable to hamsters. The evidence that the cell lines contain herpesviruses or herpesvirus antigens is still preliminary.

HOST RESPONSES

HOST RESPONSES TO HERPESVIRUSES – A REVIEW

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In a discussion of immune responses to herpes-group viruses, it is important to differentiate between primary infections, ensuing latent carrier states, activation of persistent infections, and possibly virus-induced malignancies. While primary immune responses can be confirmed serologically with some assurance, it is far more difficult to prove on a serological basis an activation of latent infections or an association of given herpesviruses with malignancies. In these two situations, determination merely of neutralizing or other antibodies to structural components of the virus may fail to provide significant information against the background of normally observed antibody levels in silent carrier states. It may be necessary, therefore, to resort to detection of antibodies to other virus-determined, but non-structural viral antigens that may not normally be present in healthy carriers.

It might be a blessing in disguise that to date no fully permissive cells have been found in which the Epstein-Barr virus (EBV) replicates with large yields of infectious progeny. The development of serological test procedures had thus to be based on various virus-determined antigens found in cultured lymphoblasts from EBV-producer or non-producer lines *per se* or after certain manipulations. As already discussed by Dr Klein[1], at least four different groups of antigens have been differentiated with the aid of selected human sera. In turn, tests for detection and titration of the corresponding antibodies have become available that have revealed a remarkable association of certain antibodies with given disease states. There is little doubt, and indeed evidence is available, that the equivalent groups of antigens can be differentiated in cells infected or transformed by other herpesviruses.

It is unusual for a human virus, for once, to serve as a model for other human as well as animal viruses. Accordingly, we shall concentrate on immune responses to EBV, not refraining from some speculation, and shall

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[1] See *Part 1*, p.294.

emphasize gaps in knowledge that need to be closed.

PRIMARY EBV INFECTIONS

EBV is the cause of infectious mononucleosis (IM) (Henle & Henle, 1972, 1973a) but not every primary infection is accompanied by clear signs of this disease. Some remain silent and others are not specifically diagnosed when only mild illnesses ensue or the patient presents with uncharacteristic features.

That IM is a consequence of primary EBV infections is clearly evident from the facts that: (*a*) the disease occurs only in individuals who previously had no antibodies to EBV; and (*b*) antibodies to some EBV-related antigens appear regularly and others at a high frequency in the course of IM. Pertinent references are to be found in a recent review (Henle et al., 1974). Briefly, IgM and IgG antibodies to EBV capsid antigen (VCA) develop and reach peak titres successively in the early acute phase of IM. Antibodies to EBV-determined cell membrane antigens (MA) and EBV neutralizing antibodies, which in part appear to be identical, arise slightly later than anti-VCA. The majority of IM patients show in addition a transitory antibody response to EBV-induced early antigens (EA), which is usually directed against the D (diffuse), rarely against the R (restricted), component of the EA complex. Antibodies to the soluble (S) complement-fixation antigen and to the EBV-associated nuclear antigen (EBNA) develop in every case but, as a rule, only during convalescence. It appears that EBNA is the major, but possibly not the sole, component in S antigen preparations (Klein & Vonka, 1974[1]; Henle & Henle[1]). The striking difference in the time of emergence of anti-VCA, anti-EA, anti-MA and neutralizing antibodies, on the one hand, and of anti-S and anti-EBNA, on the other, requires an explanation that will be discussed later. Finally, the transitory response in heterophil antibodies is practically pathognomic for IM, but it fails to materialize in about 10% of adult patients, more frequently in children, and in nearly all infants. The exact role of EBV in eliciting these heterophil IgM antibodies is unknown.

PERSISTENT VIRAL CARRIER STATE

Primary EBV infections lead regularly to a persistent viral carrier state. A remarkably stable equilibrium between the virus and host defences is suggested by the remarkably constant titres of anti-VCA, neutralizing antibodies and anti-EBNA over observation periods of many years (Henle & Henle, 1970; Henle et al., 1971; Hewetson et al., 1973). It is rare to find anti-R, and even rarer to find anti-D in healthy carriers and then only at low titres among those who maintain relatively high anti-VCA titres (Henle & Henle, 1973b).

ACTIVATION OF THE VIRAL CARRIER STATE

The usually stable equilibrium between EBV and host defences may be upset by a variety of malignant and non-malignant diseases. As compared to appropriate controls, an over-representation of high anti-VCA titres has been observed among patients with Hodgkin's disease (HD), other lymphocytic lymphomas, certain leukaemias or various carcinomas, as well as among patients with sarcoidosis, systemic lupus erythematosis or rheumatoid arthritis (Henle & Henle, 1973b). The elevated antibody titres might possibly

[1] Unpublished data.

be accounted for by the immunosuppressive effects of these diseases or their therapy, which may activate persistent infections and lead to enhanced antibody production by committed lymphocytes. High anti-VCA titres may be accompanied by anti-D and/or anti-R at low and occasionally at substantial titres. It will be important to correlate the serological data with evidence for immunosuppression, as is discussed later by Drs Levine[1] and Johansson[2].

EBV-ASSOCIATED MALIGNANCIES

It is possible that the generally high titres of antibodies to EBV-related antigens in Burkitt's lymphoma (BL) of African children and in anaplastic or poorly differentiated nasopharyngeal carcinoma (NPC) are in part also accounted for by immunosuppressive effects of these malignancies. However, the association of EBV with BL or NPC does not rest solely on serological evidence, as in the other malignant and non-malignant diseases mentioned in the preceding section. EBV DNA and EBNA have been found at high frequency in African BL biopsies and in the carcinoma cells of NPC biopsies, but not in biopsies from patients with HD, other lymphomas or, with rare exception, other carcinomas of the head and neck region (zur Hausen et al., 1970, 1974; Nonoyama et al., 1973; Wolf et al., 1973, 1974; Lindahl et al., 1974; Reedman et al., 1974; Klein et al., 1974; Huang et al., 1974). Thus, African BL and NPC stand in a class by themselves.

The EBV-related serological reactivities of BL and NPC patients also differ considerably from those seen in patients with the other diseases as to the regularity of detection of antibodies to EBV, as well as their spectra and titres. There is a remarkable difference also between BL and NPC in that antibodies to the EA complex in BL are predominantly directed against the R and in NPC against the D component (Henle et al., 1971). The antibody spectra and titres increase with the stage of the disease to some extent in BL (Magrath et al.[3]) and to a considerable degree in NPC (Henle et al., 1970, 1973a). BL patients who have been brought to remission have a good chance to become long-term survivors if they have no anti-R or show a gradual decline in its titres, whereas persistence or development of high anti-R and/or anti-D titres foreshadows multiple and ultimately fatal relapses (Henle et al., 1973b). Successful therapy of NPC is followed by a gradual decline of anti-VCA titres to lower levels and loss of anti-D (Henle et al., 1973a). Thus, the EBV-related serology may serve to monitor progression of these malignancies (increases in antibody titres and spectra) as well as the effectiveness of therapy (declines in titres and loss of certain antibodies), that is, provide some prognostic information.

COMPARABLE ANTIGEN-ANTIBODY SYSTEMS OF OTHER HERPESVIRUSES

MA, EA and LA (late antigens equivalent to VCA) have been differentiated in herpesvirus saimiri (HVS)-infected cells (Pearson et al., 1972, 1973; Klein et al., 1973). As with EBV, EA appears to be composed of two components yielding different types of immunofluorescent staining (Klein et al., 1973). In the natural host, silent

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[1] See p.225.

[2] See p.238.

[3] Unpublished data.

carriers of the virus maintain anti-LA and neutralizing antibodies but not anti-EA. In primary HVS infections of marmosets or owl monkeys, a transitory anti-EA response is observed when no tumours develop, and persistent, rising anti-EA titres when the animals develop lymphoproliferative malignancies. These observations show striking similarities with EBV infections of man. A nuclear antigen, equivalent to EBNA, has not yet been demonstrated in HVS-transformed cells, possibly for technical reasons, since it is likely to exist.

EA has been differentiated also in herpes simplex virus (HSV)-infected cells by interruption of the infectious cycle by cytosine arabinoside-C but corresponding antibody titrations have failed to reveal differences in incidence and titres when sera from donors with recurrent herpes, cervical dysplasia, carcinoma *in situ* or invasive carcinomas were compared (Koldovsky et al.[1]). A nuclear antigen that may be equivalent to EBNA has been found recently in HSV-transformed cells (Chang et al., 1974), but its application to serological surveys has not yet been reported. HSV non-virion antigens have been isolated from cells within a few hours after infection with the virus and an apparently identical antigen from squamous-cell carcinomas (Tarro & Sabin, 1970; Hollinshead et al., 1973; Aurelian et al., 1973). There seems to be no counterpart among known EBV-related antigens, since the corresponding complement-fixing antibodies to HSV non-virion antigens are found almost solely in patients with squamous-cell carcinomas, including NPC. It has not yet been determined whether these antigens are coded for by the virus or represent tissue-specific or embryonal components derepressed, among others, by herpesviruses.

ANTICELLULAR IMMUNE RESPONSES

Information on immune responses to cells transformed by EBV (or other herpesviruses) is as yet very limited. That EBV-transformed cells arise in the course of IM and remain present in the ensuing persistent infection may be deduced from the establishment of EBV genome-carrying lymphoblast lines from patients and healthy carriers and the differential appearance of anti-VCA, anti-MA, anti-EA and neutralizing antibodies, on the one hand, and anti-S and anti-EBNA, on the other. It has been suggested (Henle et al., 1974) that the two groups of antigens become available for stimulation of antibody synthesis under different conditions. VCA, EA, MA and virus particles are obviously derived from productively infected cells (whatever they may be) and these invariably degenerate with consequent release of the respective components. If EBNA synthesis were part of the lytic cycle, it would be produced in insufficient amounts, as a rule, to evoke early antibody responses. EBNA is present, however, in all EBV-transformed cells that are viable and divide presumably *in vivo* as they do *in vitro* (Reedman & Klein, 1973). There is evidence that some of the cells transformed by EBV during primary infections enter the blood stream and that these are the cells that regularly grow in culture into permanent lymphoblast lines. Indeed, colonies may be obtained from peripheral leukocytes of IM patients when seeded directly in soft agar (Hewetson[2]). It is noteworthy in this respect that virus excreted into the oropharynx readily transforms lymphoid

[1] Unpublished data.

[2] Unpublished data.

cells *in vitro* (Gerber et al., 1973; Miller et al., 1973) but has failed in our hands to induce EA synthesis in lymphoblasts from non-producer lines. The time elapsing before anti-EBNA becomes detectable may depend, therefore, on the time required for development of an effective anticellular immune response resulting in partial destruction of the transformed cell population and, with it, in the release of EBNA for antibody stimulation.

It appears that EBV-transformed cells are never completely eliminated, since EBV-carrying lymphoblast lines can be established regularly from lymph-nodes of anti-VCA positive donors (Nilsson et al., 1971) and, at variable frequencies, from their peripheral blood, depending on the numbers of leukocytes placed in culture (Diehl et al., 1969; Gerber & Monroe, 1969; Moore et al., 1967). In the absence of other known virus reservoirs, the sources of antigens for continued maintenance of anti-VCA and neutralizing antibodies are presumably EBV-transformed cells after spontaneous induction; these would be too limited in number, as a rule, to yield enough EA also to maintain anti-D and/or anti-R at detectable levels. EBNA for persistent antibody stimulation would be derived from transformed cells during the continuing, but never completed, process of their elimination by host defences. EBNA appears to be the equivalent of the T-antigens of papova- or adenovirus-transformed cells. Animals bearing tumours induced by these viruses have antibodies to the specific T antigens, but they disappear after removal of the tumours. The persistence of anti-EBNA in healthy viral carriers might thus be taken as evidence for the continued presence of EBV-transformed cells rather than as an indication that EBNA is not a T antigen.

If release of EBNA for maintenance of the corresponding antibodies depended on cell-mediated immune reactions, the anti-EBNA titres observed in patients with various malignant diseases might reflect the degrees of immunosuppression evoked by these conditions. Pertinent studies are in progress with as yet limited results. In NPC, the anti-EBNA titres are usually higher than in controls. In BL, they may vary from >1:640 to <1:2 in moribund patients, but a few long-term survivors may also show unusually low levels. The situation in these two malignancies may be complicated by the possible release of EBNA from necrotic tumours. Thus, very high anti-EBNA titres would not necessarily imply highly effective anticellular immune reactions, and very low anti-EBNA levels could denote blocking of antibodies or of effector cells by antigen rather than a decline in cell-mediated immunity. This problem would not arise in malignancies free of EBV genomes. To assess the influence of immunosuppression, patients with organ transplants are being studied in collaboration with Drs Hauw The and Milan Fiala. It appears that the incidence of very low anti-EBNA titres is greater in such patients than in appropriate controls. It is too early, however, to draw definite conclusions.

As to actual anticellular reactions, none of the various antibodies discussed might be expected to be cytotoxic, except anti-MA. This proved not to be the case. However, the recent differentiation of early and late MA components (Ernberg et al., 1974; Silvestre et al., 1974) suggests further studies, since the late MA might be the equivalent of surface antigens on lytically HSV-infected cells that are lysed by antibodies in the presence of C' (Roane & Roizman, 1964; Ito & Barron, 1972; Smith et al., 1972).

A C'-dependent, heat-labile antibody that is cytotoxic for cultured lymphoblasts has been found in many

human sera (Herberman & Nam, 1971). This antibody proved unrelated to EBV, however, since it was found at similar frequencies among anti-VCA negative and positive sera (Yang & Hewetson, 1974).

Preliminary observations (Hewetson[1]) indicate that a humoral anticellular factor may develop in IM. Colony formation by peripheral leukocytes of patients could be reduced in some instances by incorporation of serum from the autologous as well as other patients into the soft agar, but not when anti-VCA negative sera were used.

Information on cell-mediated immune responses is equally sketchy. Peripheral lymphocytes from BL or IM patients or from healthy viral carriers showed no significant cytotoxicity for BL biopsy cells or cultured lymphoblasts of various origins, according to one report (Hewetson et al., 1972), but others have recorded positive, though fluctuating results (Rosenberg et al., 1974). Cells from draining lymph-nodes of two BL patients proved highly cytotoxic, indicating that effector cells might be found more readily near or at sites where transformed cells are concentrated (Hewetson et al., 1972).

Exposure of peripheral lymphocytes *in vitro* to cultured lymphoblasts of various origins converts them within 4-6 days into efficient attacker cells, as determined by the ^{51}Cr release or colony inhibition techniques (Hardy & Steel, 1971; Golub et al., 1972a, 1972b; Steel et al., 1973; Svedmyr et al., 1974). This was found true also for autologous confrontations, i.e., when cultured BL or IM lymphoblasts were used to stimulate lymphocytes of the autologous donors. The antigen-stimulated (AS) lymphocytes were active against a broad spectrum of cultured lymphoblasts from EBV-producer and non-producer lines of BL, NPC, IM or healthy donor origin or obtained by *in vitro* transformation of lymphoid cells. In fact, the AS lymphocytes were cytotoxic also for cultured lymphoblastoid cells *free* of EBV genomes, but not for phytohaemagglutinin-stimulated blastoid cells (Svedmyr et al., 1974). These results indicate that the *in vitro* cytotoxicity of AS lymphocytes is directed in part, if not totally, against surface antigens of lymphoblasts that are not directly related to EBV and may represent, among others, organ-specific or embryonal components. It is currently unknown whether AS lymphocytes may in addition recognize EBV-specific cell-membrane antigens.

CONCLUDING REMARKS

It is all too apparent that much remains to be learned and clarified. Further differentiation of distinct, virus-determined antigens and development of procedures for detection of the corresponding antibodies may prove useful not only for EBV but also for other potentially oncogenic herpesviruses in linking them to given malignancies. Of greatest importance will be a continued search for anticellular immune responses of viral or other specificities, whether humoral, cell-mediated or a combination of both. Once sensitive and reproducible test procedures become available, answers may be obtained to questions such as: what mechanisms arrest the unrestrained growth of EBV-transformed cells in the course of IM and the subsequent viral carrier state? Are the mechanisms involved not elicited, defective or blocked in patients with EBV-associated malignancies? The same questions apply to other potentially oncogenic herpesviruses.

1 Unpublished data.

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HUMORAL AND CELLULAR IMMUNITY TO EBV AND LYMPHOID CELL LINE ANTIGENS IN HUMAN LYMPHOMA

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Elevated antibody levels to the Epstein-Barr virus (EBV) have been demonstrated in several lymphoproliferative diseases, but only in one, infectious mononucleosis (Henle & Henle, 1972), has the antibody level been shown to have etiological implications. Several explanations have been advanced for the high titres in human lymphoma: (1) EBV has an etiological relationship; (2) the high titres reflect an imbalance of humoral immunity in the presence of depressed cellular immunity; and (3) the high titres reflect an inappropriate response to EBV not directly linked to (1) or (2) above but possibly linked to a state of susceptibility to cancer. In studies focusing on the second possibility, we have attempted to compare cellular immunity (CMI) to a variety of antigens with EBV titres in lymphoma patients and controls to determine whether there was a relationship between CMI and humoral antibodies to EBV. In a review of studies on 48 patients whose general CMI was monitored by skin tests (Levine & Reisher, 1973), we concluded that the elevated EBV titres were not the result of general CMI depression. In this report, we shall summarize our extended studies on skin testing (these have recently included antigens more relevant to lymphoproliferative tumours) and

compare the EBV titres to *in vitro* as well as *in vivo* measurements of CMI.

MATERIALS AND METHODS

All patients entered into our studies were skin tested with a battery of standard antigens to measure their general immune status. Recall antigens used in these studies included streptokinase/streptodornase (SKSD), Candida, mumps, Trichophyton and intermediate tuberculin purified protein derivative (PPD). Primary sensitization with keyhole limpet haemocyanin, *Brucella abortus* and dinitrochlorobenzene (DNCB) was used when possible (Hesse et al., 1973).

Membrane preparations of several lymphoid cell lines (Table 1) were prepared using a modification of the method of Davies (Oren & Herberman, 1971). Protein concentrations for all skin tests were adjusted to 0.1 mg/0.1 ml. The absence of infective EBV in the preparation was monitored by confirming its failure to induce early antigen in the Raji cell line. All skin test preparations were monitored for micro-organisms prior to inoculation. Intradermal inoculation of 0.1 ml of the membrane extracts as well as the standard antigens was used in skin tests. Inoculation of the membrane extracts was restricted to cancer patients with incurable disease or untreated patients with antibody to EBV. The presence of 5 mm or more of induration at 48 hours was considered to be a positive skin test (ST).

A ^{51}Cr-release assay for lymphocyte-mediated cytotoxicity, an *in vitro* assay utilized as a measurement of CMI (McCoy et al., 1973; Rosenberg et al., 1974), was performed using the F265 cell line as the target. Antibodies to the EBV capsid antigen (VCA) and early antigen (EA) were measured by standard fluorescent techniques as

Table 1. Source of antigens

Cell line	Source	EBV membrane antigen	EBV genome[a]
Raji	Burkitt's lymphoma (African)	-	+ (60)
Onemus	Burkitt's lymphoma (African)	+	+
Maku	Burkitt's lymphoma (African)	+	+
HKLY28	Nasopharyngeal carcinoma	+	+
F265	Normal	-	+ (100)
NC37	Normal	-	+ (80)
P3HR-1[b]	Burkitt's lymphoma (African)	+	+

[a] Numbers in parentheses are genome equivalents. Source: Pagano (personal communication).

[b] Used only in *in vitro* studies.

previously described (Henle et al., 1972). The P3HR-1 cell line was used as a source of VCA antigen, and chemically treated (BUDR and IUDR) Raji cells served as a source of EA.

RESULTS

The addition of a greater number of patients to the studies on the relation between EBV antibodies and the immune status of the patient, as measured by delayed hypersensitivity to standard antigens (Table 2), continued to demonstrate that reactive individuals often have EBV titres as high as anergic cases. Different patterns were apparent, with Hodgkin's disease and nasopharyngeal carcinoma (NPC) cases having slightly higher VCA titres if they were anergic, and non-Hodgkin's lymphoma and other solid tumour cases having higher VCA titres

Table 2. EBV titres (VCA and EA) for cancer patients and normals with positive and negative skin tests to a battery of standard antigens

Disease group	VCA titres				EA titres			
	Reactive[a]		Anergic[b]		Reactive[a]		Anergic[b]	
	Number[c]	GMT[d]	Number[c]	GMT[d]	Number[c]	GMT[d]	Number[c]	GMT[d]
Hodgkin's disease	17/18	222	4/5	380	12/18	37	4/5	34
Other lymphomas	23/24	298[e]	7/7	98[e]	19/24	20	4/7	34
Nasopharyngeal carcinoma	11/11	455	2/2	640	11/11	59	2/2	57
Other solid tumours	19/19	425	2/2	320	18/19	24	2/2	10
Normals	11/13	82	1/1	80	4/13	12	1/1	80
Totals	81/85	270	16/17	199	64/85	28	13/17	32

[a] Reactive (≥5 mm induration 48 hours after skin testing) to at least one standard antigen.

[b] Anergic (not reactive) to all standard antigens utilized.

[c] Number with positive EBV titres (≥ 1:5)/number tested.

[d] Geometric mean titre for those with positive titres.

[e] Difference between reactive and anergic group is statistically significant ($P < 0.05$).

if they were reactive. However, the number of cases in any one subgroup was small, and only the VCA titres of the non-Hodgkin's lymphoma group demonstrated statistically significant differences ($P < 0.05$) between reactive and anergic cases. The importance of using antigens prepared from tissue culture lines is shown in Table 3, where patients with lymphoma, acute lymphocytic leukaemia (ALL) and NPC demonstrated greater reactivity to antigens from tumour-derived cell lines than to antigens from cell lines derived from the peripheral blood of normal individuals. Extracts from the cell lines rarely elicited positive delayed hypersensitivity reactions in patients with soft tissue sarcomas or carcinomas other than NPC, although they reacted to the standard ST antigens as often as the lymphoma, ALL and NPC patients. Utilizing the more disease-related antigens associated with the cell lines (Table 4), the pattern was similar to that observed with the standard antigens. The non-Hodgkin's lymphoma patients with positive skin tests had significantly higher levels of VCA and EA antibody than the non-reactive group, and solid tumour patients also showed a similar pattern that was less pronounced. In contrast, reactive Hodgkin's disease and NPC patients had lower titres than patients who

Table 3. Patient reactivity to extracts of lymphoid cell lines

Disease group	Cell lines							
	Raji		HKLY28		F265		NC37	
	Number[a]	(%)	Number[a]	(%)	Number[a]	(%)	Number[a]	(%)
Lymphoma	16/36[b]	(44)	3/14	(21)	4/26	(15)	0/10	(0)
Hodgkin's disease	5/11	(45)	1/5	(20)	0/5	(0)	0/4	(0)
Non-Hodgkin's lymphoma	11/25	(44)	2/9	(22)	4/21	(19)	0/6	(0)
NPC[c]	2/16	(13)	9/17	(53)	1/17	(6)	1/17	(6)
Solid tumours[d]	0/53	(0)	9/46	(20)	1/46	(2)	5/46	(11)
ALL[e]	26/65	(40)	-	-	1/54	(2)	-	-

[a] Number of patients with ≥5 mm induration/number tested.

[b] Includes 12/22 positive for Onesmus and/or Maku.

[c] Nasopharyngeal carcinoma.

[d] Carcinomas and soft-tissue sarcomas other than NPC.

[e] Acute lymphocytic leukaemia.

were skin-test negative, but the number of patients was too small for firm conclusions to be reached.

Studies with the lymphocyte-cytotoxicity test (LC) indicated a relationship between the assay and general CMI, since cancer patients generally had lower values than simultaneously tested clinically healthy individuals (Fig. 1). There was no clear relationship between LC and EBV titre (Fig. 2). Most cancer patients had lower than normal levels of cytotoxic reactivity, but there were as many patients with low EBV VCA antibody levels in the group with apparently depressed cytotoxicity values as there were patients with high EBV antibody levels. In the individuals without cancer, as in the cancer patients, low EBV VCA antibody levels were more frequent in those with low values in the LC assay. The correlation between EBV VCA titre and LC response was not complete, however, since there were individuals with cytotoxic reactivity against the F265 cell line who had no antibody to EBV and there were also individuals with high titres to

Table 4. EBV titres (VCA and EA) for cancer patients with positive and negative skin tests to tumour-derived cell lines

Disease Group	VCA titres				EA titres			
	Positive[a]		Negative[b]		Positive[a]		Negative[b]	
	Number[c]	GMT[d]	Number[c]	GMT[d]	Number[c]	GMT[d]	Number[c]	GMT[d]
Hodgkin's disease	5/5	160	2/4	905	3/5	50	2/4	69
Other lymphomas	8/8	698[e]	9/10	105[e]	8/8	24[e]	6/10	11[e]
Nasopharyngeal carcinoma	7/7	307	6/6	806	7/7	47	6/6	76
Other solid tumours	4/4	708	17/17	364	4/4	40	16/17	19
Totals	24/24	405	34/37	318	22/24	36	30/37	24

[a] Positive skin test to at least one of the tumour-derived cell lines shown in Table 1.

[b] Negative skin tests to all of the tumour-derived cell lines.

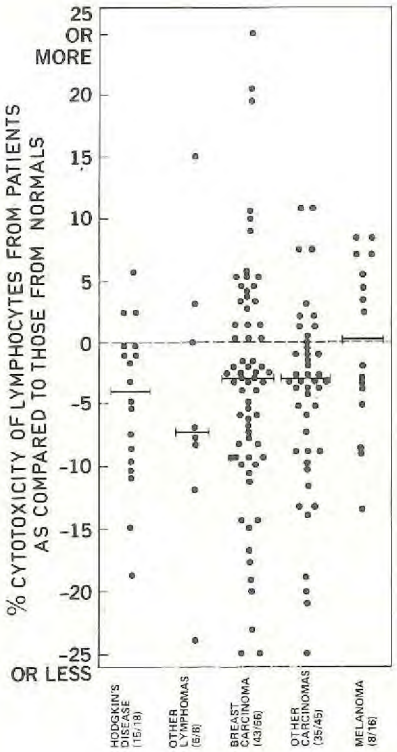
[c] Number with positive EBV titres/number tested.

[d] Geometric mean titre for those with positive titres.

[e] Difference between reactive and anergic group is statistically significant ($P < 0.05$).

FIG. 1. CYTOTOXICITY OF LYMPHOCYTES FROM CANCER PATIENTS

Results of ^{51}Cr-release assay of lymphocyte cytotoxicity to F265 cells using lymphocytes from cancer patients. Figures in parentheses are values of the ratio No. ≤0/No. tested. Position of median values is shown by horizontal bars.



EBV who had low levels of cytotoxicity. The independence of the *in vitro* measurement of CMI (LC) and the *in vivo* ST assay was demonstrated by the rarity of a positive ST response (7.8%) to F265 in contrast to an LC response rate of 95%.

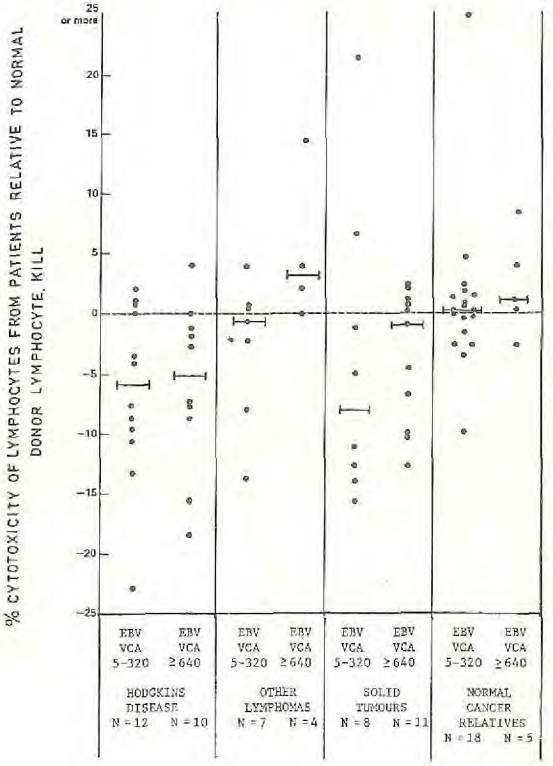
DISCUSSION

It has been generally observed that the height of the antibody level to the EBV VCA remains stable in normal individuals, probably as the result of host control mechanisms (Klein, 1975). In an area where immunological abnormalities due to infection are common, such as Uganda, reversions from antibody-positive to antibody-negative status and marked fluctuations in titre are common (de-Thé et al.[1]). In view of the growing number of situations where

[1] See p.11.

FIG. 2. CYTOTOXICITY OF LYMPHOCYTES FROM CANCER PATIENTS COMPARED WITH EBV TITRES

Results of ^{51}Cr-release assay of lymphocyte cytotoxicity to F265 cells using lymphocytes from cancer patients relative to normal donor lymphocyte kill. Position of median values is shown by horizontal bars.



elevated EBV VCA titres are linked to cancer (Henle et al., 1969; Levine et al., 1972; Ablashi et al., 1974; Levine et al., 1970; Johansson et al., 1970; Levine et al., 1971a; Levine et al., 1971b; Johansson et al., 1971; Levine et al., 1974), an analysis of the clinical and immunological features in each such situation is necessary in order to begin to understand the mechanisms for antibody control and the significance of the titres, particularly since it is unlikely that high titres have the same meaning in each disease state and in all normal individuals.

An evaluation of these results should be preceded by a comment about some of the major problems affecting studies such as ours. The observation of diverging patterns in Hodgkin's disease and non-Hodgkin's lymphoma emphasizes the importance of accurate diagnosis in each patient. Standardization of antigens utilized in CMI assays is also a critical factor, both

for *in vivo* (Eltringham & Kaplan, 1973) and *in vitro* (Hersh et al., 1974) studies. Thus the categorization of patients into "anergic" and "reactive" groups, as has been done in many studies, including this one, must be understood to be somewhat arbitrary. Finally, most studies are limited by their cross-sectional character, which does not allow evaluation of the kinetics of CMI and humoral immunity. Longitudinal studies are now under way at the National Cancer Institute.

Despite the problems noted above, it is possible to draw several conclusions from these studies. The data provided by our skin testing evaluation of 88 cancer patients and 14 normal individuals (Table 2) clearly indicate that general CMI depression, as measured by skin-test reactivity to a battery of antigens, is not responsible for the elevated EBV titres in most individuals. Similar conclusions have been reached in our *in vitro* studies (LC), although further work is necessary to determine to what extent these findings are applicable to each disease state and to "high-risk" normal individuals. In studies with antigens derived from lymphoid cell lines that appeared to be tumour-related (*in vivo* studies were restricted to cancer patients), a comparison of delayed hypersensitivity and EBV titres (Table 4) indicates that in most cases the high EBV titres are not the result of cellular immunosuppression. This does not preclude the possibility of such a relationship, however, in one or more specific disease groups.

The finding of a high percentage of skin test-positive patients in the lymphoma and NPC groups, two groups with generally high EBV titres, is of great interest. Our findings in these NPC patients, most of them Caucasian, are similar to those of Ho et al.[1], who found 13/25 (52%) NPC patients reactive to HKLY28 by skin testing as compared to only 2/23 (9%) solid tumour controls. A second observation we have made is that antigens prepared from long-term tissue culture lines show promise for further *in vivo* studies of CMI, as reactions to these antigens appear to be disease-related. The reaction of NPC and lymphoma patients against tumour-derived cell lines, but not those derived from normal individuals, suggests that they may be more useful in CMI studies in lymphoma patients than standard non-tumour-related antigens. In a cross-sectional study on 27 lymphoma, 13 NPC and 21 solid tumour patients, no correlation between EBV titre and CMI to these lymphoid-related antigens has been observed, but longitudinal studies are in progress. Since EBV is unlikely to cause disease in all patients with elevated titres, it is important to evaluate the mechanisms operating in different groups. The possibility that high titres indicate a general susceptibility to cancer that is independent of a direct effect of the virus has been suggested by our observation of elevated antibody levels in a number of families where multiple cases of cancer have occurred (Levine et al., 1974; Li et al., 1974). The inability of EBV antibody to prevent the appearance of BL (Magrath & Henle[2]) also raises the possibility that the antibody levels are an ancillary phenomenon. While the emphasis in studies on the viral etiology of human tumours may have shifted to detection of the virus within the tumour (Lindahl et al., 1974; Reedman et al., 1974) as opposed to sero-epidemiology, the apparent relationship of EBV antibody to stage of disease and even, perhaps, susceptibility to cancer, needs to be further evaluated.

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[1] Personal communication.

[2] See p.277.

SUMMARY

Although elevated antibody levels to the Epstein-Barr virus (EBV) have been reported in a number of lymphoproliferative neoplasms, it has not been possible to determine whether these antibodies were the result of a specific response to an oncogenic agent (EBV), whether they were a non-specific humoral compensation for depressed cell-mediated immunity (CMI), or whether a different mechanism was responsible. We have previously shown in a group of lymphoma patients that depressed cellular immunity to a number of standard antigens (Candida, SKSD, etc.) is not associated with an increase in antibody to EBV. In this study, we tried to compare CMI to possible EBV and lymphoid cell line antigens with humoral antibody to EBV. The two basic CMI assays utilized were lymphocyte cytotoxicity (LC) and skin testing (ST) for delayed hypersensitivity. In the LC assay, an EBV-containing cell line (F265) was used as the target. Reactivity against F265 was stronger in normal individuals than in cancer patients, suggesting a relationship to general cellular immune competence. ST studies showed that membrane extracts from lymphoid cell lines derived from patients with Burkitt's lymphoma and nasopharyngeal carcinoma (NPC) were more likely to elicit a delayed hypersensitivity in lymphoma and NPC patients than cell lines derived from normal individuals. Patients with ST reactivity against the membrane preparations from the tumour-derived cell lines were as likely to have elevated EBV antibodies as patients without such reactivity. The data strongly indicated that the elevated EBV titres in lymphoma patients are not related to a specific or non-specific depression of CMI.

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EPSTEIN-BARR VIRUS (EBV)-ASSOCIATED ANTIBODY PATTERNS IN RELATION TO THE DEFICIENCY OF CELL-MEDIATED IMMUNITY IN PATIENTS WITH HODGKIN'S DISEASE

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It has been shown that Hodgkin's disease (HS) is often associated with elevated titres against Epstein-Barr viral (EBV) capsid antigens (VCA) and EBV-determined cell membrane antigens (EA), but the antibodies are not always present and the mean titres are lower than in Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC) (Johansson et al., 1970; Levine et al., 1971; Levine, 1972). When the patients were divided into subgroups in relation to clinical and histological data of prognostic importance,

an inverse relationship was found between the frequency of lymphoid cells in the tumours and the EBV-associated serological reactivity. However, if all available data on EBV and HD are taken together, they fail to support a direct oncogenic role of EBV in the majority of HD cases, but do not exclude it in all. Since high serological anti-EBV reactivities have mainly been found in those histological subtypes of HD often associated with poor cellular immunity, it has been suggested (Henle & Henle, 1973; Hesse et al., 1973) that elevated antibody titres might be related to a depression of cell-mediated immunity through a compensatory activation of EBV-carrying lymphoid cells not involved in this immunity. The elevated antibody levels would then reflect the disease process but would not be of etiological importance. Support for this assumption is provided by a recent observation that the elevated EBV titres in two patients with congenital T-cell deficiency became normalized after thymus grafting (Businco et al.[1]). However, Hesse et al. (1973) found no correlation between the impairment of cell-mediated immunity measured by skin tests in untreated HD patients and the anti-EBV titres.

Since January 1973, we have included a characterization of the immune defect in the initial evaluation of all untreated HD patients. Sera from the same patients have been examined for antibodies to VCA and to the D and R components of the early antigen (EA) complex. Data on the relation between the immune defect and the anti-VCA titre in 43 unselected patients will be presented and discussed here. As only 15 (35%) sera had positive anti-EA titres, these antibodies will not be taken into consideration.

[1] Unpublished data.

MATERIALS AND METHODS

Immunofluorescence test for antibodies to VCA

The preparation of acetone-fixed smears of EB-3 cells, a producer line of BL origin, for anti-VCA titration, and the indirect immunofluorescence technique used have been described (Henle et al., 1969). Geometric means were calculated for the anti-VCA titres. Titres of 1:80 or less were classified as low, titres of 1:160 or more as high.

DNA synthesis and mitogens

Highly purified lymphocytes from defibrinated venous blood were used. Incorporation of ^{14}C-thymidine was used as a measure of DNA synthesis. Spontaneous DNA synthesis was measured during the first 24 hours of incubation. DNA synthesis in lymphocytes stimulated by different concentrations of Concanavalin A (Con A), phytohaemagglutinin (PHA), purified tuberculoprotein (PPD) and pokeweed mitogen (PWM) was quantitated by incorporation of ^{14}C-thymidine in 72-hour cultures. Details of the methods have been described previously (Mellstedt & Holm, 1973). Healthy controls were included in each experiment. Incorporation of ^{14}C-thymidine below the range obtained in the controls was classified as decreased, values within the range as normal, and values above the range as increased.

Delayed cutaneous hypersensitivity reaction

Delayed cutaneous hypersensitivity reaction was carried out with PPD, 0.1 ml of PPD (2 TU) being given

intradermally after collection of blood for the *in vitro* tests. A dermal induration of 10 x 10 mm or more after 48 hours was considered as positive.

Assays for T lymphocytes and non-T lymphocytes

Highly purified lymphocytes were tested for lymphocytes forming rosettes with untreated sheep red blood cells as an assay for T lymphocytes. B lymphocytes carrying surface immunoglobulins (Ig) were identified by indirect immunofluorescence using a polyvalent rabbit antihuman Ig serum followed by treatment with fluorescein-conjugated sheep antirabbit Ig serum. Lymphocytes with surface receptors for complement (C 3) were counted after forming rosettes with sheep red blood cells coated with antiserum and human complement. The methods have been described in detail previously (Jondal et al., 1972; Holm et al., 1974).

RESULTS

Of 43 sera, 22 (51%) had high anti-VCA titres, and the mean anti-VCA of all sera was 1:123. These values are in agreement with those given in previous reports (Johansson et al., 1970; Henle & Henle, 1973). Many patients were lymphopenic due to subnormal levels of T lymphocytes and the lymphocyte stimulation, and skin tests showed defferent degrees of impairment in a considerable number of the patients. Detailed information on the cellular immunity defects in relation to different clinical and histological parameters will be presented elsewhere.

Concanavalin A

Con A in soluble form has been shown to activate T lymphocytes in mouse and man (Andersson et al., 1972; Hedfors, 1974). In the present material, 23 (56%) cases were found to have a considerably decreased response to Con A (Table 1). Fifteen (65%) sera from these patients had high anti-VCA titres and the mean anti-VCA level was 1:204. The corresponding values in cases with a normal lymphocyte response to Con A stimulation were 32% and 1:66 respectively.

Phytohaemagglutinin

Soluble PHA stimulates T lymphocytes in man and mouse (Meuwissen et al., 1968; Rockling et al., 1970; Janossy & Greaves, 1972). However, activation of B lymphocytes may also occur (Philips & Weisrose, 1974). Of sera from patients showing a highly decreased lymphocyte response to PHA, 12 (71%) had anti-VCA titres with a mean value of 1:188 (Table 2). In the group of patients with a normal response, 10 (38%) cases had high titres and the mean anti-VCA level was 1:93.

Purified tuberculoprotein

PPD stimulates T lymphocytes from normal individuals who have been previously exposed to the antigen. The same persons normally show a positive delayed hypersensitivity reaction after intradermal application of the antigen. In Sweden, about 90% of healthy individuals have a positive skin test due to the vaccination programme against tuberculosis in this country. The absence of a positive reaction therefore usually reflects a decreased functional activity of the T lymphocytes.

The *in vivo* and *in vitro* tests with PPD were concordant in the majority of cases, but discordant results were obtained in a few patients. However, those patients with a negative skin reaction who were

Table 1. Anti-VCA levels in relation to lymphocyte response to Con A stimulation in 42 HD cases

Anti-VCA level	Normal response: 19 (44%) cases	Decreased response: 23 (56%) cases
≤1:80	13 (68%)	8 (35%)
≥1:160	6 (32%)	15 (65%)
Mean	1:66	1:204

classified as normal in the *in vitro* test with PPD were very close to, or on the borderline between, normal and decreased response in this test. Patients who had a decreased or a negative response in either of the two tests or were abnormal in both tests were therefore classified as having a decreased response to PPD stimulation; 31 (72%) fell into this category (Table 3). Among them, 20 (65%) had high anti-VCA titres. The mean anti-VCA level was 1:164. In those cases that responded normally, only two (17%) had high titres and the mean anti-VCA was 1:60.

Pokeweed mitogen

After stimulation of blood lymphocytes by PWM, two types of blasts can be distinguished by ordinary light microscopy (Chessin et al., 1966). The morphological features suggest that PWM stimulates both T and B lymphocytes. This suggestion has recently been confirmed and it has been shown that a low dose of PWM induces DNA synthesis mainly in B lymphocytes (Biberfeld & Mellstedt, 1974; Mellstedt, 1974). In the present material, only 13 (30%) cases had a decreased response to PWM at low dose (Table 4). Neither the percentage of high anti-VCA titres nor the mean anti-VCA level was significantly different in the two groups of patients, namely 50% and 54%, and 1:113 and 1:152.

T-lymphocyte level

It has been known for a long time that HD patients are often lymphopenic,

Table 2. Anti-VCA levels in relation to lymphocyte response to PHA stimulation in 43 HD cases

Anti-VCA level	Normal response: 26 (60%) cases	Decreased response: 17 (40%) cases
≤1:80	16 (62%)	5 (39%)
≥1:160	10 (38%)	12 (71%)
Mean	1:93	1:188

Table 3. Anti-VCA levels in relation to lymphocyte response to PPD stimulation in 43 HD cases

Anti-VCA level	Normal response: 12 (28%) cases	Decreased response: 31 (72%) cases
≤1:80	10 (83%)	11 (35%)
≥1:160	2 (17%)	20 (65%)
Mean	1:60	1:164

and it has been shown that the lymphopenia is due to a decreased level of circulating T lymphocytes. Nineteen (44%) patients had T-cell counts below the normal range (832-3 040/mm^3) found in the controls (Table 5). Twelve (63%) of them had high anti-VCA titres and the mean anti-VCA level was 1:172. The corresponding values in the group of patients with lymphocyte counts within the normal range were 43% and 1:94.

Spontaneous DNA reactivity

Crowther et al. (1969), who studied the morphology and tritiated thymidine uptake of unstimulated lymphoid cells from the peripheral blood in HD patients, reported that the lymphoid cell population in HD differs from normal blood in that large reactive lymphoid cells are present. Table 6 shows that there was no obvious difference in the anti-VCA titres in patients with increased spontaneous DNA reactivity (high values: 59%; mean anti-VCA level: 1:129) as compared to cases with normal reactivity (high values: 44%; mean anti-VCA level: 1:115).

Degree of T-cell defect

An attempt was made to quantify the T-cell defect in the individual cases. Accordingly, the patients were subdivided into three groups depending on the number of T-cell tests (Con A, PHA, PPD and T-cell level) that were abnormal. Group A included patients who were normal in all tests, group B patients abnormal in 1-2 tests, and group C patients

Table 4. Anti-VCA levels in relation to lymphocyte response to PWM stimulation in 43 HD cases

Anti-VCA level	Normal response: 30 (70%) cases	Decreased response: 13 (30%) cases
≤1:80	15 (50%)	6 (46%)
≥1:160	15 (50%)	7 (54%)
Mean	1:113	1:152

Table 5. Anti-VCA levels in relation to the T-lymphocyte level in 43 HD cases

Anti-VCA level	Normal level: 24 (56%) cases	Decreased level: 19 (44%) cases
≤1:80	14 (58%)	7 (37%)
≥1:160	10 (42%)	12 (63%)
Mean	1:94	1:172

abnormal in 3-4 tests. As seen from Table 7, there was a striking difference in the anti-VCA reactivity among the three groups, with the highest anti-EBV reactivity in group C and the lowest in group A, while group B was intermediate in reactivity.

DISCUSSION

The chain of evidence pointing to an etiological role of EBV in BL has grown steadily in recent years. High titres of antibodies against EBV-determined antigens are regularly found in sera from BL patients. It has been demonstrated by molecular hybridization tests that the large majority of African BL cases are positive for EBV DNA (zur Hausen, 1970, 1974; Nonoyama et al., 1973; Lindahl et al., 1974; Pagano et al., 1974). Recently, a complement-fixing EBV-determined intranuclear antigen (EBNA) was demonstrated by anticomplementary immunofluorescence (Reedman & Klein, 1973). EBNA is present not only in virus-producing but also in non-producing EBV-carrying cell lines and has been shown to express the presence of the EBV genome. All African BL biopsies (with the same few exceptions found to be negative in the hybridization tests) contain EBNA (Reedman et al., 1974). These observations show that, in most cases, African BL represents the neoplastic proliferation of an EBV-carrying lymphoid cell clone.

A direct oncogenic effect of EBV in malignant lymphomas other than BL

Table 6. Anti-VCA levels in relation to the spontaneous DNA reactivity in blood lymphocytes from 42 HD patients

Anti-VCA level	Normal response: 25 (60%) cases	Increased response: 17 (40%) cases
≤1:80	14 (56%)	7 (41%)
≥1:160	11 (44%)	10 (59%)
Mean	1:115	1:129

Table 7. Anti-VCA levels in relation to the number of T-cell tests that were abnormal in 43 HD cases

Anti-VCA level	A Normal response: 6 (14%) cases	B Decreased response in 1-2 tests: 18 (42%) cases	C Decreased response in 3-4 tests: 19 (44%) cases
≤1:80	6 (100%)	10 (56%)	5 (26%)
≥1:160	0 (0%)	8 (44%)	14 (74%)
Mean	1:40	1:92	1:230

would seem likely if a similar close association could be demonstrated between the malignant cells and the virus. The absence of detectable amounts of the EBV genome in HD tumours has been reported by zur Hausen et al. (1970), Pagano et al. (1973), Lindahl et al. (1974) and Reedman et al. (1974). These results fail to support a direct oncogenic role of EBV in HD, so that the following question arises: why should a tumour patient develop high antibody titres against EBV-determined antigens if the viral genome is not present in the tumour? Among different possible explanations, it has been suggested (Henle & Henle, 1973) that a depression of cell-mediated immunity in HD may favour the proliferation of EBV-carrying B cell lines. Impaired immune responses in patients with HD have been described by many investigators (for a review, see Crowther, 1973). The antibody response usually remains intact in these patients until late in the disease (Aisenberg & Leskowitz, 1963), but defects in cellular immunity, including delayed skin-homograft rejection and diminished delayed hypersensitivity response, are found early. Furthermore, there are data showing that EBV has a remarkable and possibly exclusive predilection for B cells. B lymphocytes, but not T lymphocytes, from healthy individuals have receptors for EBV and are the target cells in experimental infections with EBV (Jondal & Klein, 1973). All established human lymphoblastoid cell lines of B-cell type, with a few exceptions, have been found to carry the EBV genome (Klein et al., 1974). A small number of cell lines with T-cell characteristics were negative in tests for EBV DNA (Pagano, 1974).

Most patients in the present study were lymphopenic, due to subnormal levels of T lymphocytes. The lymphocyte-stimulation tests and the cutaneous PPD reaction showed different degrees of impairment in a considerable number of patients. The distribution of the immune defect within different histological subgroups of HD and the correlation with clinical parameters, as well as its prognostic significance, will be discussed elsewhere; the present study deals only with the relation between EBV-determined anti-VCA titres and the T-cell defect.

The finding that sera from patients whose lymphocytes responded poorly

to mitogens known to activate T lymphocytes had a higher anti-VCA reactivity than sera from patients whose lymphocytes responded more normally, shows that there is a correlation between the T-cell defect in HD and the anti-VCA titres. This correlation becomes even more striking when patients with different degrees of immune defect are compared. Those patients whose reactivity was decreased in three or four of the T-cell tests showed a considerably higher serological anti-VCA reactivity than those who were normal in all four tests, while cases depressed in only one or two tests showed intermediate reactivity. The correlation becomes more significant with the observation that there is no difference in the anti-VCA titres as between patients with normal and decreased lymphocyte response to low concentrations of PWM, which is known to activate mainly B cells.

The results presented here support the above-mentioned suggestion by Henle & Henle (1973) that the elevated anti-VCA titres in HD may be due to compensatory activation of EBV-carrying B lymphocytes. Furthermore, this suggestion is in line with observations in sarcoidosis, a benign disease, where both elevated antibody titres against EBV-associated antigens (Hirshaut et al., 1970; Wahren et al., 1971) and depression of cell-mediated immunity (Hedfors, 1974; Hedfors et al., 1974) have been found. However, more studies on this point will be necessary before any conclusions can be drawn. The present material is too limited for it to be possible to extend the comparison between serological anti-EBV reactivity and immune impairment to the histological subgroups of HD, which would be of importance. We therefore propose to increase the number of patients and also to include studies on titres of antibodies against EA and EBNA.

SUMMARY

Sera from unselected and untreated patients with Hodgkin's disease (HD) were examined for antibodies to Epstein-Barr viral (EBV) capsid antigens (VCA). Delayed cutaneous hypersensitivity reactions were carried out with purified tuberculoprotein (PPD). Highly purified blood lymphocytes of the same patients were studied morphologically and classified for cell surface markers. Incorporation of ^{14}C-thymidine was used as a measure of spontaneous DNA synthesis and DNA synthesis after exposure to different concentrations of three mitogens (PHA, Conconavalin A and pokeweed mitogen) and PPD.

The distribution of EBV titres was in good agreement with previous reports. Most patients were lymphopenic, due to subnormal levels of T lymphocytes. The lymphocyte stimulation and skin tests showed different degrees of impairment in a considerable number of the patients. The results in 43 patients indicated that a relation exists between the immune defect and the anti-VCA titres. High serological anti-VCA reactivity was related to a poor cutaneous response to PPD, a decreased level of T lymphocytes in the blood and a depression of mitogen-induced DNA synthesis.

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IMMUNOGENETIC ASPECTS OF NASOPHARYNGEAL CARCINOMA (NPC) III. HL-A TYPE AS A GENETIC MARKER OF NPC PREDISPOSITION TO TEST THE HYPOTHESIS THAT EPSTEIN-BARR VIRUS IS AN ETIOLOGICAL FACTOR IN NPC

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Suggestions that genetic factors might be important in the development of NPC have been based largely on incidence and relative frequency data (Shanmugaratnam, 1971; Ho, 1972). One approach to identification of the putative genetic factors was to determine whether NPC patients have a restricted heterogeneity in type of one or more of the known immunogenetic systems. The rationale for this approach has been previously presented (Simons et al., 1973, 1974a). Although red cell group studies (ABO, Rhesus, MNS) have so far proved unrewarding (Hawkins et al., 1974), marked

differences in HL-A type have been detected between 144 patients and 236 controls (Simons et al., 1974b, 1974c). A schematic representation of the major histocompatibility complex (MHC) genes is presented in Fig. 1. The gene system within the MHC that has been most studied in man is the HL-A system. This comprises at least three serologically detectable loci, of which SD1 and SD2 are the best characterized. The SD system is highly polymorphic, comprising some 14 alleles at the SD1 locus, and at least 18 at SD2. The HL-A antigen profile associated with a high risk for NPC consisted of an increased frequency of HL-A2 at the SD1 locus and a deficit of antigens detected at the second locus (SD2). The proportions of NPC and non-NPC Chinese who have both of the high-risk HL-A factors (HL-A2 and the blank) are shown in Table 1.

The phenotypic association of the two high-risk factors is given in Table 2 for the total NPC patient group and the subgroups of Cantonese patients and non-Cantonese patients. The association between HL-A2 and the blank was much more pronounced in the Cantonese patients than in the Teochew and Hokkien. Table 3 shows that the non-Cantonese NPC patients differed from normal Chinese subjects in that the frequency of HL-A2 and the blank was higher in the NPC group. It would seem from these data that the relationship of HL-A with NPC is different in populations of different incidence (Table 4), thus strengthening the hypothesis that a major part of the variation in incidence is determined by genes close to the SD genes.

The most likely explanation for the disease association with the second locus "blank" was that an additional antigen or antigens existed, undetected by the reagents used, that was associated with high risk for NPC. Alternatively, the deficit of detectable antigens may have been a consequence of the disease (disease artefact), or it may have been due to homozygosity of detected second-locus antigens. To distinguish between these possibilities, the families of nine Singapore Chinese NPC patients who had a second locus blank were HL-A typed. The results clearly indicated that the "blank" segregated in the manner of an HL-A antigen (Simons et al., 1975).

To identify the putative anti-HL-A activity corresponding to the SD2 blank, sera from more than 600 parous Singapore women were screened using lymphocytes most likely to display the "blank" antigens. Two sera were identified that appeared to specify a new antigen, designated as Singapore-2 (Sin-2). The relatively higher frequency of Sin-2 in NPC patients than controls (Table 5) suggests that the occurrence of Sin-2 may be associated with a high risk for NPC. If this finding is confirmed in larger numbers of patients, then the relative risk for NPC associated with HL-A2-Sin-2 will be approximately 3-5.

The high-risk type of HL-A2 is not present in all NPC patients. Conversely, some individuals who do not have NPC are HL-A2 and Sin-2 positive. Thus it is unlikely that the HL-A genes *per se* are primarily associated with NPC. Current concepts favour the view that HL-A genes are linked to disease susceptibility (DS) genes (McDevitt & Bodmer, 1974). It is a reasonable assumption that the DS genes are in some way involved in immune reactions. That NPC patients have altered immunological function is suggested by a high frequency of antinuclear antibodies (Yoshida et al.[1];

[1] See p.261.

FIG. 1. SCHEMATIC PRESENTATION OF MAJOR HISTOCOMPATIBILITY GENE COMPLEX

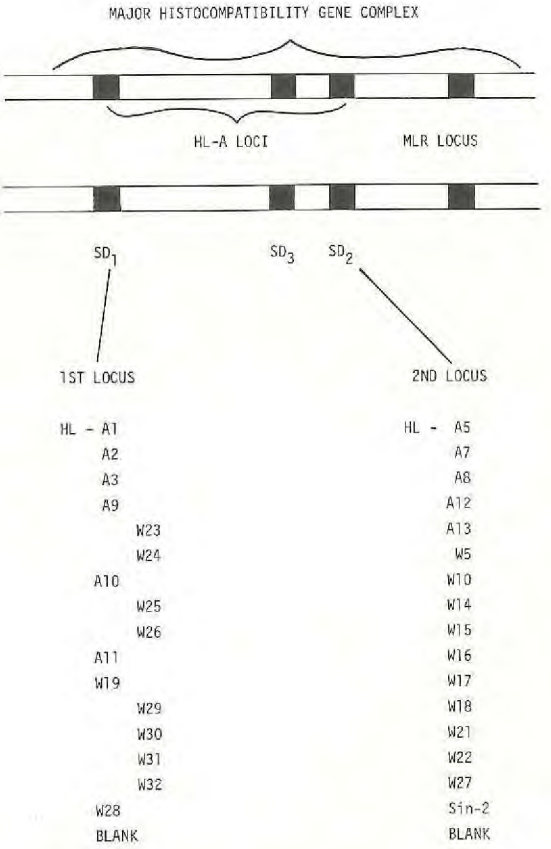

Table 1. NPC and non-NPC Chinese who have both high-risk HL-A factors (A2 and blank)[a]

Group	A2-blank	Not A2-blank	Total
NPC	76	68	144
Non-NPC	68	168	236
Total	144	236	380

[a] $\chi^2 = 21.8$; $P < 0.001$

Yoshida & Simons[1]), by lymphocyte hyporesponsiveness to phytohaemagglutinin (PHA) stimulation *in vitro* (Chan et al., 1975), and by diminished delayed skin hypersensitivity to purified protein derivative (PPD) challenge *in vivo* (Chan et al.[1]). Antibodies to several Epstein-Barr virus (EBV)-directed antigens are increased in titre in NPC patients. Complement-fixing (CF) antibody levels to soluble antigens of four herpesviruses in 50 Singapore Chinese NPC patients and 50 controls are shown in Table 6. Highly significant differences in antibody titres to EBV and CF soluble antigen were detected but no differences were observed in herpes simplex virus types 1 and 2 (HSV-1, HSV-2) or cytomegalovirus (CMV) CF antibody levels. The apparent selectivity of this immunological difference, occurring in patients with non-specifically depressed cell-mediated immunity, weighs against the possibility that the high EBV antibody levels reflect a non-specific humoral compensation. Taken together with the evidence for EBV genomes in NPC tumour cells from patients in Singapore and elsewhere (Pagano et al.[2]; Desgranges et al.[3]), strong support is given to the hypothesis of a special and possibly etiological role for EBV in NPC development.

The high frequency of HL-A2 and Sin-2 in NPC promises to be the first clear demonstration of an association between HL-A type and a human carcinoma. The HL-A pattern associated with NPC risk also promises to be a powerful tool in testing the hypothesis that EBV is etiologically involved in NPC. In all studies of cancer etiology, a major problem is to determine the relationship between the time of action of suspected causative factors and the onset of malignancy. With a genetic marker, it is almost certain that the high-risk characteristic existed prior to exposure to the putative oncogen. Intra-uterine infection with EBV seems to be a rare event, if it occurs at all. Thus, by HL-A typing babies at birth, it is theoretically possible to identify those with high risk for NPC, to follow them through

[1] Unpublished data.

[2] See p.180.

[3] See p.192.

Table 2. Association of both high-risk HL-A factors (A2 and blank) in Chinese NPC patients

A. Total NPC patient group[a]

Group	Blank	No blank	Total
A2	76	22	98
Not A2	26	20	46
Total	102	42	144

[a] $\chi^2 = 6.70$; $P < 0.01$.

B. Cantonese[b]

Group	Blank	No blank	Total
A2	29	5	34
Not A2	5	12	17
Total	34	17	51

[b] $\chi^2 = 15.9$; $P < 0.001$.

C. Non-Cantonese (Teochew, Hokkien)[c]

Group	Blank	No blank	Total
A2	47	17	64
Not A2	21	8	29
Total	68	25	93

[c] Not significantly different.

Table 3. Comparison of non-Cantonese Chinese NPC and non-NPC who have both high-risk HL-A factors (A2 and blank)[a]

Group	A2-blank	Not A2-blank	Total
Non-Cantonese NPC	47	46	93
Non-NPC	68	168	236
Total	115	214	329

[a] $\chi^2 = 13.8$; $P < 0.001$

the period of first exposure to EBV, and then to determine whether the pattern of immune response differs from those lacking both HL-A2 and Sin-2. The sero-epidemiological study that established the age-dependency of EBV immunity acquisition in various ethnic groups and geographical areas, and that revealed differences in type and specific antibody response between Chinese and Indian children during the early age period (de-Thé et al.[1]), would serve as a source of basic data for planning such a follow-up study.

At present, it is uncertain whether the crucial event for NPC development occurs at the time of primary infection with an agent such as EBV, or in association with recurrent infection. There are, however, two leads suggesting that the period from childhood through the teenage years is the time when the NPC-causing event(s) is/are taking place. Firstly, some cases of NPC occur before 20 years of age, mainly in the intermediate-risk areas (Tunisia, Sudan). Secondly, the incidence curve for NPC in Singapore Chinese males declines after 50 years of age. This curve is very similar to that for carcinoma of the cervix in Sweden, and it is accepted that the development of cervical carcinoma is related to the age of first sexual exposure. Another possible way of testing the EBV/NPC hypothesis is to screen adults in the NPC age-range for the NPC-associated HL-A type. The township of Toa Payoh in Singapore, where the sero-epidemiological survey was conducted, has a population of approximately 160 000. This number includes some 40 000 Chinese over the age of 30, among whom arise 12-15 cases of NPC per year. If blood samples were obtained and screened for HL-A2, Sin-2 at the rate of 150 per working day, it would be possible to survey this population in one year. In three years, 36-45 individuals would have developed NPC from among the estimated 2 000 with the NPC-HL-A marker. Thus in three years, and possibly less, it should be possible to establish whether the HL-A gene marker, and other gene markers within the MHC (*vide infra*) are in fact high-risk factors for NPC.

[1] See p.6.

Table 4. Relationship between the HL-A NPC association and the incidence of NPC in different populations

Group	NPC incidence rate/100 000 year age-standardized to world population (males)	Type of association with HL-A type	
		1st locus A2	2nd locus blank
Cantonese Chinese (Singapore)	29	+ (in association)	+
Teochew Chinese (Singapore)	17	+ (independent)	+
Hokkien Chinese (Singapore)	14	+ (independent)	+
Malays (Singapore)	4	-	+
Tunisians	4	-	+ ?
Indians (Singapore)	0.9	-	-

A third study would be that of the family members of NPC patients, among whom the genetically determined risk factor (DS genes) can be expected to occur in higher frequency than in the general population. Preliminary results from HL-A studies of multiple NPC case families from Hong Kong support this expectation (Ho et al.[1]). If the number of families studied were sufficient to include persons covering the age-range 5-20 years, it might be possible to determine whether the type of immune response to primary EBV infection segregated with the high-risk HL-A type. This would be the strongest evidence that could be obtained in support of the hypothesis of genetic predisposition to NPC development, mediated by abnormality of immune response to EBV infection.

In Fig. 1, the MLR locus is shown on the side of the SD2 opposite to that of SD3 and SD1. In mice, genes controlling immune responses to a range of antigens occur in the same chromosomal segment as the major MLR locus (McDevitt & Bodmer, 1974). Since the MHCs of mice and man are similar, it is suspected that some immune-response (Ir) genes in man will be in strong linkage disequilibrium with the MLR locus. Gene typing of the MHC region in the chromosomal direction of the putative NPC-DS locus should result in progressively increasing restriction of marker gene heterogeneity in the NPC patients. If the NPC-DS genes are

[1] Unpublished data.

Table 5. Frequency of HL-A second-locus antigen Singapore-2 in Singapore Chinese NPC patients and non-NPC controls

Patients	No. tested	Sin-2 positive
NPC	31	9 (29%)
Non-NPC	30	1 (3%)

Ir genes in the region of the MLR locus, then an even stronger association might be expected between an MLR allelic type and NPC than has been found with HL-A2 and Sin-2. This approach has been successful in identifying DS genes in multiple sclerosis (Jersild et al., 1973). In the next few months, the goals are to find the HL-A2-Sin-2 homozygous cells required for MLR typing, and to establish a microlymphocyte culture assay system adaptable to screening large numbers of subjects. The sooner MHC gene-marker typing can be introduced as a screening procedure for high NPC risk, the better, because one way of reducing mortality from NPC is by achieving earlier diagnosis.

SUMMARY

HL-A typing of 144 NPC patients and 236 controls revealed an increased frequency of 1st locus HL-A2 (relative

Table 6. Complement-fixing antibody titres (GMT) to herpes-group viruses in Singapore Chinese NPC patients and non-NPC controls

Herpes-group virus	NPC	Non-NPC	Difference (NPC-Non-NPC) in dilutions[a]
EBV	45.6	11.2	2.03 ± 0.30 (P <0.001)
HSV-1	35.8	50.0	-0.48 ± 0.65 (NS)
HSV-2	18.3	30.6	-0.74 ± 0.44 (NS)
CMV	46.8	38.3	0.29 ± 0.61 (NS)

[a] ± Standard error. NS: not significant

risk = 2.24) and an increased frequency of unidentified antigens at the 2nd locus (relative risk = 2.60) in the NPC patients. HL-A2 and the 2nd locus "blank" appeared to act together (HL-A2 blank haplotype) in determining NPC risk in highest-risk Cantonese, whereas in relatively lower-risk non-Cantonese Chinese (Hokkiens, Teochews) they appeared to act independently. Only the "blank" had an increased frequency in Malay NPC patients. Thus there was an indication that the strength of the HL-A association with NPC reflected the 30-50-fold difference in incidence between highest-risk Cantonese and lowest-risk Indians. In Singapore, HL-A segregation patterns in families of nine Chinese NPC patients confirmed that the "blank" was a genetic phenomenon. A new 2nd locus antigen (Singapore-2) has recently been detected. Singapore-2 occurs more frequently in NPC patients and appears to be associated with a high risk for NPC. Since HL-A2 and Singapore-2 are not the risk factors, it is likely that the HL-A association with NPC reflects the existence of disease-susceptibility (DS) genes in linkage disequilibrium with alleles of the HL-A loci. It is proposed that NPC-DS genes may determine differences in immune responsiveness to environmental agents, and thereby determine differences in NPC incidence. If the known altered immune responsiveness of NPC patients to EBV reflects the function of DS genes linked to the high NPC risk HL-A type, then the hypothesis that Epstein-Barr virus has an etiological role in NPC can be tested by several types of prospective studies.

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AUTO-ANTIBODIES IN THE SERA OF PATIENTS WITH NASOPHARYNGEAL CARCINOMA

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In the decade since Epstein et al. (1964) revealed the association of Epstein-Barr virus (EBV) with cultured Burkitt's lymphoma (BL) cells by means of electron microscope studies, a considerable body of data has accumulated from international research in the field of virology, including molecular biology and immunology. Three different diseases, namely Burkitt's lymphoma, infectious mononucleosis (IM) and nasopharyngeal carcinoma (NPC), have been related to the EBV genome by the techniques of tumour immunology and virology including molecular biology (Henle, G. et al., 1968; Kawamura et al., 1970; Klein, G. et al., 1966, 1969; Klein, G. et al.[1]; Niederman et al., 1968; Nonoyama & Pagano, 1971; Nonoyama et al., 1973; Reedman et al., 1972; Reedman & Klein, 1973; Yoshida et al., 1969; zur Hausen & Schulte-Holthausen, 1970; zur Hausen et al., 1970). The oncogenicity of EBV in marmosets and owl monkeys has been demonstrated very recently by the experimental induction of lymphoreticular malignancies (Epstein et al., 1973; Shope et al., 1973).

[1] Unpublished data.

During the course of studies on the etiology of NPC, antinuclear antibodies (ANA) in the sera of NPC patients have been found to act as auto-antibodies and iso-antibodies. It was confirmed that these ANA were heterophilic by testing with the nuclei of other cultured cell lines in both humans and animals (Yoshida et al., 1971; Yoshida, 1971). The results summarized in this paper suggest that the evidence for auto-antibodies to normal tissue in the sera of NPC and other cancer and IM patients may be considered from the following three points of view: (1) impairment of immune surveillance and immune response in the course of EBV infection and tumour development; (2) presence of common antigens shown by EBV and other infectious agents, malignant transformed cells, and normal tissue components; and (3) the genetic background involved in immune surveillance, response and tissue antigens.

MATERIALS & METHODS

Cells

Human cultured cells derived from NPC biopsy materials, embryos, adult omentum, and P3HR-1 and Raji cells, and animal cultured cells derived from newborn mice, rats and rabbits, and monkey kidney were used (Yoshida et al., 1971; Yoshida, 1971).

Sera

Sera were collected from patients with NPC, BL and other tumours and from normal healthy people among Chinese, African and Japanese (Yoshida et al., 1971; Yoshida, 1971, 1974; Yasuda & Yoshida, 1974).

Purification of an antigen corresponding to speckled pattern ANA

The procedure is summarized later in Fig. 7 (Yasuda & Yoshida, 1973, 1974).

Immunofluorescence tests

Antinuclear antibodies have been detected with acetone-fixed monolayer cultured cells on the cover glass by indirect and direct immunofluorescence procedures using a Tiyoda fluorescence microscope, model FM 200 A and B (Yoshida et al., 1971; Yoshida, 1971).

Blocking tests were performed in which the target cell sheet was preincubated with various ANA-positive NPC and other cancer sera. After the sheet had been washed with phosphate-buffered saline (PBS), it was reacted with the fluorescein isothiocyanate (FITC)-conjugated IgG of auto-immune diseases (Yasuda & Yoshida, 1973, 1974).

Absorption tests were carried out according to the procedure described by Yasuda & Yoshida (1973, 1974).

RESULTS

High incidence of non-specific antinuclear antibodies (ANA) in the sera of patients with NPC

Our early work showing the presence of ANA in the sera of patients with NPC was performed using cultured cells from human embryo lung, NPC-12, NPC-79B and Raji as target cells, the sera of 97 Chinese cases who came to the clinic for initial treatment, and the sera of 50 Chinese out-patients at the National Taiwan University Hospital Clinic. The results of the experiments are summarized in Table 1. It was finally also confirmed that the auto-antibody was heterophil with rat, mouse and rabbit fibroblast cells and monkey kidney cultured cells (Yoshida et al., 1971; Yoshida, 1971).

The results of tests for ANA in the sera of tumour patients among Chinese, in addition to the cases

Table 1. Results of tests for antinuclear antibodies in the sera of nasopharyngeal cancer patients who visited the Clinic for their initial treatment (untreated NPC), and of patients treated at the Clinic (treated NPC)

Patients	Test results	Pattern in nucleus						Positive cases / Total cases	%
		I	II	III	IV	V	VI		
									
Untreated NPC	Positive	3	11	3	1	2	6	26/97	28
	Weakly positive	4	10	3	0	0	0	17/97	18
	Total	7	21	6	1	2	6	43/97	45
Treated NPC	Positive	1	1	7	5	1	5	20/50	40
	Weakly positive	0	1	4	0	0	1	6/50	12
	Total	1	2	11	5	1	6	26/50	52
Healthy controls		0	0	0	0	0	0	0/50	0

presented in early reports, among Japanese and Africans, are given in Table 2.

The immunofluorescence studies on the sera of all NPC patients suggested that 40-50% of all patients with NPC were ANA-positive; positive results were not found to be related significantly to clinical stage, treatment, or race. At present, it appears that the sera of BL patients do not have detectable ANA, but 5-10% of the cases of malignant lymphoma and leukaemia, and 20-30% of the cases of cancer, were ANA-positive.

Immunoglobulin class and titres of ANA in the sera of NPC patients

IgG and IgM have been found as ANA in the sera of patients with NPC. The titre of ANA in the sera of patients with cancer was not high, usually between 1:10 and 1:160, with very rare cases showing between 1:640 and 1:2 560 (Yoshida, 1971; Yoshida & Simons[1]).

Fluorescence pattern of ANA in the nuclei of human cultured cells

The six basic patterns of immunofluorescence staining of the sera from NPC and other cancer patients within the nuclei of monolayer-cultured cells at a resting stage have usually been called "diffuse" (pattern I), "dotted" (pattern II), "speckled" (patterns III and IV), "nucleolar" (pattern V), and "nucleolar combined with speckled" (pattern VI), as shown in Table 1, and Figs. 1, 2, 3, and 4. In the nucleolar pattern (pattern V), there were both diffuse and speckled types, the two types being clearly identifiable.

[1] Unpublished data.

FIG. 1. DOTTED TYPE (PATTERN II) OF ANTINUCLEAR ANTIBODIES (x 400)

FIG. 2. SPECKLED TYPE (PATTERN IV) OF ANTINUCLEAR ANTIBODIES IN THE SERUM OF MPC-12

This was the first observation of this type in cultured cells from biopsy material stained by the patient's own serum (April 1969). (x 100)

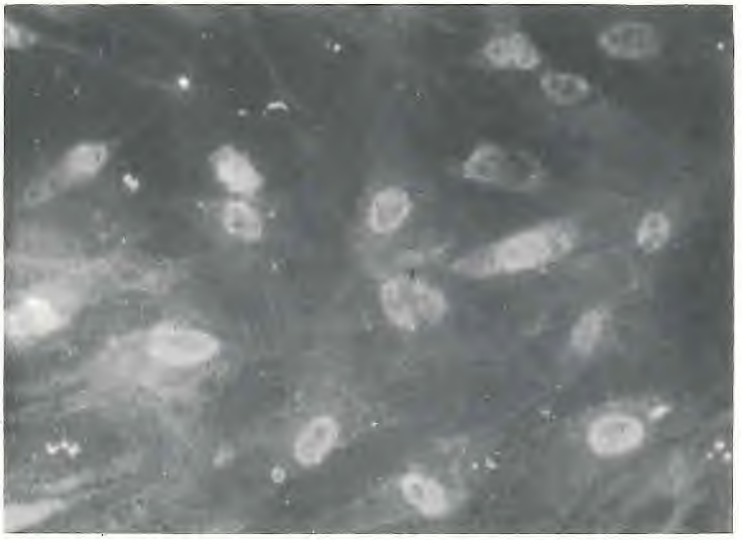


FIG. 3. SPECKLED TYPE (PATTERN IV) OF ANTINUCLEAR ANTIBODIES IN CULTURED CELLS

The localization of the antigen corresponds to that of speckled-type ANA at the metaphase of cultured cells. (x 400)

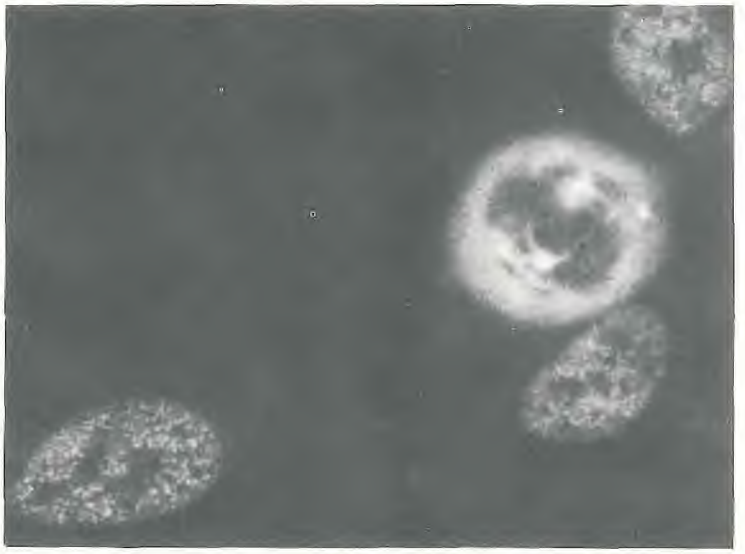


FIG. 4. NUCLEAR COMBINED WITH SPECKLED TYPE (PATTERN VI) OF ANTINUCLEAR ANTIBODIES IN CULTURED CELLS (x 400)

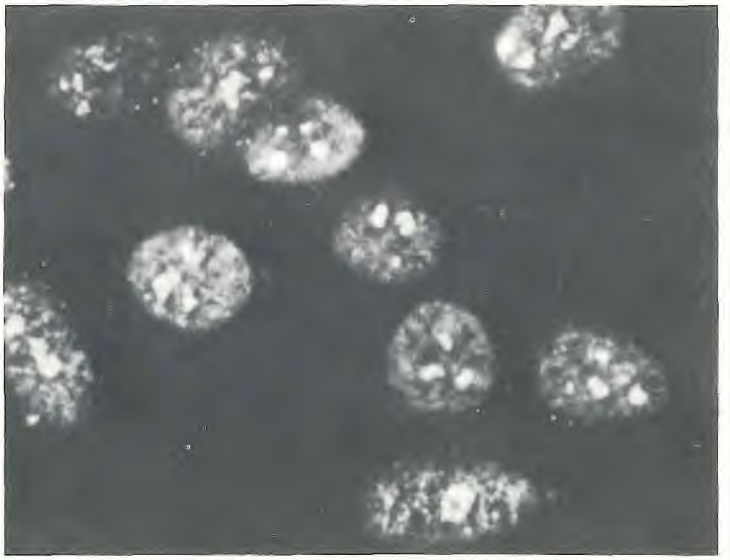


Table 2. Summary of results of tests for antinuclear antibodies in the sera of tumour patients (Chinese in Taiwan; Japanese in Japan; and Africans in Africa)

Patients	Tumour	Test cases	Positive	Weak positive	%
Chinese	NPC without treatment	97	26	17	44
	NPC with treatment	114	42	25	57
	Leukaemia	34	3	6	9
	Lymphoma	17	0	1	6
	Other cancer	27	4	6	37
	Normal healthy controls (40 years of age approx.)	50	0	0	0
Japanese	NPC with treatment	29	9	7	55
	Leukaemia	38	1	4	13
	Gastric cancer	150	34	16	33
	Breast cancer	75	12	6	24
	Cervical cancer	18	3	1	22
	Other cancer	39	5	3	21
	Normal healthy controls: 7-10 years	30	0	0	0
	45-70 years	190	5	16	11
African	BL	41	0	2?	0-5
	NPC	76	38	2	53
	Other cancer	60	6	4	17
	Tonsillitis as control	20	0	0	0

Mixed staining patterns of ANA, namely patterns II and III, patterns II and IV, and patterns II and VI, have sometimes been observed in the sera of patients with NPC.

The mixed type and the single type of staining pattern were observed in the sera of different stages in the clinical course of follow-up cases with NPC, as shown in Table 3 (Yoshida et al.[1]). These data suggest that the titre of ANA varied during the clinical course of NPC. In addition, our studies on the localization of antigens detected by ANA at different stages of cultured cells suggest that there are various antigens corresponding to ANA, as shown by the staining patterns and by Tan's paper (1967), which dealt with the relationship between the staining patterns of ANA in the sera of systemic lupus erythematosus patients and the corresponding antigens in the nucleus. Furthermore, the staining data suggest

[1] Unpublished data.

Table 3. Data on antinuclear antibody patterns at pretreatment stage, during treatment and at post-treatment stage in the clinical course of nasopharyngeal cancer in patients in Africa

Constant				Variable	
Single pattern	No. of cases	Double pattern	No. of cases	Single and double pattern	No. of cases
I	1	II, III	2	VI→III	1
II	2	VI, II	1	II, III→II, VI	1
III	4			IV→IV, II→IV	1
IV	1			V→VI→V	1
Total	9		3	Total	4

that patients differ in their immune response capacity to various auto-antigens in the nuclei in the course of tumour development (Yoshida, 1971; Utsumi et al., 1973).

A soluble acidic protein corresponding to ANA pattern IV, as described earlier (Yasuda & Yoshida, 1973, 1974; Yasuda-Yasaki & Yoshida, 1975), is able to diffuse into the cytoplasm through the nuclear membrane at metaphase, the antigen being passed back into both daughter nuclei at telophase.

Auto-antibodies to various tissue components in the sera of tumour patients

Apart from ANA, a 10-20% positive reading of anticytoplasmic antibodies (ACA) with "diffuse", "fibrous", "brashed", "speckled" or "dotted" patterns, together with their various combinations, in the sera of patients with NPC and other tumours has been observed in cultured cells, and has also been detected by immunofluorescence. Of the ACA-positive sera, one-third contained ANA, and the remainder contained only ACA. One strongly ACA-positive serum of the "brashed" type (Fig. 5), KY-343' of African NPC, also reacted with nerve fibres.

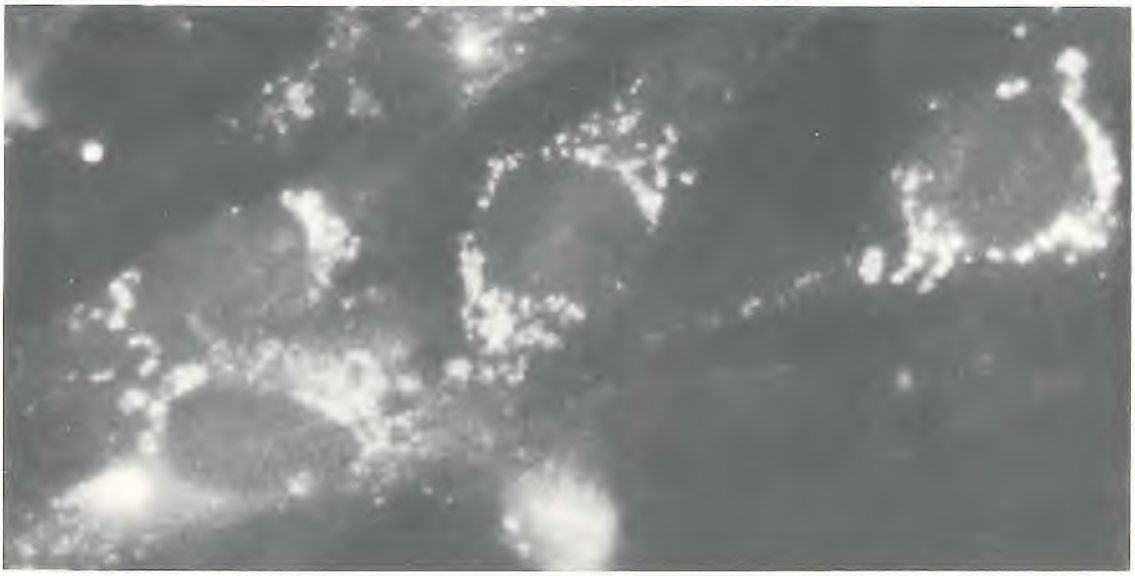
Cross-reactivity of ANA in the sera of patients with NPC and auto-immune diseases

Two methods, blocking tests and absorption of ANA with purified antigenic substance, have been applied to show the cross-reactivity of ANA in the sera of patients with NPC and auto-immune diseases. Selected sera showing typical patterns were used for these experiments.

Blocking test by the direct immunofluorescence technique. The results, shown in Fig. 6, suggested that both auto-antibodies were able to cross-react with the same antigenic determinant(s) in the cell nuclei, since the original patterns of auto-immune sera were blocked by pretreatment of cancer patients' sera (Yasuda & Yoshida, 1973, 1974).

FIG. 5. EXAMPLE OF "BRASHED" PATTERN

Example of "brashed" pattern (serum No. KY-343') of anticytoplasmic antibodies stained by the immunofluorescence procedure.



Absorption test with purified antigenic substance. Absorbed ANA-positive sera of cancer and auto-immune disease patients with the purified antigenic substance (Figs. 7 and 8) showed staining patterns in which the speckled pattern was specifically absent from the nuclei. Nuclear speckled fluorescence of pattern III and pattern IV was found to represent the same and/or partially the same antigenic substance. The nucleolar staining (pattern V) and the rather large dot of pattern II remained clear after absorption (Fig. 9).

The results of both blocking tests and absorption clearly suggest that cross-reactivity of ANA exists, and an immunological phenomenon occurs in two different diseases (Yasuda & Yoshida, 1973, 1974; Yasuda-Yasaki & Yoshida, 1975).

Beck (1961, 1962) and Lackmann & Kunkel (1961) also suggested that the staining patterns of ANA in the sera of auto-immune diseases were correlated with various antigens in the nucleus.

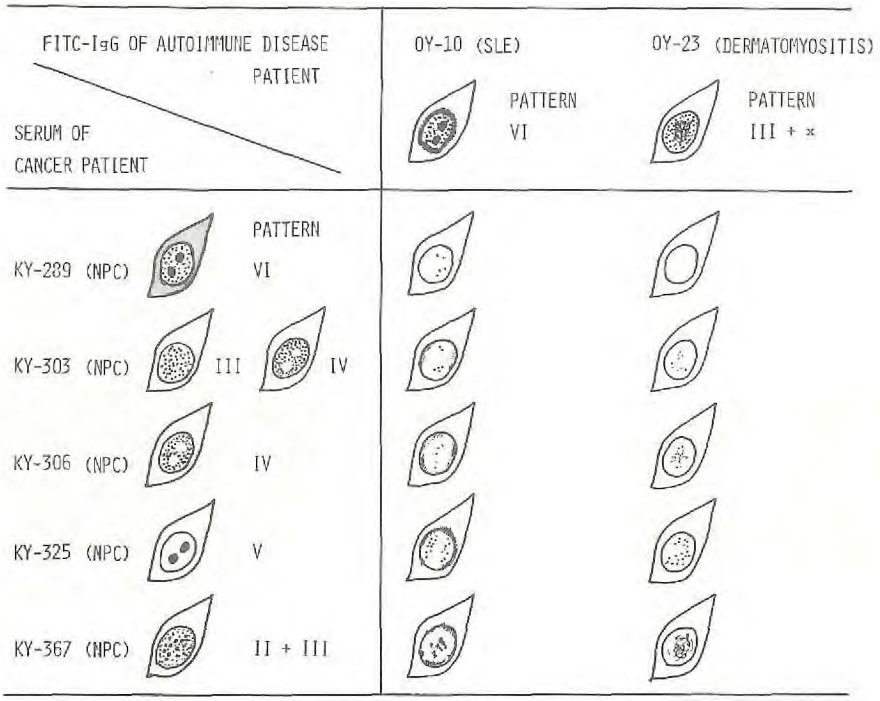
Attempts to find anti-Epstein-Barr (virus) nuclear antigen (EBNA) antibody in the sera of patients with NPC

Twenty sera of BL patients and 29 sera of NPC patients in Africa, and 1 serum of a BL patient and 10 sera of NPC patients in Taiwan were used to find antibody against EBNA (Klein et al.[1]; Pope et al., 1969; Reedman & Klein, 1973), using the absorption

[1] Unpublished data

FIG. 6. SUMMARY OF RESULTS OF BLOCKING TESTS BY THE DIRECT IMMUNOFLUORESCENCE PROCEDURE

III + x : pattern III + unknown pattern.

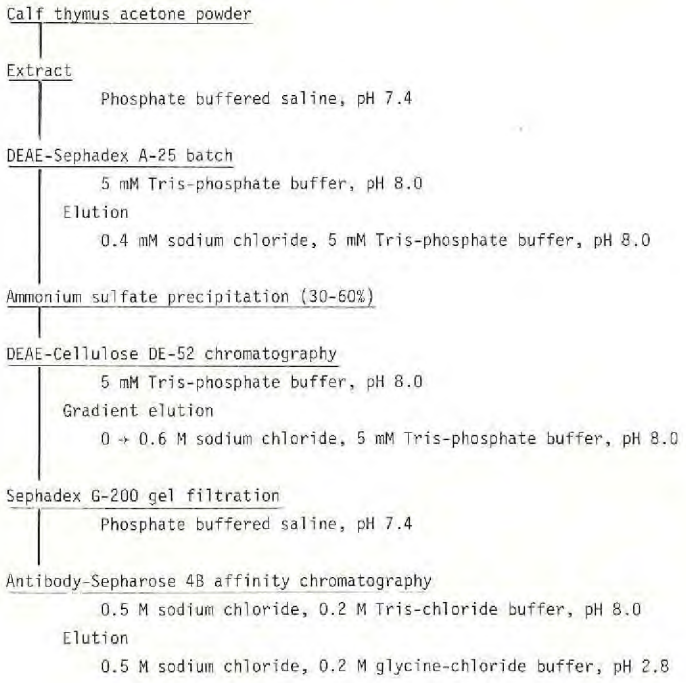


procedure (Fig. 4) with partially purified antigen from calf thymus just before Sephadex G-200 filtration, and the purified antigen from calf thymus corresponding to speckled type ANA of patterns III and IV, and as target cells, the acetone-fixed Raji and human embryo cells stained by a complement immunofluorescence procedure. The antibody to EBNA has not so far been observed in the absorbed sera of NPC patients. Heterophil antibodies, ANA and specific antibody, and anti-EBNA antibody, will be identified in various ways in the future.

DISCUSSION

Many questions are raised by the observations reported. Why, for example, is EBV related to three different diseases, namely, an infectious disease (IM), a lymphoma (BL) and a carcinoma (NPC)? Why do two of these diseases, namely IM (Kaplan & Tan, 1968) and NPC, produce ANA, but not BL? Is ANA related in any way to the hypothetical infectious agent or agents that may induce malignant transformation and subsequently provide a stimulus for auto-antibody

FIG. 7. PURIFICATION PROCEDURE FOR ANTIGENIC SUBSTANCE FROM CALF THYMUS CORRESPONDING TO SPECKLED PATTERN



formation in the impairment of immune surveillance and response? Why is an alteration of ANA incidence related to the individual patient's immunogenetic background?

EBV has been known to infect and to convert normal human and other primate B lymphocytes into abnormal cells in the case of IM and BL (Klein, E. et al., 1968). In the case of IM, it seems that the T lymphocytes proliferate and form morphologically atypical lymphocytes (Epstein & Achong, 1973). In NPC biopsy materials, lymphocytic infiltration was very commonly observed in the histopathological findings. The diagnosis of NPC was formerly lymphoepithelioma, but at present it is not known whether the infiltrating lymphocytes are T lymphocytes. The anti-early antigen (EA) antibody in the sera of patients with BL, IM and NPC has been described as restricted (R) in BL, diffuse (D) in IM, and mixed R and D in NPC, when the immunofluorescence procedure is used on EBV-infected Raji cells (Henle, G., 1971).

It is conceivable that the immunodeficiency, and particularly the abnormality of T cell antigen recognition, results in the appearance of atypical T cells in patients with IM due to the infection of the B cells by EBV (Miller et al., 1972; Shope & Miller, 1973). However, while that

FIG. 8. PROCEDURE FOR CHARACTERIZATION OF ANTIGENIC SUBSTANCE CORRESPONDING TO SPECKLED PATTERN

Phosphate buffered saline soluble
Molec. wt. 2.5×10^5
Isoelectric point 5.0

Heat stability ~60 C°and pH 7.4: 15 min
pH stability 2.6 ~ 8.5 and 20°C: 2 h

Sensitive:
Trypsin
Papain
Pronase
Ribonuclease
Alkaline phosphatase
Resistant:
Deoxyribonuclease
Neuraminidase
β-Galactosidase

Sensitive:
Sodium periodate (0.01 M, 5°C, 15 min)
Resistant:
Ethylenediamine tetraacetic acid
Cysteine, dithiothreitol
Iodoacetate, *p*-chloromercuribenzoate

LOCALIZATION

Interphase
nucleus (speckle)

Metaphase:
cytoplasm (diffuse)
spindle fibre

Human embryo
cultured cell

might occur in the course of NPC development, such an abnormality of T-cell antigen recognition did not occur in that of BL. A further possibility is that, as a result of the abnormality of T-cell antigen recognition, auto-antibodies were produced in patients with IM and NPC. Yet another possibility is that there are biologically different viral variants in BL, IM and NPC.

From our observations of ANA and auto-antibodies in the sera of other cancer patients (Yoshida, 1971, 1974), and other reports (Whitehouse & Holborow, 1971), it would appear that, even if the causative agents, whether viruses or chemicals, are different, the abnormality of T-cell antigen recognition generally occurs in the course of tumour development.

We have also observed similar characteristic ANA in the sera of patients with auto-immune diseases, such as systemic lupus erythematosus, scleroderma, polydermatomyositis, rheumatoid arthritis, juvenile rheumatoid arthritis, lupoid hepatitis, Sjögren's syndrome, and periarteritis nodosa.

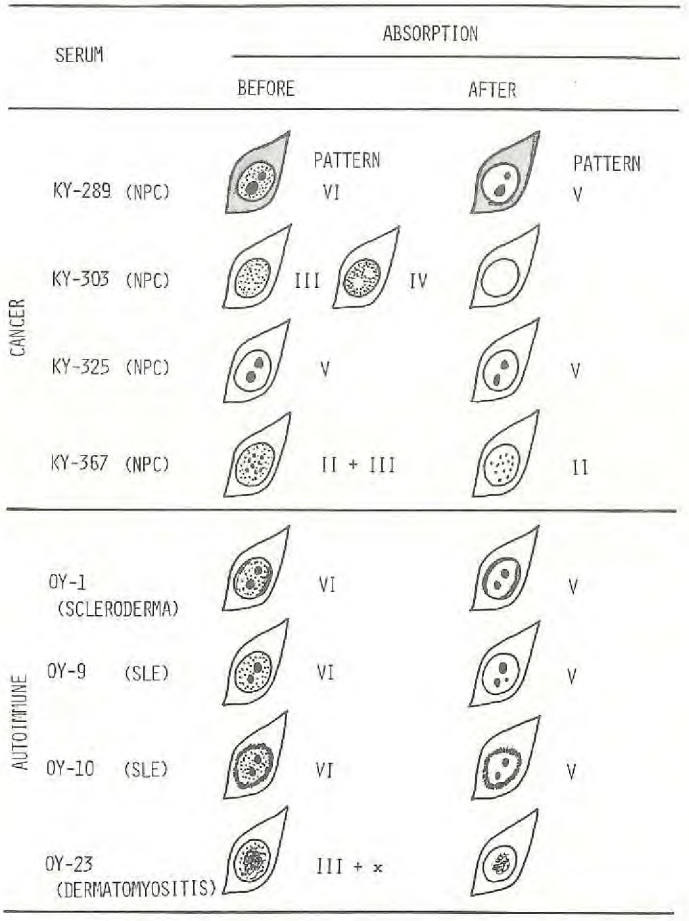
The correlation between the appearance of ANA in cancer patients with EBV infection and other viral infections, and auto-immune diseases must be carefully investigated in relation to the immunogenetic background and aging.

Of 157 Chinese cases with NPC, three were complicated by the well-known ANA-producing auto-immune disease, polydermatomyositis, and some gastric cancer patients in Japan have had dermatomyositis.

Cachexia may be related to immunological disorders in the course of

FIG. 9. SUMMARY OF RESULTS OF ABSORPTION TEST

The sera of patients with nasopharyngeal carcinoma and various auto-immune diseases were absorbed with the purified substance, soluble acidic protein. III + x : pattern III + unknown pattern.



tumour development, one of which is auto-antibody formation (Yoshida, 1971, 1974).

In animal experiments, Cannat & Varet (1972) found that ANA appears in (C x B6) F_1 mice after inoculation with Graffi and Rauscher leukaemogenic viruses, and their incidence is correlated with the infectivity of the viral inoculum and its leukaemogenesis.

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CHANGES IN ANTIBODIES TO EPSTEIN-BARR VIRUS-ASSOCIATED ANTIGENS WITH THE DEVELOPMENT OF BURKITT'S LYMPHOMA

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In recent years, serological associations of Epstein-Barr virus (EBV) with a wide range of diseases have been recorded. These include infectious mononucleosis (Henle & Henle, 1973), chronic diseases such as systemic lupus erythematosis (Evans et al., 1971), sarcoidosis (Byrne et al., 1973), and several malignant tumours, including Burkitt's lymphoma (Henle et al., 1969), nasopharyngeal carcinoma (Henle et al., 1970), Hodgkin's disease (Johansson et al., 1970), and lymphocytic lymphoma (Johansson et al., 1971). Some of these associations are weak or disputed (Stevens et al., 1972), while others extend beyond serology; the EBV genome, for example, has been detected in nearly all Burkitt's lymphomas (Nonoyama et al., 1973), and in the epithelial cells of nasopharyngeal carcinoma (Wolf et al., 1973). This somewhat confusing situation has been partly clarified by advances in the understanding of EBV-host relationships. Following primary infection, EBV persists in host tissues for a prolonged period, perhaps indefinitely (Miller et al., 1973). It would be surprising if the balance between virus and host were not disturbed in host-compromising situations, and indeed, increased pharyngeal excretion of putative virus has been observed in the immunosuppressed recipients of renal allografts (Strauch et al., 1974).

Some at least of the elevations in EBV antibody titres that have been observed in apparently distinct disease entities may be related to immunological disturbances that are, in fact, prominent in all of the chronic disease processes with which the virus has been associated.

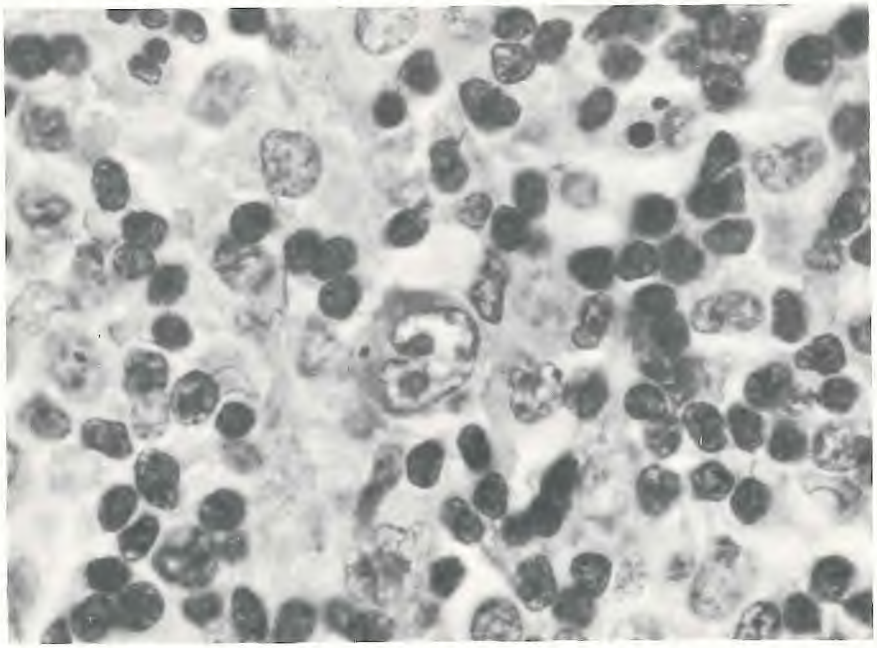
In the context of the two malignant diseases in which the EBV genome

is uniformly present in tumour cells, two outstanding questions have been at issue for some time. Firstly, do these tumours result from primary EBV infection? Secondly, and irrespective of the answer to the first question, though pertinent to it, to what extent do the serological differences between patients and controls relate to the observed immunological impairment associated with the tumour load (Magrath, 1974)? The case history of a unique patient seen at the Lymphoma Treatment Centre in Kampala, Uganda, in whom we were able to start to measure antibodies to EBV-associated antigens serially nine months before the onset of Burkitt's lymphoma, is of particular relevance to these questions, and, hopefully, will serve as a basis for discussion.

CASE HISTORY

The patient, an eight-year-old Ugandan boy of the Lango tribe, presented to the Lymphoma Treatment Centre in May 1972, with a five-year history of unilateral cervical mass unassociated with any symptoms. Biopsy and cytological examination of the tumour (Fig. 1) was consistent with mixed cellular Hodgkin's disease, and investigations revealed no evidence of tumour elsewhere. The patient at this time had positive cutaneous reactions to three of four microbial recall antigens. In the absence of a radiotherapy facility in Uganda, he was treated with the four-drug combination chemotherapy known as MOPP (mustine, vincristine, procarbazine and prednisone). Six complete courses were

FIG. 1. HISTOLOGICAL APPEARANCE OF THE CERVICAL MASS
(Haematoxylin and eosin - x 1 000)



given with no complications and the cervical mass became impalpable. About a month after completion of the therapy, the patient began to experience nasal obstruction, and at his next visit, nine months after the initial diagnosis of Hodgkin's disease, he was seen to have multiple jaw tumours, bilateral renal enlargement with additional abdominal masses, and cerebrospinal fluid malignant pleocytosis. Biopsy of a jaw tumour (Fig. 2) and cytological examination of tumour and cerebrospinal fluid cells (Fig. 3) confirmed a diagnosis of Burkitt's lymphoma. 50-60 genome equivalents of EBV were present per tumour cell (T. Lindahl[1]). Cutaneous reactivity at this time was similar to that recorded at the diagnosis of Hodgkin's disease, but phytohaemagglutinin reactivity of peripheral blood cells was also tested and shown to be markedly impaired, with no increase in thymidine uptake of stimulated cells compared to control cells. Cyclophosphamide and intrathecal drug therapy was commenced, and although a complete remission was rapidly induced, this was sustained for only three months, after which time the patient suffered a succession of neurological relapses and finally died at home, one year after the onset of Burkitt's lymphoma. Necropsy was not done.

Titres of antibodies to EBV-associated antigens measured serially from the patient's first presentation to the Lymphoma Treatment Centre are shown in Fig. 4. Anti-VCA (viral

[1] Personal communication.

FIG. 2. HISTOLOGICAL APPEARANCE OF ONE OF THE MAXILLARY SWELLINGS (Haematoxylin and eosin - x 400)

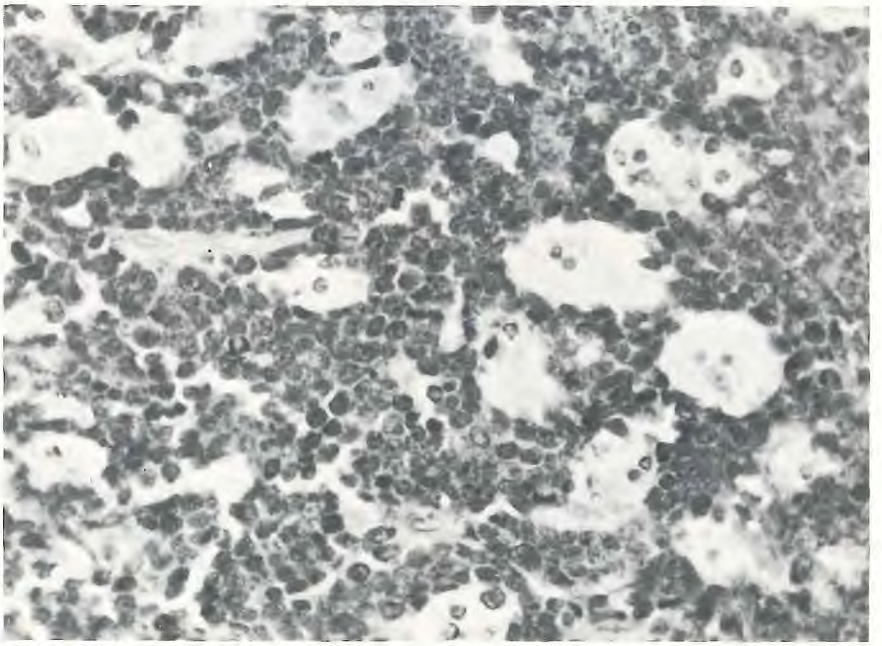
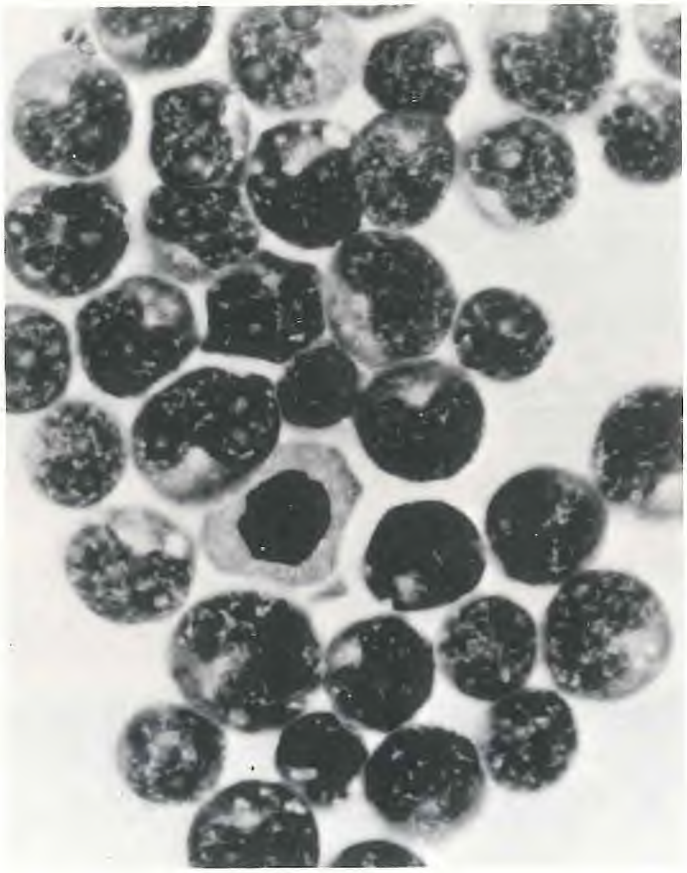


FIG. 3. CYTOLOGICAL APPEARANCE

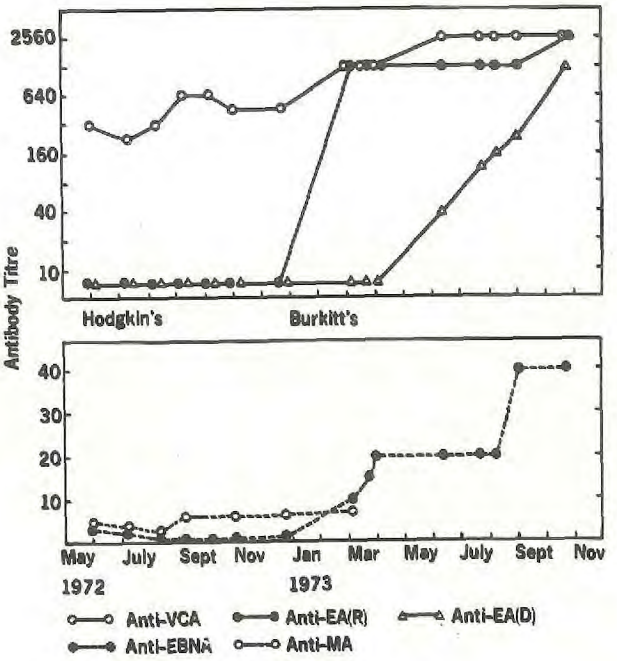
Cytocentrifuge preparation of cerebrospinal fluid cells at presentation with Burkitt's lymphoma. (x 1 000)



capsid antigen) was present in high titre (1:320) at the time of original presentation with Hodgkin's disease. The titre increased slowly during the period of study to a titre of 1:1280, the clinical development of Burkitt's lymphoma appearing to increase the rate of climb. Antibodies to early antigens (EA) were absent initially, but increased sharply with the onset of Burkitt's lymphoma, the R component rising first, in parallel with the clinical features, the D component a little later, during the post-treatment period of clinical remission. Antibodies to the EBV-determined nuclear antigen (EBNA) were present in low titre at the first presentation, but became negative until the onset of clinical Burkitt's lymphoma, when they rose rapidly to a titre of 1:60.

FIG. 4. ANTIBODY TITRES TO EBV-ASSOCIATED ANTIGENS

Antibody titres to EBV-associated antigens from presentation with Hodgkin's disease (May 1972) until diagnosis of Burkitt's lymphoma (February 1973) and beyond.



Anti-membrane antibodies (anti-MA) were positive throughout the observation period, but showed no changes relating to the clinical course.

DISCUSSION

Regardless of the presence or absence of Hodgkin's disease, there can be no doubt that, in this patient, antibodies to VCA, EBNA, and MA were present at least nine months before the diagnosis of Burkitt's lymphoma, although the possibility that Hodgkin's disease altered the serological pattern precludes attempts to define more precisely the time of primary EBV infection. The changes in antibody titres associated with the clinical development of Burkitt's lymphoma may be the result of an alteration in the host-virus relationship brought about by tumour cell proliferation. Immunosuppression, or increased antigen production by tumour cells (which are presumably rapidly lost from the tumour-cell population) are possible explanations for this, and either or both may be responsible, to a greater or lesser extent, for changes in individual antibody titres. This does not argue

against an etiological role for EBV, but the occurrence of Burkitt's lymphoma in an already seropositive individual indicates that, if EBV is causally related, either the latent period between primary infection and clinical tumour can be at least nine months, or Burkitt's lymphoma results from the interaction of co-factors with lymphoid cells that harbour the EBV genome. Such co-factors may differ in individual patients, and the possibility that, in the present case, Hodgkin's disease or its therapy with immunosuppressive agents provided a co-factor is worthy of consideration.

The data presented are also consistent with the possibility that EBV is not etiologically related to Burkitt's lymphoma, although its presence in the tumour cells may be responsible for some of the serological changes related to tumour proliferation. If that is so, then interest must centre on the question why at least 10% of patients with Burkitt's lymphoma are not seronegative, as in controls (Henle et al., 1969). Much depends upon the sensitivity of immunofluorescence techniques. It is possible that some or all of the individuals currently referred to as "seronegative" actually possess antibodies, but in quantities too small to be detectable at present. It is clearly of considerable importance that this issue should be resolved.

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SPECIFIC IgM RESPONSE AND CHARACTERISTICS OF THE LYMPHOBLASTOID CELL LINES OBTAINED FROM AN INFANT WITH PROBABLE MIXED CYTOMEGALOVIRUS AND EPSTEIN-BARR VIRUS INFECTION

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Cytomegalovirus (CMV) is a member of the herpes group of viruses. It has been shown to transform hamster fibroblasts (Albrecht & Rapp, 1973), stimulate host cell DNA synthesis (St. Jeors et al., 1974) and induce neo-antigens on the surface of infected cells (The & Langenhuysen, 1972). It has not been shown to date to transform cord-blood leukocytes and, to our knowledge, permanent lymphoblastoid cell lines have not been established from infants with congenital cytomegalovirus infection. This paper summarizes the characteristics of two lymphoblastoid cell lines established successively from an infant with the characteristic sequelae of congenital cytomegalovirus infection. The specific IgM and IgG rresponse of this infant suggests mixed cytomegalovirus and Epstein-Barr virus (EBV) infection.

MATERIALS AND METHODS

Patient material

The infant from whom the leukocyte cultures were obtained was a 2 027 g male infant born with a petechial rash

and periventricular calcifications. Total serum IgM level was 84 mg % at birth and 160 mg % at age five months. Characteristic inclusion bodies were observed in the cells of the urinary sediment and cytomegalovirus was isolated on human embryo fibroblasts from three successive urines collected from birth to age five months. The virus was identified as CMV by complement fixation, using the supernatant of human embryo fibroblasts infected cells and uninfected cells as antigen-positive and antigen-negative material with a known CMV antibody-positive and antibody-negative human serum (Lennette & Schmidt, 1969).

Leukocytes cultures

Leukocytes cultures were established from 10-20 ml heparinized blood collected in a Becton-Dickinson vacutainer tube (Moore et al., 1968). Lymphoid cells were separated from red blood cells and polymorphonuclears using the Ficoll-Isopaque gradient technique (Froland & Natvig, 1970). Tests for EBV-S (soluble antigen) were carried out on the supernatant of the established cell lines using supernatants of BHK21 cells as antigen-negative control material and supernatants of Raji cells as antigen-positive control, with a known positive and negative human serum (Vonka et al., 1972). Tests for EBV-VCA and EBV-EA antigens were done by indirect immunofluorescence on acetone-fixed cells using known EBV-VCA and EA-positive, VCA-positive EA-negative, and VCA-negative EA-negative human sera (Joncas et al., 1973, 1974a). The above tests were also carried out on the established cell lines five days following activation by bromodeoxyuridine (BUDR) (25 µg/ml) (Gerber, 1972). Tests for Epstein-Barr virus-determined nuclear antigen (EBNA) were carried out by the complement-immunofluorescence method (Reedman & Klein, 1973; Henle et al., 1974) on cells from both lines using known EBNA-positive and EBNA-negative human sera. EBNA-positive Raji cells and EBNA-negative Molt cells were used as antigen control cells. The lymphoblastoid cell lines obtained were also examined for the presence of CMV antigens by indirect immunofluorescence using known CMV-EBV antigen-positive human serum and CMV-negative, EBV-positive human sera. CMV-positive, EBV-negative human serum could not be readily found. The lymphoblastoid cell lines (supernatant and cell pellet) were also examined before or after BUDR activation for virus particles by negative staining (Joncas et al., 1973) and thin-sectioning (Berthiaume & Joncas, 1973) with a Philips 200 electron microscope. The examination of the cells for immunoglobulin synthesis was done by direct immunofluorescence of unfixed and acetone-fixed cells using fluorescein-conjugated antihuman IgG (heavy and light chain-specific) goat antiserum (Hyland) (Klein et al., 1968). The identity of the immunoglobulin produced and the specificity of the test were confirmed by blocking tests with Hyland's specific anti-IgM, IgG, IgA and IgD antisera. Rosette formation with sheep red blood cells was used to determine the presence of T-cells and their percentage in the two established cell lines (Jondal et al., 1973). Karyotype analysis was carried out by the method of Moorhead et al. (1960).

Serological tests

Sera taken from the mother and the baby on three different occasions, namely at birth, and when the baby was four and nine months old, were tested for CMV and EBV IgG and IgM antibodies by indirect immunofluorescence, as described in previous publications (Joncas et al., 1974a, 1974b). The sera were also tested for EBNA antibodies (Reedman & Klein, 1973; Henle

et al., 1974). Confirmation of the EBV and CMV IgM antibody results was obtained from two reference laboratories.[1]

Molecular hybridization tests

The first lymphoblastoid cell line obtained was examined for deoxyribonucleotide sequences hybridizable with RNA complementary to EBV DNA (Wolf & zur Hausen, 1972).[2]

RESULTS

The two lymphoblastoid cell lines established from the infant were found to have B-cell characteristics, the percentage of immunoglobulin-producing cells in both cell lines exceeding 15%. The first cell line, however, obtained at age four months, produced IgG whereas the second line, established at age nine months, produced IgM exclusively. Lymphoid cells forming rosettes with sheep red blood cells present early after establishment of the cell lines in the proportion of 2% were no longer seen after three months in culture. Karyotype analysis carried out on both cell lines disclosed a diploid pattern with 46 chromosomes, including an easily recognizable Y chromosome. The percentage of polyploidy, however, reached 18% in both cell lines, exceeding the percentage of polyploidy usually encountered in similar lymphoblastoid cell lines. CMV antigens or virus particles could not be detected in cells from either cell line, even following bromodeoxyuridine activation. Both cell lines, however, were positive for EBV-S by complement-fixation tests and for EBNA by immunofluorescence. The first lymphoblastoid cell line obtained at age four months was negative for EBV-VCA and EA antigens, even following BUDR activation, but was positive for the EBV genome by molecular hybridization.[3] The second lymphoblastoid cell line, obtained at age nine months, was found to be positive for EBV-EA antigens spontaneously without activation. In order to determine this with certainty, the cells had first to be blocked by the goat antihuman IgM antiserum. This procedure eliminated the direct fluorescent staining of the IgM-producing cells by the antihuman immunoglobulin conjugate.

Unfortunately, it was not considered either possible or ethical to draw 10-15 ml of heparinized blood from the infant at birth or shortly afterwards in order to establish a cell line as early as possible in the life of this infant. A very small amount only of a serum collected at birth was available. Serology results are shown in Table 1.

DISCUSSION AND CONCLUSIONS

The establishment of permanent lymphoblastoid cell lines with B-cell characteristics from this infant, the significant rise in EBV and CMV IgG antibodies noted after birth and the presence of both CMV and EBV IgM antibodies suggest a mixed CMV-EBV infection rather than a simple serological cross-reaction. The discrepancy between the infant's and the mother's CMV and EBV IgG titres in the sera taken at birth was reproducible in duplicate experiments and seen with two different tests, namely complement fixation and fluorescence. A tentative explanation for this phenomenon could be that specific IgG antibodies, possibly transferred from the mother,

[1] Courtesy of Drs B. Hanshaw and H. Schmitz.

[2] Courtesy of Dr H. zur Hausen.

[3] Courtesy of Dr H. zur Hausen.

Table 1. CMV and EBV IgG and IgM antibody titres of successive sera from mother and infant[a]

Source	Sera	CMV			EBV			
		IgG		IgM	IgG			IgM
		FA	CF		VCA	EA	NA	
Infant	First	ND	4	ND	40	<5	ND	20
	Second[b]	8 000	256	160	160	<5	2[c]	20
	Third	8 000	256	20	160	<5	10	40
	Fourth	-	-	-	-	-	10	-
Mother	First	≥1 000	64	-	≥1 280	<5	10	-
	Second	≥1 000	64	-	≥1 280	<5	10	-
	Third	≥1 000	128	-	≥1 280	<5	10	-
	Fourth	-	-	-	-	-	10	-

[a] ND: Not done: insufficient amount of serum; CMV: Cytomegalovirus; EBV: Epstein-Barr virus; VCA: Viral capsid antigen; EA: Early antigen; NA: Nuclear antigen; First serum: At birth; Second serum: When the infant was 4 months old; Third serum: When the infant was 9 months old; Fourth serum: When the infant was 12 months old.
The specific IgM antibody tests were done on whole sera and on corresponding IgM fractions separated by sucrose density gradients, except for the first serum of the infant, which was available in insufficient amount to separate into fractions.

[b] CMV IgG and IgM antibody titre confirmed in two other laboratories by Dr B. Hanshaw and Dr H. Schmitz; EBV VCA and IgM antibody titre confirmed in Dr H. Schmitz's laboratory.

[c] The EBNA test kindly performed by Dr Werner Henle on the second serum was positive at a titre of 1/10.

are used up in the neutralization of virus and at the same time limit, by a feedback mechanism, the active synthesis of specific IgG antibodies by the infant. This phenomenon, observed for CMV and for EBV antibodies as well, would also suggest a mixed infection. The possibility that the infant was infected with EBV postnatally cannot be completely ruled out, since a leukocyte culture was not taken at birth. If this is a case of congenital CMV infection alone, the detection of EBV IgM antibodies in the first serum at a titre of 20 could be the result of a serological cross-reaction between CMV IgM and EBV IgM antibodies, previously noted in patients with mononucleosis by at least two investigators (Schmitz et al., 1972; Hanshaw et al., 1972). This cross-reaction, however, has not so far been reported in patients with CMV infections. The possibility that the infant's leukocytes have been transformed by CMV in addition to EBV will be determined by the molecular hybridization technique (Huang & Pagano, 1974).[1] It is interesting to note that the expression of the EBV genome is limited early in life to one antigen of the virus (EBV-S), spontaneously, in this case, or secondary to transformation of cord-blood leukocytes by exogenous EBV in cases from the literature (Miller et al., 1973). EBV-EA and EBV-VCA antigens are more readily expressed in cell lines obtained from older children and adults. At the age of nine months, a cell line from this infant spontaneously expressed EBV-EA antigens but not VCA. This phenomenon, and its significance in terms of host-cell control mechanisms of viral genome expression, deserves further study. Other explanations for this phenomenon should also be considered.

[1] Courtesy of Drs J. Pagano and E.S. Huang.

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ANALYSIS AND DESCRIPTION OF PROCEDURES USED IN THE STUDY OF THE RELATIONSHIP OF HERPES SIMPLEX VIRUS "NON-VIRION" ANTIGENS TO CERTAIN CANCERS

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The implication of type-1 herpes simplex viruses and especially of type-2 strains in certain human cancers is based on a body of findings ranging from the higher incidence of antibodies to herpes simplex virus (HSV) in the sera of patients with cervical cancer than in control sera (Rawls et al., 1968; Nahmias et al., 1970; Rawls et al., 1973; Aurelian et al., 1973) to the presence of antibodies to virus-induced antigens only or mainly in cancer patients (Hollinshead et al., 1973; Sabin & Tarro, 1973). Further proof of the potentially oncogenic nature of these viruses (Duff & Rapp, 1971, 1973) is provided by the presence of HSV-specific antigens in some tumour cells (Hollinshead & Tarro, 1973; Aurelian, 1974) and also of viral DNA in one cervical carcinoma (Frenkel et al., 1972).

Since the occurrence of antibodies to the HSV virion suggests only an association between the virus and the cancers in question, and the biochemical studies are neither significant nor numerous enough, the "non-virion" (NV) antigens and their specific antibodies might be the appropriate field for research aimed at demonstrating the relation between HSV and selected tumours, because they provide the kind of evidence whereby virus-free experimental tumours can be proved to have been originally induced by oncogenic DNA viruses.

MATERIALS AND METHODS

Preparation of antigen

The methods used for the culture of guinea-pig kidney (GPK) and rabbit kidney (RK) primary cells and of the HEp-2 continuous cell line have already been described (Tarro & Sabin, 1970) as well as that used for propagating human fetal kidney. The HSV type-1 and HSV type-2 (HSV-1 and HSV-2) strains used in this work have been listed elsewhere (Tarro & Sabin, 1973). The procedures for the infection of the cell cultures

and the preparation of the different HSV antigens, both structural and non-structural, have been previously reported in detail (Tarro & Sabin, 1970; Sabin & Tarro, 1973). A summary is shown below:

1. GPK cells harvested at three hours (start of cytopathic effect [CPE]). RK and HEp-2 cells harvested at 24 hours (complete CPE).
2. Cells scraped, washed and centrifuged at 1 500 rpm for 10 minutes. Cell sediment resuspended in basal medium (Eagle) (BME), recentrifuged.

   Cell sediment → 10% in BME for antigen preparation
   Cell sediment → 10% in distilled water for virus stock.
3. 10% cell suspension frozen and thawed, sonicated for 30 seconds.
4. Virion antigens: suspension stored at 4°C for 2-3 weeks before use. Non-virion antigens: suspension stored at -80°C, used within two days of preparation.

Specimens for virion antigens are stored at 4°C in a refrigerator for 2-3 weeks to inactivate the non-virion (NV) antigens and then used for absorption of virion antibodies or, after further storage at -80°C, for measurement of the virion antibody. Longer storage at 4°C of virion antigen for absorption purposes (Sabin, 1974) is not advisable because virion titres are also reduced and it is necessary to increase the quantities of antigen for absorption to remove the virion antibody: NV components might be present in insufficient concentration in the "stored" 10% specimen to be detected by specific antiserum, yet in sufficient concentration to remove specific antibody with further absorption. For the same test, the virion antigen used for measurement of virion antibody was deliberately obtained from infected cells harvested after the same number of hours as those used for NV antigen, as previously described (Tarro & Sabin, 1970, 1973); thus "stored" HSV-infected GPK cells harvested at three hours were used when a "fresh" similar sample was tested for NV antigen, so as to ensure that the absorbed serum contained no antibody to a three-hour stable virion antigen not present or present only in small amounts at 24 hours. Another reason for using such an antigen was to avoid problems of anti-complementarity (AC') activity of the given absorbed serum to be tested with different kinds of antigen (Sabin, 1974).

Specimens to be used for testing NV antigen were stored at -80°C just after harvesting, frozen and thawed once and sonicated; they were used as soon as possible, usually after two days. These preparations contain both NV and virion antigens, but the absorbed serum used for monitoring contains no measurable virion antibody.

Preparation of absorbed antisera

The preparation of specific antisera to identify the HSV NV antigens has been previously described (Tarro & Sabin, 1970) and is summarized here:

A. *Guinea-pig antisera*:

1. From guinea-pigs immunized with three-hour harvests of HSV-infected GPK cells, grown in guinea-pig serum.
2. Absorbed with stored virion antigen till no residual complement-fixing (CF) antibody found against HSV-1 and HSV-2.
3. Supernatant, after centrifugation at 37 000 rpm, inactivated at 60°C.

B. *Human sera:*

1. From control and cancer patients.
2. Diluted 1:4, absorbed three times with virion antigen.
3. Processed as above (A.3) and used if not containing any measurable virion antibody.

Complement-fixation test

The method used in our tests is the same as that used previously (Sabin & Koch, 1964; Tarro & Sabin, 1970; Sabin & Tarro, 1973). The points to be noted on the CF test are as follows:

1. Anticomplementary (AC') activity was determined before the CF test, so as to establish the least amount of complement (C') giving complete haemolysis in the presence of each antigen and of each serum used in the CF test.
2. 1.5 Units of C' were used, depending on which component (antigen or serum) showed the greater binding activity for C' in the titration of C', and were found sufficient to control:

   (*a*) the sensitivity of sheep red blood cells (SRBC) to haemolysin, depending on the duration of storage in Alsever's solution[1] and as a washed suspension at 4°C;

   (*b*) different values for one unit of C' when different lots of haemolysin were tested with the same SRBC;

   (*c*) increased binding of non-specific CF activity of two AC' components.
3. Completeness of virion absorption of sera was checked with stored virion antigen that was not anti-complementary.

The observation that a mixture of two AC' components bound less complement (C') than the more strongly AC' component by itself (Sabin, 1974) cannot be considered here since both the virion and NV antigens used showed approximately the same AC' activity as described above and elsewhere (Tarro & Sabin, 1970, 1973).

DISCORDANT FINDINGS

A total of 33 human cancer sera "positive" for herpesvirus NV antibody were retested and found negative, as well as 14 negative control sera (Sabin, 1974); these data might have a bearing on the present search for HSV-1 and HSV-2 NV antigens to determine whether these viruses play a part in the causation of certain human malignancies. In comparison with the 194 coded sera previously tested (Sabin & Tarro, 1973) (56 positive, from urogenital cancers and other head and neck cancers, and 138 negative, from controls and other types of cancers), the above-mentioned study (Sabin, 1974) also shows a total of 475 human sera tested, i.e., an increase of 281, namely 125 "positive" sera from 14 types of cancer (skin, lip, oro- and nasopharynx, larynx, kidney, bladder, prostate, penis, cervix uteri, vulva, vagina, anus and rectum) and 99 negative sera from persons without cancer or with cancers of cervix uteri, vulva, or larynx after surgery. The CF titres were not given but were presumably low (Sabin & Tarro, 1973). When immunized guinea-pig and human sera have been retested within a few months with negative results (1-2 dilutions of difference), as compared with different data obtained over a period of five

[1] Sodium chloride: 0.42%; trisodium citrate (dihydrate): 0.8%; glucose: 2.05%; adjusted to pH 6.1 with 10% citric acid solution; 120 ml of solution should be used per 100 ml blood.

years (Hollinshead & Tarro, 1973; Hollinshead et al., 1973, 1974a; Sabin & Tarro, 1973; Tarro & Sabin, 1970, 1973), these discordant findings can be explained only by the following: (1) the use of absorption materials as a source of virion antigen that might still contain NV components preserved for longer periods in less strongly AC' cell suspension may have resulted in the removal of the specific NV antibody from the absorbed sera; (2) the same applies to the standard virion antigen used to check the completeness of absorption; sera can be considered as incompletely absorbed when they are tested with virion antigen that has not been properly stored so as to ensure disappearance of NV antigens; (3) the conclusion that the absorbed serum yields positive results only when tested at a higher dilution of C' is based on selective data (Sabin, 1974) and is also contrary to other findings (Hollinshead & Tarro, 1973; Tarro & Sabin, 1970, 1973), including the fact that unabsorbed cancer sera with no detectable antibody for virus did react with HSV NV antigen (Hollinshead & Tarro, 1973); (4) in the CF tests reported as negative (Sabin, 1974), there was a lack of proper positive controls because the serum specimens were not tested simultaneously with AC' virion antigen and a higher dilution of C' was not then used with GPK antigens to confirm the "positive" results previously obtained; (5) furthermore, in the elimination of the "unconfirmed" data, the appropriate antisera were not used, since none was made in guinea-pigs according to the precise methodology using the appropriate three-hour harvested HSV-infected cells; (6) finally, in the same paper (Sabin, 1974), the results of a CF test employing a small number of sera (12) are used to deny the prevalence of antibodies to HSV at higher titres in urogenital and oral cancer patients as compared with other types of cancer, despite the evidence provided by the publications in this field (Rawls et al., 1968, 1973; Nahmias et al., 1970; Aurelian et al., 1973; Lehner et al., 1973).

DISCUSSION

It is not yet known whether the NV antigen, induced early as a protein product of the repressed herpesvirus genetic information, is really non-structural or can be found in properly disintegrated, highly purified HSV particles. It is certainly not a newly synthesized or modified pre-existing host antigen: the herpesvirus specificity of the positive reactions for the NV antigens was established by the failure of the NV antibody to react with material in cells similarly infected with vaccinia virus and by the lack of absorption with large numbers of intact, trypsinized, human embryonic kidney cells or with large numbers of freshly harvested HEp-2 cells (Sabin & Tarro, 1973; Tarro, 1973). Studies are required to determine which of the many non-structural antigens obtained from herpesvirus-infected cells (Honess & Roizman, 1973) corresponds to the NV antigens detectable by absorbed guinea-pig sera. It is also important to find out whether or not the NV antigens are analogous to the early four-hour antigens (AG-4) produced by HSV-2 in HEp-2 cells that react in CF and in immunofluorescence tests with the antibodies of sera from cervical cancer patients (Aurelian et al., 1973). The multiplicity of NV antigens suggests that different components are responsible for different antigenicities and presumably for different uses from a practical point of view. The CF reactivity of AG-4 seems to correlate well with the clinical stage of the neoplastic disease (Aurelian et al., 1973;

Aurelian, 1974), while other studies showed little difference between pre-operative patients, and those clinically cured (Hollinshead et al., 1973, 1974b), in line with the observed prolonged immune cellular abnormalities in squamous-cell cancer patients (Twomey et al., 1974).

Recent data on the presence in cervical cancers of antigens requiring complement to be detected in immunofluorescence assay (Del Buono et al., 1974) indicate the possibility that these antigens may be similar or identical to NV antigens or at least are part of them: they too can react with NV guinea-pig antisera (Giordano[1]; Nahmias[2]).

[1] Unpublished data.

[2] Personal communication.

SUMMARY

After a brief review of the relationship of herpes simplex viruses to certain human cancers, a description is given of procedures used in the study of herpesvirus "non-virion" antigens, and various points on which disagreement currently exists are discussed. Complement-fixation tests on these markers with both cancer and control sera as well as with "non-virion" antisera, which did not react with non-anti-complementary stored virion antigens, are described, and it is suggested that another parameter is now available for studies of squamous-cell carcinomas. The finding in certain tumours that expression of a repressed viral genome takes place proves that there is a continuing release of virus-specific messages and would indicate an important role for the virus in the development of the tumour.

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DEMONSTRATION OF HIGH LEVELS OF CYTOLYTIC ANTIBODIES REACTIVE WITH HSV-1 AND HSV-2 INFECTED CELL MEMBRANES IN SERA OF GENITAL TUMOUR PATIENTS

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Antigenic modification of cytoplasmic membranes is directed by the genome of herpes simplex viruses (HSV) in productively infected mammalian cells. Herpes simplex virions belonging to serotype 1 (HSV-1) and serotype 2 (HSV-2) produce similar but not identical antigenic changes in the membranes of these cells. These new antigens have been subdivided into two classes: (1) type-specific antigens; and (2) type-common antigens. The number of antigenic elements in each class is unknown. Complement-fixing antibodies that are reactive with these antigens have been experimentally produced in rabbits (Roane & Roizman, 1964) and have been shown to be present in the sera of humans who have experienced natural infections with HSV-1 or HSV-2 (Smith et al., 1972; Yang et al., 1973). This report describes the results of assays in which the relative levels of cytolytic antibodies in sera from tumour patients, non-tumour patients and healthy persons were measured. The effect of addition of antiglobulins to sensitized infected cells is also described.

MATERIALS, METHODS AND RESULTS

A method for the assay of cytolytic antibody activity in the presence of complement was developed by Roane & Roizman (1964) using rabbit sera. In the present investigation, human sera were usually diluted 1/2, 1/8, 1/32 and 1/64 after absorption with uninfected HEp-2 cell suspensions and decomplementation by heating at 56°C for 30 minutes. Artificial mixtures containing approximately 10^5 HSV-MP infected cells and 10^5 HSV-2 infected cells per ml were prepared 24 hours after infection. Individual mixtures received 0.1 ml of diluted serum and 0.1 ml of guinea-pig complement (diluted 1/3 in veronal-buffered saline [VBS]). In some experiments, 0.1-ml volumes of heavy chain-specific antihuman IgA, IgG or IgM were added The final volume was always adjusted to 1.5 ml with VBS. The reaction

mixtures were incubated at 37°C for 1 hour with intermittent shaking. After this period, aliquots of the reaction mixtures were dispensed into duplicate cultures of confluent uninfected HEp-2 cells. Maintenance medium containing 0.2% pooled human gamma globulin was added and the cultures were incubated for 44-48 hours in a humidified atmosphere containing 7% carbon dioxide. After this period, the monolayers were fixed, washed and stained with Brom Phenol Blue for visualization and counting of plaques.

In the first experiment, we measured the levels of antibody reactive with HSV-2 specified antigens present on the surfaces of HEp-2 cells 24 hours post infection. Serum from a healthy female was included as a control. Each serum was titrated for cytolytic activity, as described above. In this experiment, 12 sera were examined. The serum from the healthy female had the lowest activity and was used as the index for expressing the relative activity of each serum. As may be seen in Table 1, there was a 500-fold difference between the most potent and the least potent sera. In the next series of experiments, artificial mixtures of HSV-MP and HSV-2 infected cells were used. The results of a representative experiment are shown in Table 2. Two features should be pointed out: (1) serum No. 2 from

Table 1. Relative cytotoxic effect of human sera[a] against HSV-2 infected HEp-2 cells

Serum No.	Clinical diagnosis	Fractional survival of infectious centre formation
1	Healthy female	1.000[b]
25	Chronic myelocytic leukaemia	0.210
194	Penile carcinoma	0.178
116	Sarcoma	0.104
107	Pharyngeal carcinoma	0.093
537	Breast sarcoma	0.045
273	Hodgkin's disease	0.040
262	Prostatic carcinoma	0.022
209	Ovarian carcinoma	0.002
352	Chronic myelocytic leukaemia	0.015
2	Cervical carcinoma	0.002
263	Pancreatic carcinoma	0.036

[a] At half dilution.

[b] Used as the reference.

Table 2. Relative cytotoxic effect of human sera[a] against HSV-1 and HSV-2 infected HEp-2 cells

Serum No.	Clinical diagnosis	Fractional survival of infectious centre formation	
		HSV-1 infected cells	HSV-2 infected cells
3	Healthy male	0.612	1.000[b]
149	Chronic myelocytic leukaemia	0.013	0.018
164	Acute myelocytic leukaemia	1.000[b]	0.697
119	Acute myelocytic leukaemia	0.067	0.093
5	Recurrent facial herpes	0.046	0.015
2	Cervical carcinoma	0.002	<0.001
7	Cervical carcinoma	0.002	0.023
6	Degenerative ovarian teratoma; invasive carcinoma of endometrium	0.007	0.023
8	Rhabdomyosarcoma of the uterus	0.002	<0.001

[a] At half dilution.

[b] Used as the reference.

a cervical carcinoma patient had consistently high activity against HSV-2 infected cells (see Table 1); (2) the test system discriminates between type-1 (MP) and type-2 infected cells.

We examined a total of 51 human serum specimens and then determined the volume of undiluted serum that was required to kill 90% of HSV-1 (MP) or HSV-2 cells. The results of this comparison are summarized in Figs. 1 and 2. It is apparent that most of the sera tested were of low potency against HSV-2 infected cells, with the exception of sera from: (i) cervical carcinoma patients (6/8); (ii) vaginal carcinoma patients (2/3); and (iii) patients with recurrent genital herpes (2/2). An arbitrary boundary between high antibody titre and low antibody titre was established at 0.01 ml of patient's sera, i.e., high antibody titres were expressed as a reduced requirement for undiluted serum to produce 90% killing of infected cells. Table 3 summarizes the distribution of antibody titres against HSV-1 and HSV-2 infected cells among the sera tested. It is apparent that the system discriminates between high and low antibody titres with respect to HSV-1 (MP) and HSV-2 infected cell membrane antigens.

In another series of experiments, we were interested in determining whether specific IgA was present in these sera. Since IgA does not fix

FIG. 1. DISTRIBUTION OF SERUM ANTIBODY TITRES AGAINST HSV-1 (MP) INFECTED HEp-2 CELLS

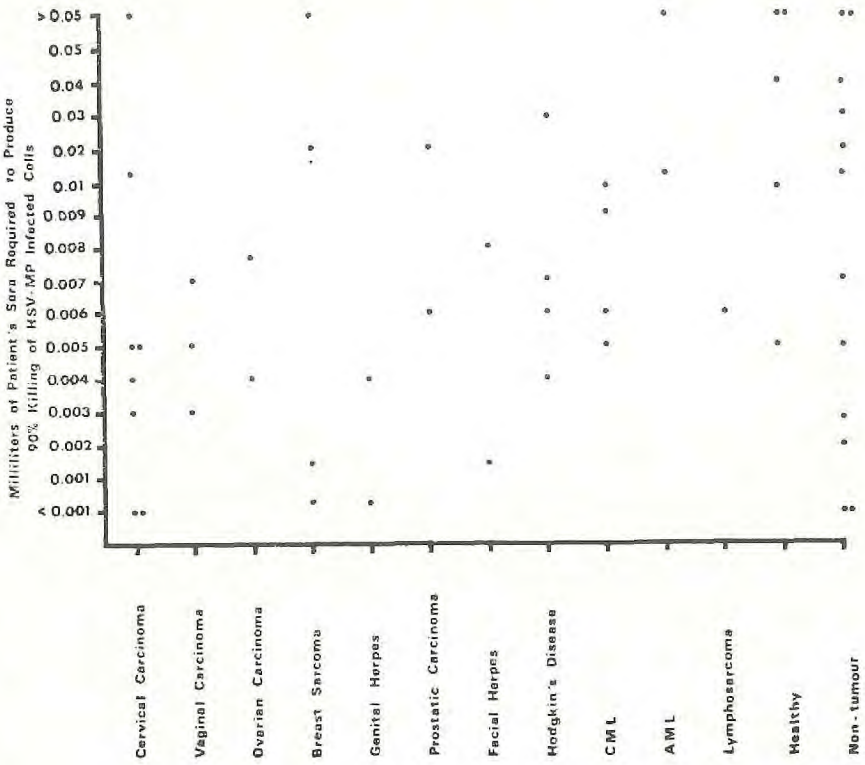


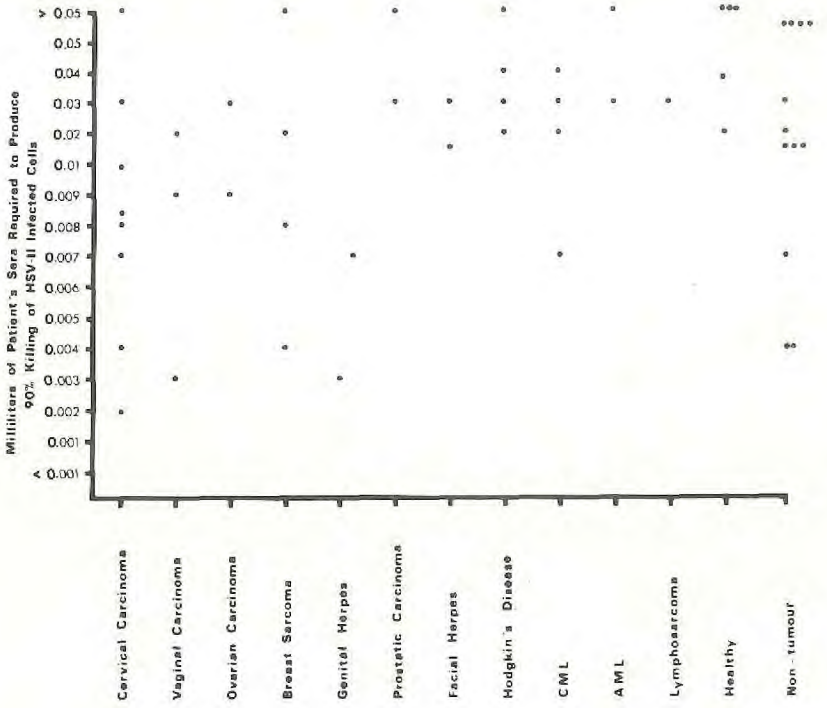
Table 3. Distribution of high and low antibody titres among the human sera tested

Infected cell membrane antigens	Low titres[a]	High titres[b]
HSV-1 (MP)	18/51	33/51
HSV-2	33/51	18/51

[a] ≥ 0.01 ml.

[b] <0.01 ml.

FIG. 2. DISTRIBUTION OF SERUM ANTIBODY TITRES AGAINST HSV-2 INFECTED HEp-2 CELLS



complement, we incorporated heavy chain-specific antiglobulins in a set of reaction mixtures containing HSV-2 infected cells and compared the number of infectious centres (plaques) formed in the presence of each antiglobulin with the number of plaques formed in the absence of the antiglobulins. The results of this experiment are summarized in Table 4. As may be seen, the addition of anti-IgA increased the killing of infected cells in mixtures containing sera from tumour patients or the serum from the herpes patient. The addition of anti-IgG or anti-IgM had a variable effect on the killing of cells.

DISCUSSION

We have presented evidence that the original cytolytic antibody assay method described by Roane & Roizman (1964) can be adapted to measure cytolytic antibodies in human sera. The method that we have used appears capable of discriminating between high and low serum antibody titres with respect to cell-surface

Table 4. Effect of antihuman immunoglobulins on infectious centre formation by HSV-2 infected cells

Human serum No.	Infectious centres formed as a percentage of number formed in absence of antiglobulins			
	C' + serum only	C' + serum + anti-IgA	C' + serum + anti-IgG	C' + serum + anti-IgM
2[a]	39.7	2.1	24.1	10.0
8[b]	25.8	3.5	29.4	8.6
9[c]	65.0	2.0	30.0	13.0
1[d]	47.4	47.0	64.0	57.0

[a] From cervical carcinoma patient.

[b] From patient with rhabdomyosarcoma.

[c] From adult male with recurrent genital herpes.

[d] From healthy adult female.

antigens specified by HSV-1 or HSV-2.

We have also observed that antiglobulins will increase the killing of sensitized HSV-infected cells. This is reminiscent of the observation of increased neutralization of sensitized herpes simplex virions by anti-IgG described by Ashe & Notkins (1966).

We have recently observed evidence of the binding of specific IgA to infected membranes by the indirect-immunofluorescence method. The observation that IgA antibodies are formed and may bind with cell-surface antigens suggest that IgA may play a role in the protection of infected cells *in vivo*.

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STUDIES OF THE NATURE OF HERPESVIRUS-INDUCED TUMOUR-ASSOCIATED ANTIGENS INDUCED BY HERPES SIMPLEX VIRUS TYPE 1 AND FURTHER ANALYSIS OF THEIR RELATIONSHIP WITH SQUAMOUS-CELL CARCINOMAS OF THE HEAD AND NECK REGION

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In this report we present further studies of the nature of herpesvirus-induced tumour-associated antigens (HSV-TAA) and report the results of further studies of complement-fixing (CF) reactivity by the antigens to sera from head and neck squamous-cell cancer patients and controls, as well as the titration of antibody levels in untreated and cured groups. In addition, we have studied the relationship between HSV-TAA antibody and sex, age, race, stage of cancer, immune status, and level of antibody in unfiltered sera stored less than five months as compared with filtered sera stored for up to three years in liquid nitrogen.

In previous reports (Hollinshead et al., 1973, 1974) we presented the results of two large-scale tests using sera from the National Cancer Institute that had been filtered and stored, and compared them with those from one large-scale test using unfiltered sera from Baylor University Medical Center that had been collected approximately two years prior to our study and stored at -35°C.

In the current test, we used 44 µg of HSV-TAA, which consists of two polypeptide bands. Details of the preparation of HSV-TAA have been presented in detail elsewhere (Hollinshead et al., 1972). A study was made of hamster embryo kidney (HEK) cells superinfected with herpesvirus, and grown in serum-free media containing labelled amino-acids. Bottles were removed at

different times and the HSV-TAA polypeptide bands sliced and separated, while some of the cultures were grown for 4+ cytopathic effect (CPE), the mature viruses harvested and purified by sucrose density-gradient ultracentrifugation. The upper band was labelled within 30 minutes both for leucine and for alanine in two separate cultures. The label for leucine continued to be present in cultures harvested at 12, 24 and 36 hours, but was not present in any of the bands separated from the mature purified virus. The alanine label persisted and was present in the purified virus. Since the upper band of HSV-TAA does not appear in that form in the mature virus structural materials, we conclude that this upper band is present in early, provirus formation, and that part of this peptide chain is used in the assembly process of one of the structural components of the mature virus. This upper band is a highly unstable peptide chain, disintegrates very easily, and is probably responsible for the disappearance of CF reactivity of the purified antigen stored at 4°C for a period of five to six days. The lower band labelled with leucine only, but not alanine, and could be separated by 33 hours, but none of the label appeared in the mature virus. Thus the lower band does not seem to be a part of the assembly process. Perhaps neither of the two polypeptides, in that form, are a part of the protein structures of the mature virus. Neither of the two bands is a major histocompatibility antigen, as measured using broadly reactive sera in the chromium-release cytotoxicity assay. It is still possible, therefore, that the lower band, at least, could be an augmented weak histocompatibility antigen "turned on" by HSV nucleic acid formation or a new protein, neoantigen, or possibly a fetal protein. The nature of these tumour-associated antigens must be studied in greater detail.

In the previously reported tests with NCI filtered sera, 71% of patients with preoperative squamous-cell cancers of the head and neck (other than larynx) had complement-fixing antibodies to the herpesvirus-induced polypeptides. Of preoperative squamous-cell cancers of the larynx, 75% had positive complement-fixation reactivity. In contrast, sera from cured squamous-cell cancer patients had CF reactivity to HSV-TAA in 57% of the specimens, while 71% of sera from patients with cured squamous-cell cancers of the larynx had positive antibody to the polypeptides. In the studies using the unfiltered Baylor University sera, 91% of sera from patients with preoperative squamous-cell cancers of the larynx were CF-positive, while 100% of sera from patients with postoperative (11-96 months post surgery) squamous-cell cancers were positive. Positivity in sera from normal individuals was 6% for the NCI sera and 5% for the Baylor sera, respectively.

We were curious about the level of activity in patients who had been cured of squamous-cell cancer (4-16 years after surgery) in the NCI group, but in the control series, 0% of preoperative non-squamous-cell and 16% of cured non-squamous-cell cancer sera had antibody to HSV-TAA (Hollinshead et al., 1973, 1974).

In the present series, 55 patients with squamous-cell carcinoma of the head and neck contributed sera; these were Millipore-filtered and stored for up to three years. Of these, 30 sera were from tumour-bearing and 25 from cured patients. Sera were obtained from an additional 55 patients, unfiltered, obtained within five months prior to CF testing; they comprised 34 sera from tumour-bearing and 21 sera from cured patients. Also included were sera less than five months old and unfiltered from 16 normal individuals as well as filtered sera up to three years old from 12 non-squamous-cell cancer patients. As shown in Table 1, a higher incidence of

Table 1. Comparison of CF-positive filtered and unfiltered sera tested against HSV-TAA in present and previous tests

A. Present tests

Sera	All titres		High titre	
	No. positive/No. tested	%	No.≥1:4/No. tested	%
Filtered (1-3 years storage)				
Untreated	17/30	56.7	8/30	26.7
Cured	12/25	48.0	2/25	8.0
Total	29/55	52.7	-	-
Unfiltered (1-5 months storage)				
Untreated	30/34	88.2	14/34	41.18
Cured	19/21	90.5	10/21	47.62
Total	49/55	89.1	-	-

B. Comparison with previous tests [a]

Sera	% Positive	
	Previous tests (NCI)	Current tests (NCI)
Filtered		
Untreated non-laryngeal cancer	71	50
Untreated laryngeal cancer	75	70
Cured non-laryngeal cancer	57	42
Cured laryngeal cancer	71	54
Unfiltered	(Baylor)	(NCI)
Untreated non-laryngeal cancer	-	88
Untreated laryngeal cancer	91	83
Cured non-laryngeal cancer	-	100
Cured laryngeal cancer	100	82

[a] See Hollinshead et al. (1972, 1973).

positive reactions was obtained with the unfiltered sera (49 out of 55 positive; 89%) than with the filtered sera (29 positive out of 55; 53%). These results were similar to those obtained in the previous study using the filtered sera from NCI and the unfiltered sera from Baylor University. The differences between untreated patients and cured patients in studies of the filtered sera proved to be spurious when the numbers of sera with higher titres to HSV-TAA of untreated versus cured sera were measured in the unfiltered sera (41 and 47% respectively). In addition, as shown in Table 1, the distribution of CF reactivity in the unfiltered sera was similar among groups with laryngeal cancer and groups with oropharyngeal tumours. This shows that the lower titres obtained with sera from oral cavity tumours in both the previous and the present study can be attributed to filtration of the sera rather than to location of the tumour.

No significant differences were detected in a study of the distribution of positive reactivity to HSV-TAA according to age, sex, or race. In this particular study, none of the normal sera were positive (0 out of 16) for CF reactivity to the antigen; however, in previous tests, using a larger number, at least 5% of normal control sera were positive. Additional sera from patients with non-squamous-cell cancers were also included in the current tests, and the same approximate percentage of positive reactors (17%) was seen, as in the previous studies of non-squamous-cell cancers (Hollinshead et al., 1973, 1974).

Of considerable interest in our analysis of the positive reactions to unfiltered sera from patients with laryngeal carcinomas, was that patients with stage I laryngeal carcinomas, which are much smaller tumours than those seen in stage I oral cavity cancers, were somewhat less CF reactive to HSV-TAA. Seven out of 11 positive stage I laryngeal carcinomas, in contrast to 17 out of 18 positive in stages II-IV, were seen ($P = 0.05$). This was in contrast to the comparison of stage I and stages II-IV oral cavity cancers, where no significant differences were seen for positive CF reactivity in the different stages. In studies in which the *in vitro* reactivity of lymphocytes from the same patients to phytohaemagglutinin (PHA) was measured, the stage I cancer patients did not differ very much from normal individuals in their PHA response, as compared with a significant response in patients with laryngeal cancers in stages II-IV.

SUMMARY

The differences seen in levels of antibody response to HSV-TAA between non-laryngeal and laryngeal cancers in cured and untreated patient populations were due to the use of filtered sera. In previous and present studies using unfiltered sera, these differences were not noted. Of unfiltered sera from patients with squamous-cell carcinomas of the head and neck, 89% (49 out of 55) were positive for CF reactivity to HSV-TAA, and 44% of these unfiltered sera had titres of 1:4 or greater to 44 µg of antigen. The upper band of the antigen appears to be present in early provirus formation and is highly unstable whereas the lower band does not seem to be part of the assembly process.

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DISCUSSION SUMMARY

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Discussion was initially directed toward recent observations on Epstein-Barr virus (EBV) antibody patterns in human lymphoma patients. Data were presented that suggested that elevated anti-EBV levels in patients with a variety of lymphoproliferative neoplasms could not be related to specific or non-specific depression of cellular immune responses. In other studies of untreated patients with Hodgkin's disease, however, a definite relationship was demonstrated between immunological defects and anti-EBV titres. In the Hodgkin's group, high anti-EBV reactivity seemed to be related to diminished cutaneous responses to PPD, decreased numbers of T lymphocytes in the peripheral blood, and depression of mitogen-induced DNA synthesis. Numerous comments emphasized the importance and necessity of developing more complete understanding of the type and mechanism of the immunosuppressive reaction that is being measured in such studies, inasmuch as lymphocytes, macrophages, or soluble mediators of immune responses may be affected at any of several points in the sequence of events leading to delayed hypersensitivity reactions.

Studies of certain immunogenetic aspects of patients with nasopharyngeal carcinoma (NPC) reveal an increased frequency of 1st locus HL-A2 antigen and a deficit of antigens at the 2nd locus, one of which has been identified as Singapore 2 antigen. These HL-A associations with NPC suggest the presence of genes associated with NPC disease susceptibility. Furthermore, the genes may also be determinants of differences in immunological responses to environmental factors. Demonstration of a high incidence of antinuclear antibodies (ANA) and anticytoplasmic antibodies (ACA) in patients with NPC has also suggested the presence of different genetic backgrounds. The importance of lymphocyte-stimulation studies of persons having these defects was mentioned and the value of prospective epidemiological investigations of these subjects and their family members in relation to the development of NPC

received emphasis. Lengthy discussion was directed to considerations of the mode of action of immune-responsiveness genes and to questions concerning methods for identifying their level of operation on the immune response, on target cells, and on immunological amplifying factors.

Questions were raised relating to the challenge of malaria on the immunological system of the host and the mechanisms by which this infection may play a role in the pathogenesis of Burkitt's lymphoma (BL).

The geographical distribution of Burkitt's lymphoma has clearly suggested a hypothesis that includes the role of some type of co-factor. However, it is not known whether the disease develops after a defined latent period following EBV infection or whether the presence of the tumour affects the titres of EBV antibody. Clinical details of an eight-year-old patient who developed Burkitt's lymphoma after completion of chemotherapy for Hodgkin's disease were presented. Anti-VCA antibodies were present at least nine months prior to onset of BL, and rose sharply with appearance of the tumour. Antibodies to early antigen (EA), undetectable prior to onset of BL, also rose sharply, and antibodies to EBV-determined nuclear antigen (EBNA) increased slowly from low pre-tumour levels. The role of previous immunosuppression by Hodgkin's disease and chemotherapy in the subsequent development of BL in this patient was not clear. It is now well recognized, however, that patients who have received renal transplants have a high incidence of lymphoma, usually reticulum cell sarcoma, and are also known to experience severe clinical infections with other herpesviruses. In such cases, reactivated EBV infection under the conditions of renal transplant and immunosuppression may be the mechanism involved in the development of lymphoma.

Additional comments were directed to the question of EBV-related tumours outside endemic areas and to the need for their extensive study by current techniques that permit identification of the EBV genome in the absence of virion production. These include nucleic acid hybridization studies utilizing strains of EBV from different sources and having different biological characteristics.

VACCINATION AND OTHER PROTECTIVE MEASURES

VACCINATION AGAINST ONCOGENIC HERPESVIRUSES – A REVIEW

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A number of protective measures are available against infectious diseases, and this is also true of herpesvirus infections. These include hygiene, management, genetic selection for resistance, and immunization, both passive and active. In farm animals, where control of the forms of husbandry and management is in the hands and the responsibility of relatively few people, methods of management and hygiene can be developed and chosen to reduce the chance of dangerous infectious agents producing disease. Genetic selection for resistance to infection or to the results of infection is also an approach that can be used, but is restricted to domestic animals. However, the interest of comparative medicine lies in the models where vaccination has been successful in controlling the effects of infection with oncogenic herpesviruses.

VACCINES

Vaccines may consist of either live or inactivated viruses. Live virus vaccines can contain: (1) pathogenic virus; (2) pathogenic virus that has been modified so as to lose pathogenicity but maintain immunogenicity; or (3) naturally apathogenic virus. Vaccines containing pathogenic viruses are used in a way that reduces their pathogenicity, e.g., administration by an unusual route or at a time when the animal is resistant to disease or, in some cases, the administration may be followed by specific antiserum. Naturally apathogenic virus can be either a virus common in the host species in which the disease occurs, or a related virus derived from another host species.

Inactivated vaccines are usually whole-virus preparations of varying purity, together with soluble antigens, inactivated by one of a number of substances that destroy the infectivity of the virus with minimum effect on its immunogenicity. The immunogenicity of such vaccines may be improved by the use of adjuvants. More recently, the possibility has arisen of using purified preparations containing no nucleic acid, but only the relevant immunizing proteins. The results of experiments with "split product" or "subunit" vaccines have been encouraging, and the prospects of overcoming the difficulties of large-scale production of single proteins have improved with recent developments

in the genetic manipulation of bacterial plasmids.

Vaccination against infection with members of the herpesvirus group is used extensively in the veterinary field. Both inactivated and live vaccines are used, although in general the latter are more effective. The live vaccines used are of all the three types described above. However, the viruses for which the vaccines are used cause their disease by productive and cytolytic infections of the parasitized cells. Oncogenic herpesviruses are non-productive in the cells they transform, and protection against this type of infection and pathogenic effect might be considered less likely. Nevertheless, the success of vaccination against Marek's disease (MD) has shown that this method of prophylaxis can be effective in controlling neoplastic disease caused by a herpesvirus.

Little work has been done on the development of vaccines for use in oncogenic herpesvirus infections other than MD. It is for this reason that I shall devote most of the remainder of this presentation to a description of the development and use of vaccines against Marek's disease, together with a synthesis of what is known of their function.

MAREK'S DISEASE

Virology

Before discussing vaccination against MD, it would be useful to briefly summarize the relevant information on the virus of the disease. Marek's disease virus (MDV) is a herpesvirus that is antigenically closely related to a herpesvirus isolated from turkeys. Cell-free MDV can be prepared from feather follicle epithelium of infected chickens (Calnek et al., 1970a), but in all other tissues in the chicken and in cell culture it is avidly cell-associated (Biggs et al., 1968). The herpesvirus of turkeys (HVT) differs in this respect because cell-free virus can be prepared from infected cultured cells (Calnek et al., 1970b).

Marek's disease viruses have been divided into three groups on the basis of their pathogenicity (Biggs & Milne, 1972): (1) those that produce the acute form of MD in which lymphoid neoplasia is the main manifestation; (2) those that produce the classical form of MD, which is more chronic and is characterized by neural involvement; (3) those that are apathogenic. The apathogenic viruses form small plaques in cultured chick kidney cells, the two groups of pathogenic viruses produce medium-sized plaques, and HVT, which is apathogenic for chickens, produce large plaques in these cells.

Although it has been known for some time that there are antigenic differences between MDV and HVT (Witter et al., 1970), more recently Bülow & Biggs, 1975a, 1975b) and Bülow et al.[1] have described quantitative and qualitative antigenic differences between pathogenic and apathogenic MDV and between these viruses and HVT using immunofluorescence, double-diffusion precipitation in agar gel and virus neutralization. They have suggested that MDV and HVT belong to a single group of herpesviruses that can be divided into three serotypes: (1) pathogenic MDV; (2) apathogenic MDV; and (3) HVT. They concluded that, of the antigens they identified in viruses of each serotype, at least one was group-specific and one type-specific.

Vaccines

Live-virus vaccines have been almost exclusively used for the control of MD. These have been of three kinds:

(1) apathogenic field MDV;

(2) modified MDV;

(3) HVT.

Apathogenic MDV - Field viruses of no or negligible pathogenicity have been isolated (Biggs & Milne, 1972; Blaxland et al., 1972; Rispens et al., 1972a; Zander et al., 1972). However, although these viruses provide protection against challenge with pathogenic virus, with the exception of the strain described and used by Zander et al. (1972), they have been modified before use as a vaccine, either to increase their growth rate in culture (Rispens et al., 1972a), eliminate low levels of pathogenicity (Rispens et al., 1972a), and/or alter their antigenic composition (Blaxland et al., 1972).

Modified MDV - In practice, most vaccines derived from MDV are modified vaccines that have been developed by:

1. Attenuation of strains of virus that produce the acute form of MD. The first vaccine was developed in this way (Churchill et al., 1969a, 1969b). Passage of the HPRS-16 strain in cultured chick kidney cells resulted in an increased rate of spread of virus in culture with the formation of large plaques, together with the loss of pathogenicity and alterations in the "A" antigen. Similar changes have been noted after passage of other strains of MDV originally capable of producing the acute disease (Nazerian, 1970; Eidson & Anderson, 1971a).

2. Attenuation of strains of MDV that produce the classical form of MD, for example attenuated the VC strain by passage in chick embryo.

3. Suitable modification of non-pathogenic MDV by passage in cultured cells (Rispens et al., 1972a; Blaxland et al., 1972).

MDV-derived vaccines could be classified according to the pathogenic characteristics of the parent virus. However, it is more useful to classify them according to their characteristics as vaccine viruses. They fall into two groups:

1. Vaccines that do not spread naturally from chicken to chicken and have "A" antigen altered in some way, e.g., attenuated HPRS-16 (Churchill et al., 1969a, 1969b), attenuated GA (Eidson & Anderson, 1971a) and the Beckenham strain (Blaxland et al., 1972).

2. Vaccines that spread naturally from chicken to chicken and have retained the "A" antigen as present in the originating virus, e.g., CVI 988 strain (Rispens et al., 1972a) and the VC strain (Bülow, 1971).

All MD-derived vaccines are used in the cell-associated form and therefore have to be stored as viable cells in liquid nitrogen after controlled freezing with dimethyl sulfoxide (DMSO) as a protective agent. The vaccines that have been or are still in commercial use were shown to be apathogenic, safe, not to revert to virulence and to provide significant levels of protection under field conditions (Biggs et al., 1970; Eidson et al., 1971; von Vielitz & Landgraf, 1971; Blaxland et al., 1972; Rispens et al., 1972a, 1972b). Providing a seed-lot system of production is used, these vaccines are immunologically stable. However, it has been reported that

attenuated MDV may lose immunogenicity after prolonged passage in cell culture (Okazaki, see Nazerian, 1973).

Herpesvirus of turkeys - Herpesviruses antigenically related to MDV were first isolated from turkeys in the USA (Kawamura et al., 1969; Witter et al., 1970). They have since been isolated in many other countries and a number of different isolates are used in vaccines.

HVT vaccines are used in either a cell-associated or, more frequently, a cell-free form. The former is stored with DMSO in liquid nitrogen and is used in the same manner as the MDV-derived vaccines, whereas the latter has the advantage that it can be stored in the lyophilized state. HVT vaccines have been shown to be apathogenic for chickens and turkeys, immunologically stable and efficacious under field conditions (Purchase et al., 1971, 1972a; Willemart, 1972).

Usage

A modified pathogenic MDV was the first vaccine to be used commercially (Biggs et al., 1970), but the advantages of more rapid growth in cell culture, ability to be lyophilized and less likelihood of a change to a pathogenic state has resulted in the almost exclusive use of HVT. Although the cell-associated form is still available, the lyophilized vaccine is most widely used.

All vaccines have been used in dosages of between 1 000 and 2 000 plaque-forming units (PFU). The minimal infective and protective dose of HVT is between 1 and 5 PFU (Patrascu et al., 1972; Purchase et al., 1972b), although if given in the presence of HVT antibodies this may be considerably higher (Patrascu et al., 1972; Churchill et al., 1973). The vaccine is given at one day of age, so that, from the second generation onwards, it will be given in the presence of homologous antibody maternally derived through the egg. Even so, there is evidence that, at the dose used under commercial conditions, the vaccine is effective (Zygraich & Huygelen, 1972, 1973). A single dose of vaccine provides protection for the 75-week commercial life of chickens.

Mode of action

There is no reason to believe that modified-MDV and HVT vaccines function in different ways. I shall therefore draw on published information on the action of both these vaccines in an attempt to understand how they function.

The relationship between the time of vaccination with HVT and the development of immunity has been examined by Eidson & Anderson (1971b) and Okazaki et al. (1971). Some protection was noted when challenge was on the same day as vaccination, but one week was required before a strong immunity had developed. Vaccine viraemia is present 1-2 weeks after vaccination and persists throughout the life of the chicken (Biggs et al., 1972; Purchase et al., 1972a; Churchill et al., 1973). Protection is highly correlated with the establishment and persistence of viraemia (Patrascu et al., 1972; Jackson et al., 1974). Jackson et al. (1974) found that, of 22 chickens vaccinated with modified MDV, four of six that never developed a demonstrable vaccine viraemia died of MD, whereas none of 16 chickens with a vaccine viraemia did so. In this study, it was found that those chickens with an early vaccine viraemia tended to have lower levels of field virus viraemia after challenge than vaccinated chicks showing no early vaccine viraemia.

Because chickens with a high level of field virus viraemia tend to die from MD (Witter et al., 1971; Jackson et al., 1974), it is probable that vaccines provide protection by reducing the level of field-virus infection in each chicken, and that the efficacy of this mechanism depends on the level of vaccine virus in the chicken.

Although vaccination appears to reduce the level of superinfection with field virus, it need not prevent field-virus infection in order to be protective. In most, if not all, individuals vaccination does not prevent superinfection with field virus (Churchill et al., 1969b; Biggs et al., 1970; Okazaki et al., 1970).

Little is known of how live-virus vaccines for Marek's disease function. It has been known for some time that passively acquired MDV antibodies offer some protection against the results of infection with pathogenic MDV (Chubb & Churchill, 1969; Jakowski et al., 1970; Ball et al., 1971). This has recently been confirmed by Calnek (1972a) and Burgoyne & Witter (1973), who found that passively acquired antibody reduced the level of virus in infected chickens and delayed the appearance of lesions. They also showed that passively acquired antibody to both MDV and HVT increased the lesion-producing $dose_{50}$ of both cell-associated and cell-free pathogenic MDV. This suggests an *in vivo* neutralization activity of circulating antibody acting both on the virus particle and cytophylically on infected cells. These observations confirm those of Calnek (1972b), who found that resistance to Marek's disease was more closely correlated with the presence of neutralizing than precipitating antibodies.

These results suggest that circulating antibodies and, in particular, neutralizing antibody is one likely mechanism of protection offered by the vaccine. It is also possible that protection that appears very soon after vaccination is mediated by interferon (Hong & Sevoian, 1971; Kaleta & Bankowski, 1972a, 1972b, 1972c). However, Purchase & Sharma (1974) found that cyclophosphamide treatment prevented the development of protection following vaccination with HVT, indicating that the most important aspect of protection is mediated through humoral and/or cell-mediated immune mechanisms. This study also suggests that protection is largely provided by bursa-dependent immune functions, which supports the observations on the efficacy of passively administered antibody in protecting chickens from MD. However, a temporary lymphoid depletion of the thymus has been described following cyclophosphamide treatment (Linna et al., 1972), and no tests of thymus function were undertaken. It is possible, therefore, that the effect on vaccine function could have been, at least in part, due to a reduction in cell-mediated immune activity.

Cell-mediated immunity is important in herpesvirus infections, perhaps because of their cell-associated characteristics. This is also true in MD. Else (1974) found that surgical bursectomy with total body irradiation did not influence protection against MD provided by an attenuated strain of MDV. He also found that there was no relationship between the development of protection and the presence of either precipitating or neutralizing antibodies. He concluded that protection induced by vaccination could develop in birds that lack an antibody-producing system. This study strongly suggests that cell-mediated immunity is the main mechanism of protection provided by vaccination. However, circulating antibody undoubtedly also plays a

part and the relative importance of these two mechanisms is not yet clear.

INACTIVATED VACCINES A COMPARATIVE VIEW

It is apparent from the paper by Melendez et al.[1] that live vaccines can be produced for other oncogenic herpesviruses. However, because of the difficulties of safety testing, can the use of a live vaccine against neoplastic disease in man be envisaged? The oncogenicity of herpesviruses, e.g., herpesvirus saimiri (HVS) , for some non-human primates and not for others indicates the difficulties that would arise in choosing a satisfactory experimental host for efficacy, and more important, safety testing. Therefore, in my view, the use of inactivated vaccines, especially purified-protein vaccines, would be more acceptable. The work in this field on oncogenic herpesviruses is limited, but the studies done so far are promising. In this context, it is important to consider as potential immunizing agents virus-dependent non-structural antigens, particularly those present in the cell membrane of infected cells, as well as antigens that are part of the virion.

Non-human primate viruses

Preliminary studies on the development of an inactivated vaccine of HVS have been reported by Laufs (1974). HVS was grown in owl monkey kidney cells (OMK) and the harvested supernatant, after clarification and inactivation, was absorbed to an aluminium hydroxygel adjuvant. The inactivated vaccine was not infectious or oncogenic for cotton-topped marmosets, but it did induce high titres of neutralizing and complement-fixing antibodies to HVS. A small number of animals were vaccinated and, together with unvaccinated monkeys, were challenged with a high dose of HVS by intramuscular inoculation. The results indicated that vaccination delayed the development of tumours. The results of further experiments are presented later (Laufs & Steinke[2]).

Marek's disease virus

A crude inactivated MDV vaccine, consisting of formaldehyde-treated cells infected with MDV, was developed in Japan (quoted by Kato, 1973) and provided some protection against MD, while Kaaden et al. (1974) found that cultured cells infected with HVT inactivated with 2-ethylethyleneimine (EEI) provided some protection. The last-named authors also found that purified HVT inactivated by heat provided no protection. However, more important was their finding that preparations of cellular membranes of HVT-infected cells emulsified in Freund's adjuvant provided a high degree of protection against challenge with pathogenic MDV. Lesnik & Ross (1974) in our laboratory have immunized chickens with detergent-soluble antigens extracted from cells infected with attenuated MDV and reduced the incidence of MD by about 2.5-fold. Both the sedimentable and non-sedimentable fractions after centrifugation at 100 000 g for 2 hours were effective when emulsified in Freund's adjuvant.

These results indicate that inactivated vaccines free of both cellular and viral nucleic acid may well be effective against the oncogenic effects of herpesviruses, and that glycoproteins solubilized from cell membranes of infected

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1 See p.353.

2 See p.346.

cells used in association with appropriate adjuvants could be effective vaccines.

CONCLUSIONS

Live vaccines have been shown to be very successful, both experimentally and in the field, against one well-recognized oncogenic herpesvirus (MDV). This work has clearly demonstrated that vaccination can be a profitable approach to the control of disease produced by oncogenic herpesviruses. Such vaccines need not prevent infection with the oncogenic virus, although they may need to keep infection at a low level to be effective. Although the use of live vaccines in man for oncogenic viruses is questionable, the study of such vaccines in animals is of comparative interest. These studies may provide important information on the mode of function of successful immunization against the oncogenic effects of herpesviruses, information that may be useful for the development of vaccines for use in man.

Perhaps of greater comparative importance than the work on live vaccines, is the recent development in the field of inactivated vaccines for oncogenic herpesviruses. It is here that animal models are of great importance. The Marek's disease model provides a readily available system for studying the potential of inactivated vaccines, the results of which could be extrapolated to the more expensive experiments with non-human primates before consideration is given to their use in man.

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CHARACTERIZATION OF A NEW SEROTYPE OF MAREK'S DISEASE HERPESVIRUS

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Isolates of Marek's disease virus (MDV) can be divided up into three categories according to their pathogenicity: (1) viruses that induce the acute disease characterized by visceral lymphoid tumours; (2) viruses that induce the classical disease characterized by lymphoid proliferation of peripheral nerves; and (3) apathogenic viruses (Biggs & Milne, 1972). However, no antigenic differences between isolates of MDV have been found. The herpesvirus of turkeys (HVT) is antigenically related but not identical to MDV (Witter et al., 1970). Because apathogenic and pathogenic isolates of MDV have been shown to differ in that the former produce small plaques and grow slowly in cultured chick kidney cells (CKC) (Biggs & Milne, 1972), it was considered worthwhile determining whether such apathogenic isolates differed antigenically from pathogenic MDV and HVT.

MATERIALS AND METHODS

Viruses and antisera

The HPRS-24 and HPRS-27 (isolate BBB 5) strains of apathogenic MDV were isolated at Houghton (Biggs & Milne, 1972). The origin of the HPRS-B14 strain of classical MDV, and the HPRS-16, JM and GA strains of acute MDV have been reviewed by Biggs (1970). The VC strain of classical MDV (Bülow, 1971), the attenuated HPRS-16 (Churchill et al., 1969) and JM strains lacking the "A" antigen, and the FC126 strain of HVT (Witter et al., 1970) have also been used for comparative studies. Stocks of cell-associated viruses were produced at low passage levels (6th-10th) in CKC cultures (Churchill, 1968). Cell-free virus was prepared from feather follicles of infected chickens (HPRS-16, HPRS-24) or from infected chicken embryo fibroblast (CEF)

cultures (HPRS-24, HVT). Virus titres were expressed in plaque-forming units (PFU).

Pathogenicity tests

Pathogenicity tests were carried out as described by Biggs & Milne (1972). One-day-old antibody-free Rhode Island Red (RIR) chickens were inoculated intra-abdominally with 500 PFU and observed for a period of 42 days.

Serological tests

Hyperimmune and convalescent sera against the various virus strains were produced in Houghton Poultry Research Station Rhode Island Red (RIR) and Reaseheath line C White Leghorn chickens reared in isolators.

Neutralization tests were carried out using a constant virus concentration (HPRS-24, HPRS-16 and HVT, 70-100 PFU/0.2 ml) and serial 2- or 3-fold serum dilutions. The plaque reduction technique in CEF cultures was used. The plaque test described by Bülow & Lorenz (1973) was employed, but agarose was used in the overlay instead of Noble agar. Plaques were counted at six days (HVT), 10 days (HPRS-24) and 12 days (HPRS-16) after inoculation. Antibody titres were calculated as the serum dilution causing a 50% plaque reduction.

Indirect fluorescent antibody (FA) tests were performed using infected CKC, CEF or duck embryo fibroblast (DEF) coverslip cultures.

Precipitin tests were performed according to the techniques described by Chubb & Churchill (1968). "A" and "BC" antigens were prepared from infected cell cultures in a manner similar to that described by Churchill et al. (1969). "A" antigens were also prepared from feather follicles of infected chickens using the technique of Calnek et al. (1970).

RESULTS

The HPRS-24 and HPRS-27 strains of MDV were chosen for study as representatives of apathogenic MDV. HPRS-27 (BBB-5) was shown to be apathogenic by Biggs & Milne (1972). Using the same test and criteria, we have found HPRS-24 also to be apathogenic (neural score 0.8, visceral score 0.1).

Behaviour in cell culture

Both HPRS-24 and HPRS-27 have been shown to produce characteristic small and slowly developing plaques in cultured CKC and to grow more slowly than pathogenic MDV and HVT in these cells (Biggs & Milne, 1972).

We have examined the behaviour of HPRS-24 in cell culture in more detail. In CKC cultures infected with the HPRS-24 strain, the rate of infectious cells at 5-6 days post inoculation never exceeded 10^3 - 3×10^3 PFU per 10^6 trypsinized cells, which was 10 times less than with HVT (10^4 PFU per 10^6 cells) and with the HPRS-16 and HPRS-16/att strains of MDV.

The HPRS-24 strain replicated significantly better in CEF than in CKC cultures. Preparations of infected cells had titres ranging from 10^5 to 4×10^5 PFU per 10^6 cells, which was as high as the titres of similar preparations of the HPRS-16/att strain or of HVT. Cell-free HPRS-24 could be extracted from cultured CEF at a rate of 2×10^2 - 3.5×10^3 PFU per 10^6 cells. This was about 1% of the virus yields usually achieved with HVT. In contrast, hardly any cell-free virus could be recovered from CEF cultures infected with HPRS-16/att (less than 10 PFU per 10^6 cells).

These findings correspond to the results of electron microscopic studies

of infected CEF. About 3.5% of 400 HPRS-24 particles were found to be enveloped, mostly at the nuclear membrane (Fig. 1). Enveloped particles were less frequent with HPRS-16 and HPRS-16/att, but about 20% of 400 HVT particles were enveloped. In contrast to HPRS-24, the majority (more than 75%) of enveloped HVT particles were present in cytoplasmic inclusion bodies, the remainder being in nuclear vesicles. This probably accounts, at least in part, for the ease with which cell-free virus can be extracted from cultures of HVT compared with those of HPRS-24 or other strains of MDV.

Protection tests

The results of protection tests (shown in Table 1) suggest an immunological relationship between the HPRS-24 and HPRS-16 strains of MDV. A significant increase in the percentage of resistant birds could be observed only in the vaccinated group challenged with HPRS-16 by contact exposure. The low rate of protection might be due to the rather low dose of HPRS-24 used for immunization (266 PFU/chicken) and to antigenic differences existing between the HPRS-24 and HPRS-16 strains of MDV.

Serological relationships between virus strains

Results of serological studies have revealed the existence of at least three serotypes in the group of Marek's disease and turkey herpesviruses (Table 2): (1) pathogenic MDV,

FIG. 1. CHICKEN EMBRYO FIBROBLAST INFECTED WITH MAREK'S DISEASE VIRUS

Chicken embryo fibroblast infected with the HPRS-24 strain of Marek's disease virus. Immature and enveloped particles in the nucleus (x 40 500)

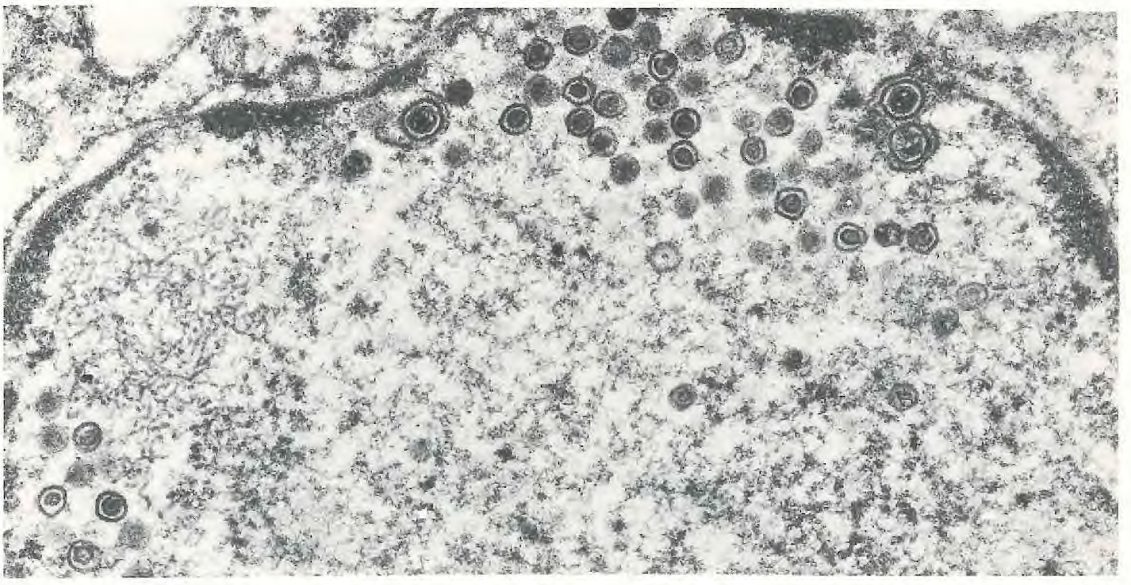


Table 1. Results of protection tests with the HPRS-24 strain of MDV

Group	Treatment	Effective No. of chicks	No. with MD	Percentage with MD	χ^2
1. Control	None	29	0	0	-
2. HPRS-24 control	HPRS-24, 266 PFU/chick at one-day old	26	0	0	
3. Challenge by inoculation	HPRS-16, 300 PFU/chick at 28-days old	30	26	86.6	2.19[a]
4. HPRS-24 + challenge by inoculation	As in groups 2 and 3	27	18	66.6	
5. Contact challenge	Exposure to HPRS-16 at 28-days old	29	21	72.4	2.92[b]
6. HPRS-24 + contact challenge	As in groups 2 and 5	26	12	46.1	

[a] Not significant

[b] Significant at the 5% level (critical value = 2.71)

typified by HPRS-16, but also including the HPRS-B14, VC, JM and GA strains; (2) apathogenic MDV, typified by HPRS-24 and including HPRS-27; (3) HVT, typified by the FC 126 strain. Representative results using the reference strains are shown in Table 3 and described below.

In the indirect immunofluorescence tests, titres of antisera were 4-8 times higher against the homologous than against heterologous virus-induced antigens (Table 3). However, there were never any significant differences in the appearance and distribution of antigen in infected cells treated with homologous or heterologous antisera. There was no difference between the results of FA tests on pathogenic strains of MDV and their attenuated (A^-) variants. Cross-reactions between different serotypes could be prevented either by absorption of antisera with the appropriate heterologous antigens, or by appropriate dilution of antisera before use in the indirect FA test.

Neutralizing antibody titres were very similar to the titres determined by indirect immunofluorescence.

Table 2. Serological classification of the group of Marek's disease and turkey herpesviruses

Serotype	Virus strains	Reference strain
Type 1	Pathogenic strains of MDV and attenuated variants	HPRS-16
Type 2	Apathogenic strains of MDV	HPRS-24
Type 3	Turkey herpesvirus and attenuated variants	FC 126

Table 3. Serological comparison between the HPRS-16 and HPRS-24 strains of MDV and the FC 126 strain of HVT

Antigen	Test[a]	Reciprocal titres of antisera		
		HPRS-16	HPRS-24	FC 126
MDV, HPRS-16	SN	966	355	186
	FA	1 280	320	160
	AGP	16	32	8
MDV, HPRS-24	SN	184	3 890	45
	FA	160	2 560	80
	AGP	8	128	4
HVT, FC 126	SN	214	148	1 550
	FA	160	640	1 280
	AGP	4	2	16

[a] SN = neutralization test; FA = immunofluorescence test; AGP = precipitin test ("BC" antigens)

Titres against the homologous and heterologous viruses usually differed by a factor of 4-8, but occasionally by as much as a factor of 100 (Table 3).

Precipitation titres against "BC" antigens were at least 10-20 times lower than the immunofluorescent and neutralizing antibody titres. Homologous and heterologous precipitin titres differed by a factor of 2-8, but only in a few cases by a factor greater than that. Precipitin bands between heterologous reagents did not appear at all when appropriate dilutions of antiserum were used.

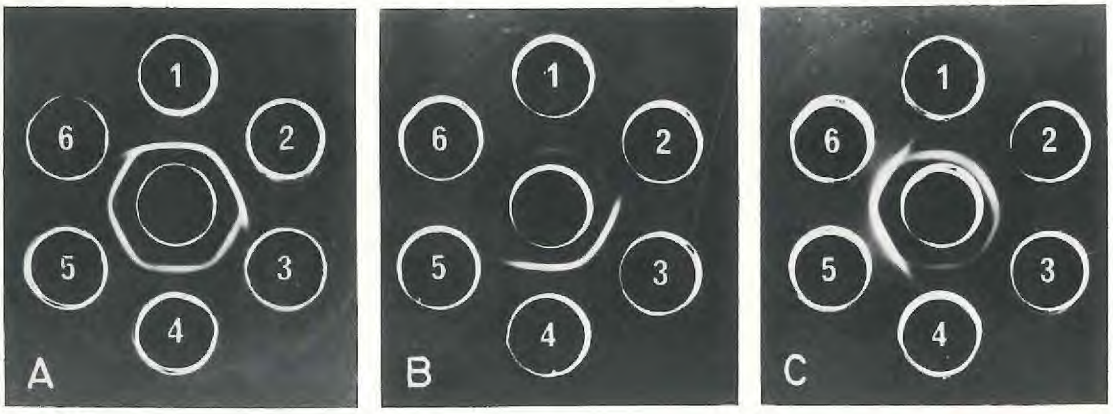
Differences between "A" antigens of the three serotypes of virus were indicated by spur line patterns of precipitin bands (Fig. 2). Similar differences between "BC" antigens have not been noticed. All virus strains had at least one "BC" antigen in common (group-specific), and one out of three "BC" antigens appeared to be type-specific.

DISCUSSION AND CONCLUSIONS

The results of this study suggest a serological division of the Marek's disease and turkey herpesvirus group into three types (Table 2). The three serotypes of virus can readily be distinguished by cross-neutralization, indirect immunofluorescence, or immunodiffusion tests. The immunofluorescence technique is likely to be preferred for differential diagnosis. The two serological types of MDV characterized by the HPRS-16 and HPRS-24 strains correspond to the pathogenic (acute and classical) and apathogenic classification of

FIG. 2. "A" ANTIGENS OF DIFFERENT SEROTYPES OF MAREK'S DISEASE VIRUS (MDV) AND A HERPESVIRUS OF TURKEYS (HVT)

The figures show the differences between "A" antigens of different serotypes of Marek's disease virus (MDV) and a herpesvirus of turkeys (HVT). Wells 1 and 2 contain HPRS-16 feather follicle antigen, wells 3 and 4 contain HPRS-24 tissue culture supernatant antigen, wells 5 and 6 contain HVT (FC 126) tissue culture supernatant antigen. The centre wells contain different antisera: A against HPRS-16, B against HPRS-24 and C against HVT.



Biggs & Milne (1972), both from their pathogenicity characteristics and their behaviour in cell cultures. However, it will be advisable to study a greater number of virus isolates before definite conclusions on serological markers of pathogenicity can be drawn.

HVT has been labelled turkey herpesvirus 1 and MDV as Phasianid herpesvirus 2 by the Herpesvirus Study Group of the International Committee for the Nomenclature of Viruses (Roizman et al., 1973). We suggest that Phasianid herpesvirus 2 be restricted to the serotype characterized by HPRS-16 and Phasianid herpesvirus 3 be introduced to describe the serotype characterized by HPRS-24.

SUMMARY

The HPRS-24 strain of Marek's disease herpesvirus was selected for closer study from a group of virus isolates that appeared apathogenic under standard test conditions. Protection studies revealed an immunological relationship between this virus strain and acute Marek's disease herpesvirus.

In chicken kidney cell cultures, the HPRS-24 strain caused small and slowly developing plaques, and the proportion of infected cells was less than with other strains. In chicken embryo fibroblast cultures, this virus multiplied rapidly, yielding a comparatively high proportion of infected cells. Electron microscopic studies revealed that infected fibroblasts contained more enveloped virus particles than those infected with other strains of Marek's disease herpesvirus. Infectious cell-free virus was extracted from cultured fibroblasts with titres high enough for use in neutralization studies.

Cross-neutralization, immunofluorescence and precipitin tests served for serological comparison of the HPRS-24 strain with turkey herpesvirus and representatives of acute and classical Marek's disease herpesvirus. Antibody titres were 4-10 times higher against the homologous than against the heterologous virus strains. Qualitative differences between precipitating "A" antigens were characterized by spur line patterns of precipitation bands. These results suggest that the group of Marek's disease and turkey herpesviruses consists of at least three serological types. One of them is represented by the HPRS-24 strain of apathogenic Marek's disease virus. The other two types comprise pathogenic strains of Marek's disease virus and their attenuated variants, and turkey herpesvirus.

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ISOLATION OF VIRUS-INDUCED ANTIGENS FROM CELLS INFECTED WITH MAREK'S DISEASE AND TURKEY HERPESVIRUSES

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Infection of chicken embryo fibroblasts (CEF) or chicken kidney cells with Marek's disease virus (MDV) results in the production of a number of virus-induced soluble antigens. Churchill et al. (1969) identified as many as six antigens in double-immunodiffusion tests (IDT) using extracts of cultured cells infected with MDV. Later, the major component (the "A" antigen), which is also released into the tissue culture supernatant, was purified by Ross et al. (1973) and identified as a glycoprotein. Cross-reactions between the precipitating antigens from MDV or turkey herpesvirus (HVT)-infected cells were described by Witter et al. (1970).

In this paper, we report on the purification and serological characterization of intracellular antigens (the "BC" complex) produced by CEF infected with MDV or HVT. Previous studies demonstrated that highly purified plasma membranes from MDV- or HVT-infected CEF contained two virus-induced antigens (VIA), as found by polyacrylamide gel electrophoresis, IDT and flotation of the plasma membrane-immunoglobulin mixture in density gradients (Kaaden & Dietzschold, 1974). Purified plasma membranes from HVT-infected CEF were able to protect chickens against experimental infection with MDV (Table 1) (Kaaden et al., 1974). However, in contrast to the high protection, only 32% of the sera taken from vaccinated chickens before challenge infection contained virus-neutralizing antibodies against infectious extracellular HVT.

For the isolation of intracellular virus-induced antigens (VIA), the GA strain of MDV (Eidson & Schmittle, 1968) and the FC 126 strain of HVT (Witter et al., 1970) were propagated in roller bottles of CEF. Details of the cell culture and virus propagation have already been described (Kaaden & Dietzschold, 1972).

In order to prepare radioactive-labelled VIA, infected cells were incubated with 5 µCi/ml ^{35}S-*L*-methionine (372 mCi/mM) in the maintenance medium for 24 hours. For labelling of cellular and viral DNA or RNA components, the cells were incubated with 20 µCi/ml of

Table 1. Immunization of day-old Rhode Island Red chickens against Marek's disease with different HVT vaccines[a]

Vaccine prepared from:	Protein content per dose (µg)	No. died from Marek's disease/ no. of survivors	Percentage with gross lesions	Reduction of specific mortality (%)
Plasma membranes from HVT-infected CEF	40	1/20	5	94
HVT intracellular virus-induced antigen	200	19/25	76	19
HVT intracellular virus-induced antigen bound to ligand (CH) Sepharose	200	29/33	87	5

[a] Groups of 20-35 chickens were vaccinated by the intramuscular route with 0.2 ml of vaccine given twice at 14-day intervals. For the challenge infection with the pathogenic HPRS-16 strain of MDV, the vaccinated chickens were exposed on day 21 of age to natural contact with three chickens showing clinical symptoms of Marek's disease.

^{3}H-thymidine and ^{3}H-uridine. The incubation of cells with 50 µCi/ml ^{3}H-L-fucose was extended to 36 hours. The maintenance medium used for radioactive labelling consisted of a 1:10 mixture of TCM 199 and Hank's solution, and 0.45% lactalbumin hydrolysate and 2% dialysed calf serum. The radioactive precursors were purchased from Radiochemical Centre, Amersham, United Kingdom. For the antigen preparation, infected cultured cells showing a distinct cytopathic effect (CPE) were scraped off the glass surface, sedimented by low-speed centrifugation and washed twice with phosphate-buffered saline (PBS). The final sediment contained a total of about 10^9 cells. VIA was extracted from the cells using 3M potassium chloride, 0.01 M mercaptoethanol and 2 x 10^{-3} M phenylmethylsulfonyl fluoride dissolved in phosphate-buffered saline, pH 7.4. Further purification steps included high-speed centrifugation, gel filtration and affinity chromatography on Concanavalin A Sepharose (Ponce de Leon et al., 1973). After these purification procedures, two virus-induced antigens were detected in the partially purified preparations when the material was allowed to react with immunoglobulins from birds that had survived an infection with MDV.

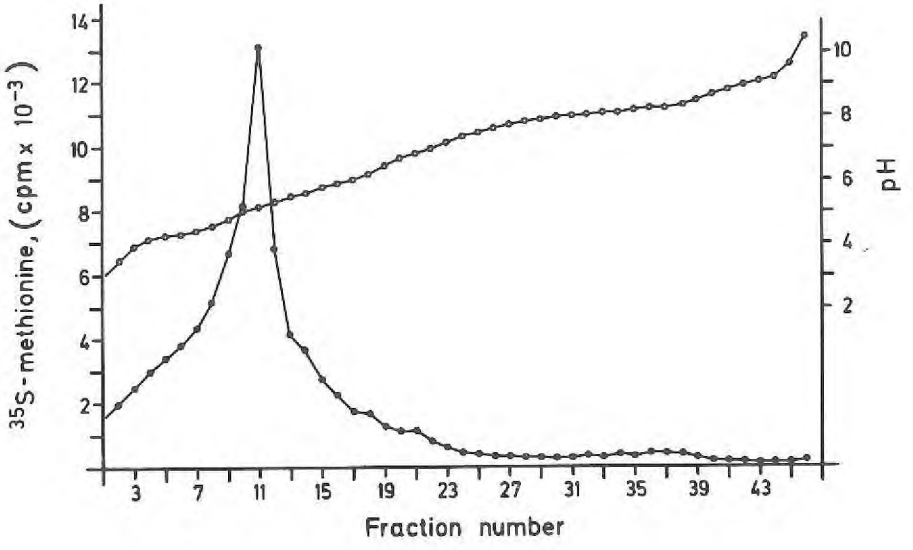
After dialysis against 1% (w/v) of glycine and glycerine, the partially purified antigen preparations were subjected to isoelectric focusing in 2% (v/v) ampholine (pH 3.5-10). A single peak of radioactivity was

found at the end of the electrophoresis, corresponding to a pH of 5.1 (Fig. 1). After dialysis against PBS, the various fractions were tested for precipitating antigens by IDT (Fig. 2). Purified HVT VIA was placed in a well adjoining wells containing HVT or MDV antisera, respectively. The precipitin lines produced by HVT and MDV (strain HPRS-16) antisera were completely identical. Purified HVT VIA preparations did not cause any precipitin line if they were allowed to react with rabbit antisera CEF extract or against normal chicken serum.

For further characterization, antisera against the isolated MDV or HVT VIA were prepared from rabbits that had been immunized by four doses of antigen given at weekly intervals. The antisera were compared by block titration with respect to their ability to bind complement-fixing antigen. Control antigens prepared from uninfected CEF and from cells infected with a pigeon herpesvirus (Cornwell et al., 1967) or an owl herpesvirus (Burtscher & Schuhmacher, 1966) were included in the tests. The reciprocal of the rabbit MDV VIA antiserum endpoint over the antigen endpoint was found to be 64/32, regardless of whether MDV or HVT VIA were titrated. The corresponding value for the rabbit HVT VIA antiserum was 128/64, thus showing a

FIG. 1. ISOELECTRIC FOCUSING OF RADIOACTIVE-LABELLED HVT VIRUS-INDUCED ANTIGEN

Distribution of radioactivity of ^{35}S-*L*-methionine-labelled HVT virus-induced antigen (VIA) after isoelectric focusing. The VIA was eluted from Concanavalin A Sepharose by 0.25 M α-methyl-*D*-mannoside and electrophoresed in 2% ampholine for 48 hours at 400 V, 7 mA.

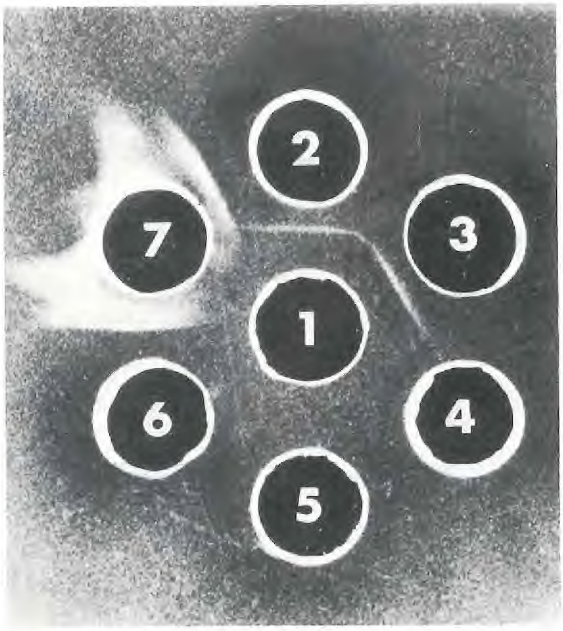


complete identity of the purified VIA. The rabbit antisera did not show any complement-fixing activity if reacted with antigens prepared from uninfected CEF or from cells infected with pigeon or owl herpeviruses.

Furthermore, purified VIA from MDV- or HVT-infected cells were tested for their ability to block the virus-neutralizing activity of HVT antisera. The blocking reaction was performed by a 60-minute incubation at 37°C of equal amounts of appropriately diluted purified MDV or HVT VIA (i.e., concentrated fractions from iso-electric focusing) with appropriate dilutions of HVT antiserum. Subsequently, about 100 plaque-forming units of extracellular infectious HVT were added. Surviving virus was determined by plaque assay in secondary CEF cultures. The blocking ability of the tested antigen preparations, expressed as the percentage reduction of the neutralizing activity of HVT antiserum, is shown in Fig. 3.

FIG. 2. DOUBLE-IMMUNODIFFUSION TEST OF INTRACELLULAR VIRUS-INDUCED ANTIGEN

Double-immunodiffusion test of intracellular virus-induced antigen against different antisera. Centre well (1): purified intracellular HVT antigen (protein concentration 4.4 mg/ml). Peripheral wells: (2) gammaglobulins from HVT antisera (protein concentration 6.7 mg/ml); (3) gammaglobulins from MDV antisera, strain GA (protein concentration 7.3 mg/ml); (4) CELO antiserum; (5) rabbit antiserum against chicken embryo fibroblasts; (6) gammaglobulins from normal chicken sera (protein concentration 6.5 mg/ml); (7) rabbit antiserum against chicken serum.



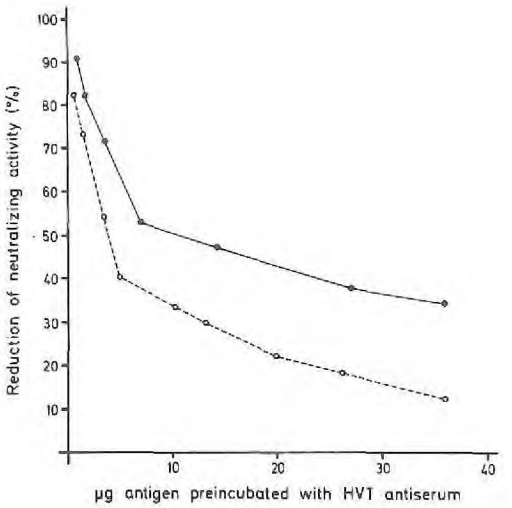
The neutralizing activity of the antiserum was reduced at the highest antigen concentrations by 60% (MDV VIA) or 80% (HVT VIA), respectively.

For the analysis of the polypeptide composition, ^{35}S-*L*-methionine or ^{3}H-*L*-fucose-labelled HVT VIA were denatured by treatment with sodium dodecyl sulfate (SDS) and dithiothreitol and subjected to electrophoresis on 8.5% acrylamide gels (Fig. 4). The radioactive profile indicates the presence of seven polypeptides. The corresponding relative molecular weights, estimated by using purified labelled vesicular stomatitis virus (VSV) proteins, were found to be: peak 1: about 200 000; peak 2: 105 000; peak 3: 95 000; peak 4: 84 000; peak 5: 74 000; peak 6: 55 000; and peak 7: 43 000 (Fig. 4A).

The electrophoretic analysis of ^{3}H-fucose-labelled HVT VIA showed that at least two glycoproteins were migrating to the same relative positions as components 2 and 3 (Fig. 4B). No differences in the polypeptide composition were observed if the purified MDV VIA was examined by SDS acrylamide-gel electrophoresis. After incubation with 10 µCi/ml ^{32}P-orthophosphate, labelled VIA was purified

FIG. 3. SERUM BLOCKING ACTIVITY AGAINST ANTISERUM TO HVT

Serum blocking activity of intracellular MDV- and HVT-induced antigens against antiserum to HVT, in terms of the residual neutralizing activity (expressed as the percentage reduction) of mixtures containing different dilutions of antigens with a constant amount of antiserum. ●——● HVT antiserum pre-incubated with MDV VIA; O-------O HVT antiserum pre-incubated with HVT VIA. The neutralization test with extracellular HVT was performed as described by Kaaden & Dietzschold (1974).

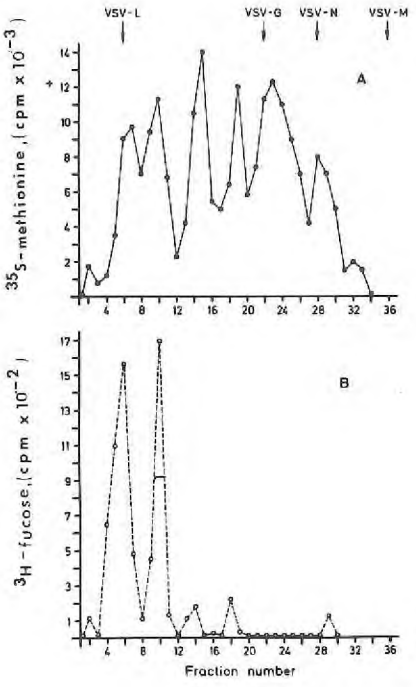


from HVT-infected CEF. The ^{32}P-label of the antigen preparation was extractable by chloroform-methanol mixture and was found to be sensitive to phospholipase C. After incubation with 50 µg/ml phospholipase C (A grade), about 83% of the input radioactivity became soluble in trichloroacetic acid (TCA). The results of experiments with ^{32}P-labelled antigen suggest the presence of a phospholipid component within the isolated HVT VIA. However, no labelled DNA or RNA components were detected within the purified VIA preparations from thymidine- and uridine-labelled CEF.

The purified HVT VIA was also used for protection experiments against Marek's disease. Two injections of vaccines prepared from HVT

FIG. 4. ELECTROPHORESIS OF RADIOACTIVE-LABELLED HVT VIRUS-INDUCED ANTIGEN

Electrophoresis of ^{35}S-*L*-methionine- (A) or ^{3}H-*L*-fucose-labelled (B) HVT VIA purified by isoelectric focusing on 8.5% acrylamide gels. The gels were sliced after electrophoresis into sections of about 1.25 mm and the radioactivity was counted, after solubilization, in a liquid scintillation counter. The positions of the reference proteins prepared from purified vesicular stomatitis virus (VSV) are indicated. The electrophoresis was run for 5 hours at 2 mA.



VIA were administered to chickens. The potency of the vaccines was expressed as the percentage reduction of the specific mortality and lesions (SM), as described previously (Kaaden et al., 1974). In a first group of chickens vaccinated with a plasma-membrane preparation from HVT-infected CEF, the SM was reduced by 94%. In contrast, the HVT VIA preparations were not able to induce a significant reduction of the Marek's disease SM, whether in their soluble or their immobilized form (coupled to ligand Sepharose) (Table 1).

Several lines of evidence suggest that the VIA isolated from MDV- or HVT-infected cells are specific for the infection of cells with these viruses. Thus the MDV and HVT antisera used for the identification of the isolated precipitating antigens failed to react with cytoplasmic extracts from uninfected CEF in the IDT. Furthermore, neither the use of labelled extracts from uninfected CEF nor that of the prepared rabbit antisera enabled host components to be detected in the purified antigen preparations.

The results of the serological comparison therefore show that the precipitating antigens isolated are specific and represent a common antigen of MDV- and HVT-infected cells.

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INDUCTION OF SERUM ANTIBODIES IN NON-HUMAN PRIMATES WITH A KILLED ONCOGENIC HERPESVIRUS (HERPESVIRUS SAIMIRI)

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The oncogenic herpesviruses derived from monkeys, herpesvirus saimiri (HVS) (Meléndez et al., 1969) and herpesvirus ateles (HVA) (Laufs & Meléndez, 1973a), can be used *in vivo* to study the problems of herpesvirus cancer vaccines. HVS regularly induces malignant lymphoma in cotton-topped (CT) marmoset monkeys (Wolfe et al., 1971; Laufs & Fleckenstein, 1973) and was therefore chosen for this study. We developed a killed vaccine from HVS and tested it for safety and efficiency in this primate model system.

The killed vaccine, HVS isolate No. S.295C (kindly supplied by Dr L.V. Meléndez, New England Regional Primate Research Center, Harvard Medical School, Southborough, Mass. 01772), was cultivated on owl monkey kidney (OMK) cells (kindly supplied by Dr M.D. Daniels, New England Regional Primate Research Center, Harvard Medical School, Southborough, Mass. 01772). The cells were propagated in Eagle's minimal essential medium (Behring-Werke, Marburg, Federal Republic of Germany) supplemented with 10% heat-inactivated bovine serum, 100 µg streptomycin/ml and 5 µg fungizone/ml. Three days before harvest, when the cytopathic effect of HVS appeared, the OMK cell cultures were washed and fed with fresh medium, without serum. After harvest, the tissue-culture supernatant was stored at 4°C as stock virus. The stock virus was centrifuged twice at 2 000 g for 10 min and the clarified fluid containing the virus was passed through Millipore membrane filters (450 nm). The filtrate was sonicated for 10 sec (Branson Sonifier B-12, micro tip, maximum output). The infectivity titre of this preparation, measured in OMK cells, was $10^{5.0}$-$10^{5.5}$ $TCID_{50}$/ml. The virus was distributed into 100-ml glass vials and heated at 56°C (temperature measured within a glass vial). Cytopathogenicity of heat-inactivated HVS was tested during the first 30 min. The inoculated OMK cell cultures were observed for cytopathic effect (CPE) over a period of 32 days. After 6 min of heat treatment, the infectivity of the unconcentrated HVS preparation (0.5-ml samples) had disappeared. However, after the virus suspension had been concentrated 100-fold by

dialysis against Aquacide (Calbiochem, Los Angeles, USA) overnight and the concentrate inoculated into OMK cells, small amounts of infectious HVS particles ($10^{0.3}$ $TCID_{50}$/ml) were found in our experiments even after an incubation period of 30 min at 56°C. In order to prepare the killed vaccine, the heat treatment at 56°C was prolonged over a period of four hours. This procedure did not significantly reduce the titre of the HVS-specific complement-fixing (CF) antigens measured in the concentrates of the virus preparation before and after heat treatment, according to standard techniques using HVS antiserum from an owl monkey (Laufs & Meléndez, 1973b). In addition to heat treatment, the virus preparation was incubated with formaldehyde (100 µg/ml, pH adjusted to 6.5, 37°C). Formaldehyde destroyed the cytopathogenicity of unheated HVS within seven hours, but infectious HVS ($10^{1.0}$ $TCID_{50}$/ml) could still be rescued from the concentrate of the virus preparation after an incubation period of 24 hours. In contrast to the heat inactivation, the formaldehyde treatment reduced the titre of the HVS-specific CF antigens. For this reason, the additional incubation of heat-inactivated HVS with formaldehyde was stopped after six days by neutralization with equimolar sodium disulfite ($Na_2S_2O_5$). The suspension was concentrated 100-fold by dialysis against Aquacide. The concentrate was centrifuged at 12 000 g for 10 min at 4°C (B 20 centrifuge, International Equipment Co., Boston, Mass.) and the supernatant, which was used for vaccination, was stored at -60°C. From each vaccine preparation (10 ml), 30% was tested for cytopathogenicity in OMK cells. No CPE appeared in these cultures during an observation period of 36 days.

The killed HVS vaccine was absorbed with aluminium hydroxide gel (10% v/v of a 3% solution, Behring-Werke, Marburg, Federal Republic of Germany) and used to immunize 42 CT marmoset monkeys. The animals received 4-6 intramuscular inoculations with 1.0 and 0.5 ml of vaccine within 4-10 weeks. All of the vaccinated monkeys developed high titres of serum antibodies against HVS and remained clinically well. The immunized monkeys did not carry the HVS genome in their peripheral white blood cells, as shown by co-cultivation with OMK cells. The titres of the serum antibodies against HVS dropped slowly during the first year and rose again after a booster injection with killed HVS (Table 1).

The protective effect of the vaccination was tested in challenge experiments. Serial 10-fold dilutions of cell-free HVS stock virus were passed through a Millipore membrane filter (450 nm) and injected into 14 vaccinated monkeys and 21 unvaccinated control monkeys. All of the vaccinated monkeys survived and have been under observation for 270 days (challenge experiment 1) or 139 days (challenge experiment 2). The unvaccinated control monkeys died 34-52 days after inoculation of malignant lymphoma, as shown both by gross pathology and histopathology (Table 2). The highest challenge dosage contained 100 $TCID_{50}$ of HVS, which corresponded to 215 LD_{50} of HVS, as calculated according to the method of Reed & Muench (1938). The vaccinated monkeys, in contrast to the tumour-bearing control animals, do not carry the HVS genome in their peripheral white blood cells, as shown by co-cultivation with OMK cells (Tables 3 and 4).

These experiments clearly

Table 1. Active immunization of 3 CT marmoset monkeys with killed HVS

Time after first immunization (days)	Infectivity titre of whole blood ($TCID_{50}$/ml)[a]	Serum antibodies against HVS	
		NI[b]	CF[c]
No. 1			
84	$<10^{0.0}$	3.0	1:4
162	$<10^{0.0}$	3.2	1:2
196	$<10^{0.0}$	2.3	AA[d]
249	$<10^{0.0}$	1.2	AA[d]
278[e]	$<10^{0.0}$	1.0	AA[d]
286	$<10^{0.0}$	3.0	1:64
No. 2			
84	$<10^{0.0}$	2.2	AA[d]
162	$<10^{0.0}$	3.7	AA[d]
206	$<10^{0.0}$	3.3	AA[d]
259	$<10^{0.0}$	1.5	AA[d]
294	$<10^{0.0}$	1.2	AA[d]
No. 3			
84	$<10^{0.0}$	3.0	1:2
162	$<10^{0.0}$	4.0	0
206	$<10^{0.0}$	3.3	0
259	$<10^{0.0}$	2.7	ND[f]
294	$<10^{0.0}$	1.5	ND[f]

[a] Determined in OMK cells.

[b] Neutralization indices.

[c] Titres of complement-fixing antibodies.

[d] Anticomplement activity.

[e] The monkey received a booster injection of killed HVS.

[f] ND: not done.

Table 2. Inoculation of serial 10-fold dilutions of cell-free HVS into vaccinated and unvaccinated CT marmoset monkeys

Dilution of HVS stock virus	Titre of HVS inoculum		Unvaccinated monkeys[b]	Vaccinated monkeys[b]
	$TCID_{50}$[a]	LD_{50}		
Challenge Experiment 1[c]				
10^{-3}	10	18	+ + +	- - -
10^{-4}	1	1:8	+ + -	- - -
10^{-5}	<1	<1	- - -	Not done
Challenge Experiment 2[d]				
10^{-2}	100	215	Not done	- - - -
10^{-3}	10	21	+ + + +	- - - -
10^{-4}	1	2	+ + + -	Not done
10^{-5}	<1	<1	- - - -	Not done

[a] Determined in OMK cells.

[b] + = Monkey died of malignant lymphoma, as shown both by gross pathology and histopathology; - = monkey remained clinically well.

[c] Monkeys have been under observation for 270 days.

[d] Monkeys have been under observation for 139 days.

demonstrate that a safe and efficient vaccine can be prepared by inactivation of an oncogenic herpesvirus. The killed HVS cancer vaccine prevents malignant lymphoma in non-human primates under laboratory conditions. The vaccinated monkeys were resistant to 215 LD_{50} of HVS. The immunity against HVS can be broken by very high challenge dosages such as $10^{3.8}$ $TCID_{50}$ of HVS (Laufs, 1974). The tumour prophylaxis with the killed HVS vaccine and the good correlation between the $TCID_{50}$ and the LD_{50} of HVS support the concept that HVS is indeed the etiological agent of malignant lymphoma in monkeys. This vaccine prophylaxis of cancer in monkeys gives rise to the hope that safe and efficient vaccines can be developed against those forms of cancer in man that may be induced by herpesviruses.

Table 3. Challenge experiments 1 and 2: vaccinated CT marmoset monkeys

Dilution of HVS stock virus used for challenge	Animal No.	Interval between first immunization and challenge (days)	Serum antibodies against HVS at the time of challenge		Period of observation after challenge (days)
			NI[a]	CF[b]	
Experiment 1					
10^{-3}	10	72	3.0	1:4	270
	11	72	3.0	AA[c]	270
	12	47	2.8	1:32	270
10^{-4}	13	47	2.0	1:16	270
	14	47	3.0	1:8	270
	15	47	2.0	1:8	270
Experiment 2					
10^{-2}	28	88	3.0	AA[c]	139
	29	88	3.0	1:32	139
	30	88	3.0	1:64	139
	31	88	3.0	AA[c]	139
10^{-3}	32	88	3.0	1:64	139
	33	88	3.0	1:128	139
	34	88	3.0	AA[c]	139
	35	88	3.0	AA[c]	139

[a] Neutralization indices.

[b] Titres of complement-fixing antibodies.

[c] Anticomplement activity.

Table 4. Challenge experiments 1 and 2: unvaccinated CT marmoset monkeys (controls)

Dilution of HVS stock virus used for challenge	Animal No.	Interval between inoculation and death from malignant lymphoma (days)	Infectivity titre of whole blood at the time of death ($TCID_{50}$/ml)[a]
Experiment 1			
10^{-3}	1	40	$10^{4.8}$
	2	42	$10^{5.3}$
	3	34	$10^{5.3}$
10^{-4}	4	44	$10^{5.5}$
	5	51	$10^{4.3}$
	6[b]	No tumour	-
10^{-5}	7[b]	No tumour	-
	8[b]	No tumour	-
	9[b]	No tumour	-
Experiment 2			
10^{-3}	16	45	$10^{5.5}$
	17	52	$10^{4.0}$
	18	44	$10^{5.5}$
	19	42	$10^{5.5}$
10^{-4}	20	52	$10^{5.3}$
	21	44	$10^{5.5}$
	22	36	$10^{4.8}$
	23[b]	No tumour	-
10^{-5}	24[b]	No tumour	-
	25[b]	No tumour	-
	26[b]	No tumour	-
	27[b]	No tumour	-

[a] Determined in OMK cells.

[b] These monkeys have now been under observation for 270 or 139 days without signs of tumour development.

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IN VITRO ATTENUATION OF HERPESVIRUS SAIMIRI BY SERIAL PASSAGE IN DOG FETAL LUNG CONTINUOUS CULTURES

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Many human and non-human primate tissues have been cultured in our laboratories since 1965. Several continuous cultures derived from them have been kept in tissue-culture medium with 10% glycerin at -186°C (liquid nitrogen).

Some of these cultures were inoculated with herpesvirus saimiri (or Cebid herpesvirus 2) (Melendez et al., 1968; Roizman et al., 1973), an oncogenic DNA virus from squirrel monkeys (*Saimiri sciureus*) (Melendez et al., 1969). The purpose of these inoculations was to try to adapt herpesvirus saimiri (HVS) to growth in these cultures and, if this were possible, to determine whether this oncogenic virus could induce some cell-layer alteration or cytopathic effect (CPE) in a non-human primate culture. The final aim of these trials was to obtain an attenuated live virus that, after serial passages, might be able to provide protection against the malignant lymphoma induced by this agent in several species of non-human primates.

This report describes the adaptation and *in vitro* attenuation of HVS in dog fetal lung (DFL) continuous cultures.

ESTABLISHMENT OF DOG FETAL LUNG CONTINUOUS CULTURES

During 1970, the inoculation of several primate cell cultures with undiluted HVS strain S-295C, stock E964E, was initiated. Of these cultures, one prepared from DFL grew very well. The cells were grown in 250-ml flasks and/or 60-mm petri dishes (Falcon).

DFL primary cultures were observed for 90 days before inoculation with HVS to rule out the presence of latent viral agents capable of inducing spontaneous CPE.

The medium employed to grow the DFL cells was Eagle's minimum essential medium with 10% fetal calf serum, heat-inactivated at 56°C for 1 hour (MEM-10). Sodium penicillin and streptomycin were added so that the medium contained 250 U and 250 mg/ml respectively.

The DFL cultures were transferred by routine trypsinization procedures (2.5% Difco trypsin in Tris buffer). The cells were transferred whenever the cultures developed a confluent monolayer. Usually the transfer splitting ratio was 1:2. Over a period of four years, we were able to make 36 transfers of DFL cultures.

VIRUS INOCULATION AND SERIAL PASSAGES

In the inoculations of HVS in DFL cells, undiluted virus was always used, 1-3 ml of virus being inoculated into each flask; after an adsorption period of 45-60 minutes, 10-12 ml of medium were added.

The first passage (E987E) was effected by inoculation of HVS in DFL cultures. No CPE was observed within 15 days. At this time the inoculated DFL cultures were trypsinized, and the cell pellet obtained after centrifugation at 900 rpm for 5 min was seeded in new flasks containing well-grown DFL cultures. This co-culture procedure was repeated nine times in a period of four months.

Parallel co-cultures at various passage levels during these first four months were observed for periods of up to 37 days to detect CPE. This developed as discrete localized foci of rounded and swollen cells. During this period, some cells grown on glass cover-slips were removed and stained with haematoxylin and eosin (HE). Ill-defined nuclear inclusions and small polykaryocytes were observed in these stained preparations (Figs. 1 and 2). For comparison, an uninoculated DFL culture is shown (Fig. 3). The presence of this ill-defined CPE led us to decide to transfer and seed these cultures over well-grown DFL monolayers. Tissue-culture fluids were also collected at this time, and those obtained from every fifth passage were stored at -86°C to be titrated in owl monkey kidney (OMK) and DFL cultures.

Tissue culture fluids from the 9th co-culture (E2774F) were collected on day 41 and inoculated after freezing and thawing in new DFL cultures. A new co-culture was carried out with this 10th virus passage in DFL cells on day 17. Thereafter seven serial passages of HVS were performed in DFL cells with the cell-free virus inoculum collected from each passage, so that an 18th passage level was reached in a period of 19½ months.

The 18th virus passage (E704H) was co-cultured on day 28 with new DFL cells to make a 19th passage because at this time a dubious CPE was observed; however, serial passages were performed with cell-free inoculum in DFL cells whenever the CPE varied from 0.5 to 1.5.

During a period of three months, a 21st passage level was reached. The inoculum for these passages was collected when the DFL cells had a CPE of 2.0-2.5. The virus was also passed serially in DFL cells as cell-free virus for another 12 months, at which time the 35th passage level was reached (E757J). The procedure is summarized in Table 1. A CPE varying from 0.5 to 4.0 as early as 10-11

FIG. 1. DOG FETAL LUNG (DFL) CELL CULTURES 37 DAYS AFTER INOCULATION WITH HVS

Numerous rounded and swollen cells can be seen. Some nuclei have nuclear inclusions, as indicated by the arrow. (x 125)

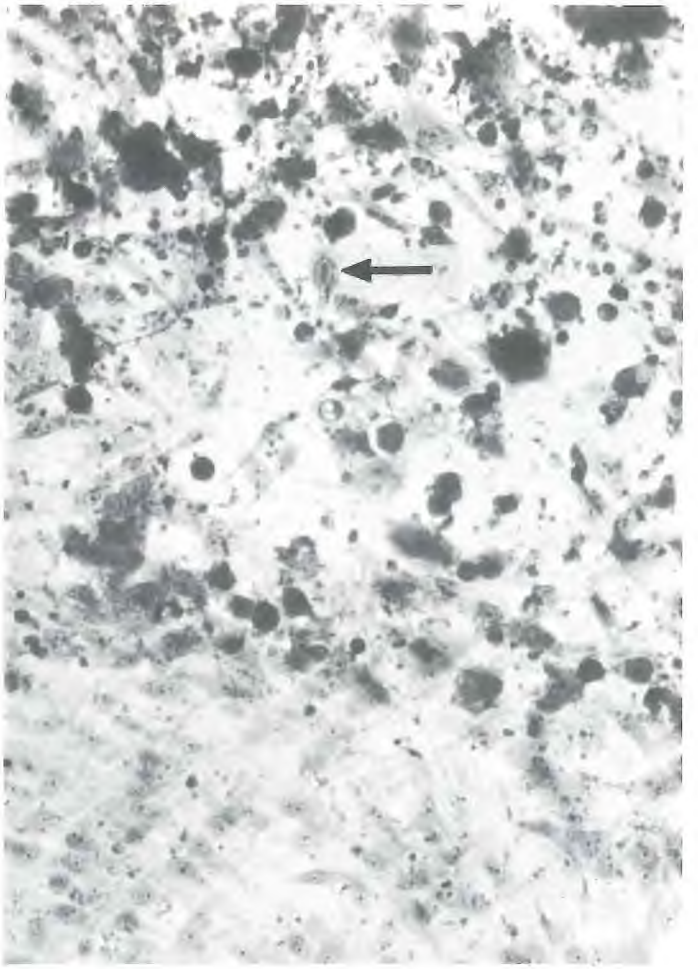


FIG. 2. ENLARGED VIEW OF CYTOPATHIC EFFECT SHOWN IN FIG. 1. Note the clearly outlined nuclear inclusions. (x 250)

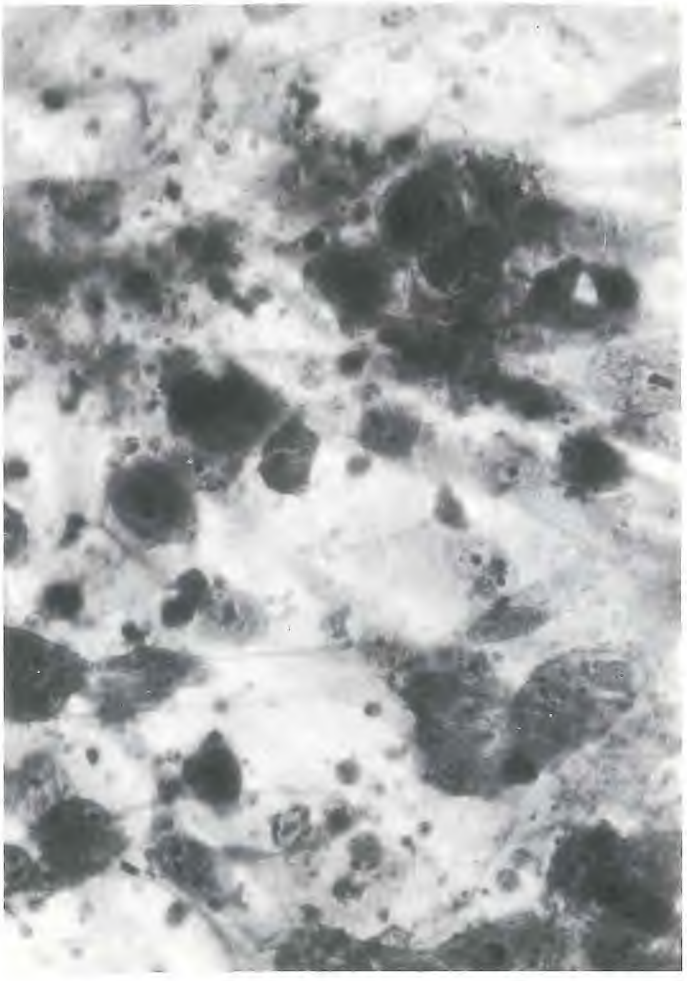


FIG. 3. UNINOCULATED DOG FETAL LUNG (DFL) CULTURES (x 125)

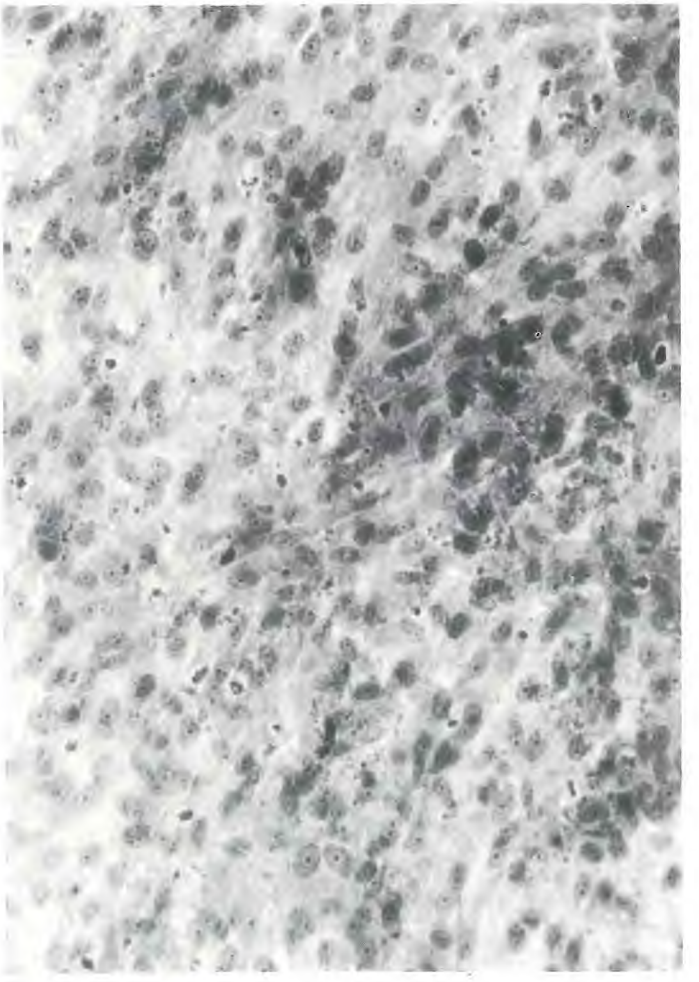


Table 1. Procedure for multiplication of HVS in DFL cultures

Step	Procedure
1	The virus was passed nine times by co-culturing inoculated DFL cultures with uninoculated cultures.
2	A 10th passage was performed with frozen and thawed tissue culture material from the previous passage.
3	A co-culture was carried out to obtain the 11th passage.
4	The 12th to the 18th passages were performed as in Step 2.
5	A co-culture was carried out to obtain the 19th passage.
6	The 20th to the 35th passages were performed as in Step 2.

days post inoculation was observed in some of these virus passages.

It was observed during these serial passages of HVS in DFL cultures that the CPE increased proportionally to the number of passages done, but that the CPE for OMK cells decreased in the same manner. This increase in CPE for DFL cultures of the virus serially passed in these cultures as well as the decrease in CPE for OMK cells are shown in Fig. 4.

A 32nd passage (E564J) in DFL cells had a titre of 5.5/ml in DFL and 1.0/ml in OMK cultures.

DFL-HVS, 32nd passage, (E564J) was neutralized by HVS antiserum prepared in goats against the prototype strain S-295C (Fraser et al., 1971) and a neutralization index (NI) of 3.5 was observed in DFL cells. The same DFL-HVS variant gave specific fluorescence with HVS antiserum.

ANIMAL INOCULATION

Owl monkeys (*Aotus sp.*) were employed to test the immunogenic and anti-oncogenic activity of the DFL-HVS.

The following passages of DFL-HVS were inoculated in owl monkeys: 5th passage (E144F): animal experiment (AE) 173; 22nd passage (E464I): AE254; 22nd passage (E464I): AE268; and a 30th passage (E710J): AE279. All these experiments will be described in a separate report.

CONCLUSIONS

The view that the DFL-HVS is a mutant of the prototype strain S-295C is supported by the following *in vitro* findings:

(1) the prototype HVS strain acquires the ability to induce definite CPE in DFL cultures after being passed for more than 30 times over a period of almost four years.

(2) the titre of DFL-HVS in DFL cultures increased from 0.0/ml to 4.5/ml after 32 serial passages, and decreased from 5.5/ml to 1.0/ml in

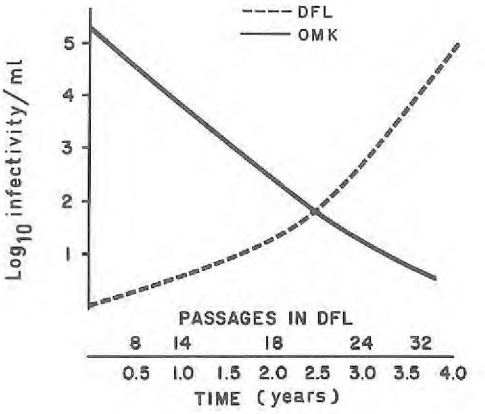
OMK cultures in the same number of passages;

(3) HVS antiserum has a NI of 3.5 for DFL-HVS, 32nd passage.

(4) DFL-HVS fluoresces specifically with HVS antiserum.

The above findings indicate that the prototype HVS strain has been attenuated in its *in vitro* behaviour, and that a new variant has been obtained under laboratory conditions. It is suggested that this variant should be called DFL-HVS.

FIG. 4. EFFECT OF NUMBER OF SERIAL PASSAGES IN DFL CELLS ON INFECTIVITY OF HVS FOR DFL AND OMK CELLS



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DISCUSSION SUMMARY

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Vaccine development for the control and prevention of cancer was the main topic discussed, and the importance of vaccines free from viral nucleic acids was emphasized. Such vaccines could conceivably be prepared from specially purified viral proteins, viral-cultured cell membranes or viral and virally-induced structural and non-structural proteins (antigens). The two viruses discussed were Marek's disease virus (MDV) and herpesvirus saimiri (HVS).

Marek's disease virus

Highly purified plasma membranes of the herpesvirus of turkeys (HVT) protected chickens upon challenge with MDV. Two HVT antigens, however, partially purified by isoelectric focusing, were found to react with HVT in immunoprecipitation tests, whereas an MDV antiserum was at least 60% less reactive. The relationship between these two findings is a topic for further research. Six MDV antigens, which could be identified by immunoprecipitation, were also discussed.

In addition, a crude, inactivated vaccine for MD has been developed in Japan; however, a purified heat-inactivated MDV vaccine is still under development because at present it fails to protect chickens. In contrast, a live, cell-free preparation, made from *in vitro* propagated HVT, was believed to be effective, stable, and suitable for large-scale production. The possible long-term effects of these various vaccines were discussed.

Studies were presented on the immunological and serological differences between strains of MDV and their relevance to the control and prevention of MD. Thus, the different strains vary in antigenic make-up, oncogenicity, and pathogenicity. Significant differences were found between the apathogenic HPR-24 strain of MDV and HVT by cross-neutralization, immunofluorescence, and immunoprecipitation tests. At least three serological subtypes of MDV have been found. One of these was the apathogenic

HPR-24 strain, while the others were pathogenic and attenuated variants of MDV and HTV.

Herpesvirus saimiri

Heat inactivation of HVS combined with formalin treatment resulted in the preparation of what seemed to be a successful vaccine for the prevention of HVS-induced tumours in cotton-topped marmosets. No HVS could be recovered from the vaccinated animals. All animals vaccinated and then challenged at 45 days with varying dose levels of HVS survived for a period of at least 260 days. Such an inactivated vaccine would be useful in preventing oncogenesis in owl monkeys or marmosets that have been in contact with squirrel monkeys, so that natural infection may have taken place.

HVS was successfully propagated in dog embryo lung (DEL) cells; the 22nd-passage DEL HVS, when inoculated into monkeys, induced neutralizing antibodies to HVS, but the animals survived longer than those animals challenged with HVS propagated in owl monkey cells. Animals given the largest challenge doses of HVS lived longer than those given smaller doses; the larger doses may have induced a greater immune response on the part of the host to the development of tumours. These results show the need for further experiments to determine how resistance may be enhanced and perhaps made complete. This may lead to an improved understanding of immunity to the oncogenic effects of HVS, and perhaps to the development of a live virus vaccine.

Passive immunization of monkeys, using antibodies to HVS prepared in goats, also delayed the onset of lymphomas after inoculation of HVS. Studies of the toxicity of immunosuppressive drugs in New World monkeys, preparatory to the use of those drugs in experiments on resistance to HVS tumour development, were also discussed.

A SUMMING-UP

A SUMMING-UP

G. KLEIN

Round the table: *P.M. Biggs, F. Deinhardt, G. de-Thé, M.A. Epstein, W. Henle, T. Lindahl, G. Miller, J. Pagano, J.H. Pope, F. Rapp, B. Roizman, V. Vonka and H. zur Hausen.*

By this time, it must be clear to all of us that a great deal of progress has been made since the Cambridge Symposium three years ago. With the exception of some introductory papers dealing with the molecular biology, genetics and latency of the lytic herpesviruses - very important in themselves - the majority of the papers were directly concerned with various aspects of proven or suspected herpesviral oncogenesis. The four main areas were Epstein-Barr virus (EBV) in man, Marek's disease herpesvirus (MDV) in the chicken, herpesvirus saimiri (HVS) and ateles (HVA) in monkeys, and the oncogenic transformation induced by defective herpes simplex (HSV) in various target cells.

EBV is now generally accepted as the causative agent of infectious mononucleosis (IM) in man. The details of the pathogenesis and the exact role of EBV-carrying cells are not clear, however. At least the majority of the atypical cells in the circulation appear to be T cells (Sheldon et al., 1973; Virolainen et al., 1973; Greaves & Brown, 1975; Denman & Pelton, 1974). Recent evidence suggests that at least some of them are specifically sensitized against EBV-carrying cells, since they kill EBV genome-carrying B cell lines but do not kill EBV genome-negative lines (Svedmyr & Jondal, 1975). This specific effect can be demonstrated only after non-specific killer cells, present in both normal and IM blood, have been removed, e.g., by virtue of their complement receptors. At the target-cell level, all EBV genome-carrying lines so far tested were sensitive to the EBV-specific killer effect, no matter whether they contained immunofluorescence-detected, EBV-determined membrane antigen (MA) or not. By analogy with the major transplantation antigen systems, it is thus possible that lymphocytes can recognize membrane antigen specificities that are not readily demonstrated by serological reagents.

It is not clear whether IM blood contains circulating B cells that carry the EBV genome and the EB-nuclear antigen (EBNA). I don't

believe that it is possible to state at present, with any degree of confidence, that IM blood is EBNA-negative. The test has not been refined to the point where a small number of positive cells can be demonstrated amongst a large number of negative lymphocytes within a highly heterogeneous cell population. I am reminded at this point of the startling paper by Dr Carter at the Cambridge Symposium (Carter, 1972). You will recall that he collected autopsy material from mononucleosis patients who died from accidents during the acute phase of the disease. Their internal organs were extensively infiltrated with atypical lymphoid cells, closely resembling a malignant lymphoproliferative disease. It would be important to know whether these were proliferating B cells, perhaps analogous to the *in vitro* transformed, "immortalized" lines, or reactive cells, perhaps corresponding to the atypical T cells of the peripheral blood.

Dr Epstein and his co-workers, Dr Rickinson in particular, presented a new view of the state of the viral genome in IM, as contrasted to Burkitt's lymphoma (BL). Opposing the view that EBV genome-carrying, potentially malignant B lymphocytes are kept under immune surveillance in post-IM patients and in normal EBV-carrying individuals, they postulate that EBV is carried in a latent state, analogous to the presence of HSV in ganglion cells. When they mixed normal cord-blood lymphocytes with the peripheral white cells of acute mononucleosis patients of the opposite sex, the emerging EBV-carrying lines frequently contained the sex chromosome marker of the cord-blood donor. This indicated that infectious virus was present in or was generated by some cells in the mononucleosis blood; following explantation, this led to the *in vitro* transformation of previously normal B-type lymphocytes. Obviously, virus is present in mononucleosis blood in some form. However, we really don't know where it is - it could be within some cells but it might also be free, at least before the appearance of neutralizing antibodies. It has been conclusively established that the throat washings of IM patients regularly contain free virus that can transform cord-blood cells; would it be hard to imagine that even the peripheral blood may contain free virus? Be this as it may, the two-step transformation that may follow the explantation of IM blood, as the Epstein experiments clearly show, can hardly be argued to prove that a similar situation must prevail when EBV-carrying cell lines are established from normal EBV-positive individuals. Even if this did happen occasionally, it would not exclude the possibility that EBV genome-carrying B lymphocytes with a neoplastic potential may lie dormant in the blood and tissues of seropositive individuals. Epstein felt that it was highly unlikely that 80-90% of the healthy adult population could walk around with a large number of potentially neoplastic cells in their bodies, yet this is exactly what seems to happen in wild mice, with regard to polyoma-transformed cells. It is known that virtually all wild mice carry polyoma virus, but they do not develop tumours when kept under observation in the laboratory. Allison has recently shown that nude mice that lack a thymus-dependent lymphocyte system, may develop as many as five primary tumours on an average, when infected with polyoma virus, and some develop ten! All tumours can be prevented by inoculating polyoma-immunized syngeneic T-lymphocyte populations. This illustrates the power of the thymus-dependent surveillance mechanism in relation to a certain kind of

potentially neoplastic cells, namely those induced by ubiquitous oncogenic viruses in their natural host species. The polyoma-mouse system may be a good model for the understanding of the relationship between EBV and man or between HVS and the squirrel monkey. A certain, but much less powerful, surveillance appears to operate in relation to Marek's disease, capable of protecting certain genetically resistant breeds of chicken whereas genetically susceptible birds are not protected efficiently. At the opposite end of the scale, the effect of HVS on the marmoset and the owl monkey represents a more or less completely unprotected situation. In this case, the susceptible animal species do not meet the virus in nature.

In all probability, surveillance evolves by the selective fixation of immune-responsiveness (Ir) or Ir-like genes, capable of influencing the efficiency and/or the promptness of the host response at multiple immunological levels. The basic genetics of Ir systems is still in a relatively early phase of its development, but it is already clear that different kinds of Ir genes exist. Some appear to act at the T-cell level, others at the macrophage level, and still others at the B-cell level or at the level of T-B cell interaction. Obviously, a strong impact by a highly lethal virus on the species will select for *all* mechanisms that contribute to resistance. If surveillance is fixed at multiple levels, one specific kind of experimentally applied immunosuppression may not be sufficient to allow the proliferation of the dormant neoplastic clone. Negative experiments are therefore less conclusive than positive. In the polyoma system, antilymphocytic serum (ALS) treatment, neonatal thymectomy, total-body irradiation or the complete T-cell deficiency of the nude mouse are all efficient, to various degrees, in breaking down the resistance of normal adult mice to the oncogenic effect of the virus. In other situations, and particularly when the target cell is of lymphoid origin, ALS may have the opposite effect, due to the fact that it can also inhibit the growth of the target cell directly. One study (Bremberg et al., 1967) has shown that ALS affects the growth of Moloney virus-induced mouse lymphomas in a way that is reproducible for each lymphoma line but unpredictable with regard to its direction, namely, inhibition or facilitation of growth. The final outcome is the end result of at least three parameters: the effect of ALS on the growth of the lymphoma cells, the effect of ALS on the immune response, and effect of the immune response on the lymphoma. Thus, the immunosuppressive agent interferes with a complex, multivariant system and the ultimate outcome depends on the interplay of the different components.

Our way of thinking about spontaneous tumour development and its relation to the immune response may have been biased by the development of high tumour strains by inbreeding and selection. Genetic analysis of AKR leukaemia (Lilly & Pincus, 1973) or C3H mammary tumour (Heston, 1973) showed the selective fixation of multiple genetic systems in these strains that favour the development of the particular tumour by different mechanisms. These include integration of the oncogenic viral genome with the germ line, fixation of "amplifier" genes that favour the multiplication of the virus, selection for immunological unresponsiveness towards the oncogenic virus and/or the neoplastic cell product, and even fixation of genes that influence the probability of neoplastic transformation at the target-cell level. While the laboratory

selection for high tumour incidence fixes multiple genes that favour tumour development, nature selects in the opposite direction, establishing multiple controls that suppress virus-induced neoplasia.

The etiological discussion concerning the possible role of EBV in BL and nasopharyngeal carcinoma (NPC) has been sharpened by the distinction between a mere serological association and the demonstrated presence of the viral genomes in the tumour cells. High anti-EBV titres can arise in the absence of a genome-carrying tumour, as reported in the paper by Dr Henle. Secondary elevation of this type occurs, in all probability, due to the relative immunosuppression (particularly T-cell suppression) that accompanies certain malignant diseases (Hodgkin's disease in particular). Conceptually at least, this should be distinguished from the more direct, primary rise of the antibody titres that accompanies the growth of an EBV genome-carrying tumour. Unless the presence of the viral genome is demonstrated directly in the tumour cells by EBV deoxyribonucleic acid (DNA) or EBNA tests, high EBV antibody titres can be very misleading by themselves. Dr zur Hausen has suggested that BL should be reclassified as EBV genome-positive and genome-negative lymphomas. It would be interesting to see whether a prospective study can reveal any clinical or histopathological differences between the two groups.

It is becoming more and more difficult to explain the presence of EBV in the genome-positive, African BL by the passenger hypothesis. According to this theory, EBV is a relatively innocuous inhabitant of lymphoid tissues. Lymphomas would develop for quite unrelated reasons, but they would carry the virus along as a passenger, with amplification of viral antigen and antibody as the result.

There are now two EBV genome-negative lymphoma lines derived from genome-negative tumours of Burkitt or Burkitt-like histology. One is Ramos, derived from an American BL. The other, BJAB, comes from one of the two exceptional EBV genome-negative African BL cases. The donors of both lymphomas were EBV-seropositive. The two lines *in vitro* are susceptible to EBV infection *in vitro* and one has been converted into a permanent genome-positive line. Thus, in spite of the fact that the donors had the virus and their lymphomas were susceptible to it, the virus did not "jump" on to the cells *in vivo*. A similar argument can be made for the numerous other genome-negative non-BL that originate in highly seropositive individuals; this argument is weaker, however, since there is no positive evidence of cellular susceptibility to the virus. Nevertheless, the fact that no EBV genome-positive lymphomas have been found so far that are *not* African BL, strongly suggests that the regular presence of the virus in African BL must have some special significance. However, I completely agree with Dr Henle that many more lymphomas should be examined outside Africa. Perhaps one should particularly look for cases with a serological picture that resembles African BL, e.g., high anti-R (restricted EA) titres.

It is easy to see why the virus does not jump. During the primary infection, as we know it from IM, neutralizing antibodies appear very quickly, in a few weeks. In infection experiments *in vitro*, even high dilutions of neutralizing antibodies prevent virus passage to new host cells very efficiently.

If these arguments are basically correct, it would follow that the vast majority of the African BL cases originate in genome-carrying cells from

the beginning. This leaves us with two main possibilities that we may call the immunological and the co-factor hypotheses. The immunological hypothesis would imply that, in contrast to what Dr Epstein thinks, healthy EBV-seropositive individuals harbour a considerable number of virally converted, potentially neoplastic cells. These are kept dormant by some control mechanism, presumably immunological in nature. This control would work very efficiently most of the time, even in Africans. In the occasional, and still relatively rare case of the African BL patient, a single clone would "sneak through" and grow to irreversible size. As you know, Dr Fialkow and his co-workers have conclusively shown that African BL is a monoclonal disease, as judged by G6PD and immunoglobulin marker studies (Fialkow et al., 1970, 1973).

The postulated escape of the African BL clone could occur for genetic or environmental reasons. The geographical distribution and the characteristic time-space clustering suggest mainly environmental factors. The question arises whether any particular type of immunosuppression would favour the outgrowth of a neoplastic clone. One wonders whether the falling anti-EBV titres in African children that Dr de-Thé postulated may reflect some relevant process. It will be very important to confirm it by horizontal studies on the same individuals. Another potentially relevant case is the patient described by Drs Henle and Magrath. They found that an EBV genome-positive BL developed in an African Hodgkin's disease patient.

It is likely that we can learn a great deal from cases where BL recurs after relatively long periods of total regression. The immunological events preceeding these recurrences may be informative. Dr Gunvén of our laboratory has obtained evidence that circulating immune complexes with EBV-determined MA specificity may appear in BL patients 4-6 months prior to recurrence (Gunvén et al., 1974). It is tempting to think that the complexes may promote recurrence, e.g., by the blocking of cell-mediated immunity.

The malaria hypothesis proposed by Burkitt (1969) does not conflict with the immunological hypothesis. It is known that chronic holoendemic malaria stimulates the proliferation of the lymphoid system. If B cells were stimulated and T cells suppressed, a situation might develop that could break down surveillance.

There is, of course, no direct evidence at present to prove the validity of the immunological hypothesis (or any other hypothesis, for that matter). One important aspect of this hypothesis is the underlying assumption that EBV-transformed lymphoid cells are potentially neoplastic, no matter whether they are found in normal people or in tumour patients.

The "co-factor hypothesis" has the opposite point of departure. It assumes that EBV-transformed cells of normal individuals are not yet malignant, although they may be premalignant. A second event would be required, induced by another virus, or by non-viral proliferative stimuli, or by an indigenous cellular change, before a fully malignant lymphoma could emerge.

The question whether Burkitt-derived and non-Burkitt-derived EBV-carrying lines differ in cellular properties is highly relevant in this connection. Dr Jarvis showed the frequent presence of the chromosome 14 anomaly, described by Manolov & Manolova (1972), in the majority of the Burkitt-derived lines tested. Dr Zech in Stockholm has obtained similar evidence on Burkitt-derived lines and also on biopsied *in vivo* BL tumours. Neither of the two

investigators found this chromosomal change in any of the EBV-carrying, non-Burkitt-derived lines that they studied. It seems, however, that the marker may be present in some EBV genome-negative lymphomas *in vivo*. If so, the marker may be more lymphoma-specific than EBV-specific. There are at least some superficial resemblances between this and the relationship of the Ph' chromosome to chronic myeloid leukaemia (CML). Obviously, comparisons on a large material are urgently needed.

Nilsson & Pontén (1975) have compared a fairly large number of normal and Burkitt-derived EBV-carrying lines with regard to several morphological, functional and growth characteristics. The Burkitt-derived lines were distinctly different. As a rule, they were more homogeneous with regard to several markers than the EBV-carrying, non-Burkitt lines. While their data are fairly convincing, it is not clear to me whether the difference may not merely reflect the fact that *in vitro* EBV-transformed or EBV-carrying normal donor-derived lines are polyclonal while the Burkitt tumour is monoclonal already *in vivo*. Could selection for monoclonality, from an originally polyclonal, EBV-transformed population, account for the difference? Alternatively, would the transformation into a BL reflect a second, EBV-unrelated event, occurring in one of the EBV-carrying cells?

Arguments can be found for and against both alternatives. It has been clearly shown that EBV-carrying lymphoid lines of non-Burkitt origin can be at least potentially malignant, since they can grow progressively as heterotransplanted lymphomas in nude mice or other immunologically crippled recipients (Southam et al., 1969; Adams et al., 1967, 1970; Levin et al., 1969). Furthermore, Leibold et al.[1] have recently found that three *in vitro* transformed lymphoblastoid squirrel monkey lines were malignant upon reimplantation to the autologous host. The chromosomal difference mentioned above would, on the other hand, argue for a true difference between Burkitt- and non-Burkitt-derived lines.

The studies reported on NPC have vindicated Dr zur Hausen's original suggestion. In contrast to what most of us used to think, in the beginning, EBV does not travel along with the large numbers of infiltrating lymphocytes in NPC; the viral genomes are localized in the epithelial tumour cells. In retrospect, it is easy to see that this is in good agreement with the serological picture. The uniform EBV seropositivity and the 10-fold elevation of the geometric mean anti-viral capsid antigen (VCA) titre in poorly differentiated NPC resembles the picture obtained with African BL, the only other known genome-carrying tumour, to the exclusion of other human neoplastic conditions so far studied. Closer scrutiny of the lymphocyte population that infiltrates the solid NPC further underlines the fallacy of the original argument. The overwhelming majority are T, not B lymphocytes (Jondal & Klein[1]). Since B, but not T lymphocytes, have EBV receptors (Jondal & Klein, 1973), the finding by Wolfe et al. (1975) of an inverted correlation between the extent of lymphocytic infiltration and the average number of EBV genomes per NPC biopsy cell makes sense. Nude mouse-passaged, human lymphocyte-free NPC cells cary much larger numbers of EBV genomes than originally suspected (Klein et al., 1974).

Where did Dr de-Thé's EBV genome-carrying, NPC-derived B lymphoid lines

[1] Unpublished data.

come from? Obviously, they must have originated from the small number of contaminating, genome-carrying B cells known to be present in both normal and neoplastic tissues in seropositive individuals. This illustrates, once again, the selective advantage of EBV-carrying B cells in tissue culture and warns against extrapolations concerning the localization of EBV genomes in tumours *in vivo* in the absence of direct evidence.

The selective advantage of EBV-carrying B cells *in vitro* is also illustrated by the earlier attempts to transform "purified" T-cell suspensions with EBV *in vitro*[1]. EBV-carrying B lines emerged after long latency periods. Apparently, subliminal B-cell contamination was sufficient to permit the selective outgrowth of the usual, immortalized B cell line. Analogous EBV-carrying B-cell contaminants have been isolated, of course, from virtually any tissue from seropositive donors that has been properly tested, including leukaemias, myelomas, normal lymph-nodes, etc. In view of the new evidence on the association of EBV with the carcinoma cell in NPC, it is probably unsafe to regard the virus carried by the NPC-derived lymphoid lines as representative of the carcinoma-associated virus, as we and others have done. If relevant viral subtype differences exist, the lymphoid lines may carry a subtype different from that carried by the carcinoma cell. The important experiments of Dr Pagano are a first approach to the question by molecular hybridization. Dr Pagano's report is at least compatible with the idea that NPC may be associated with a subtype different from BL-EBV, but the limited material does not yet allow a distinction between this and the more trivial alternative of random virus strain differences.

Dr de-Thé and others raised the important question whether EBV is as generally and uniformly associated with NPC as with African BL. The regularity of the association with NPC in different geographical areas, or in relation to various histological types or different ethnic groups is very incompletely known. Serological evidence (de Schryver et al., 1969) suggests that the association is limited to poorly differentiated or anaplastic NPC and excludes well differentiated squamous-cell carcinomas. As to geography, EBV genomes have been found in Chinese, African and Tunisian cases (see the reports of Dr de-Thé and of Dr Pagano at this Symposium). It may also be recalled that the association between EBV and NPC was first discovered by the serological study of American cases (Old et al., 1966). This suggests that even the relatively rare Western cases may carry the genome (see also de Schryver et al., 1974). Obviously, much more will have to be done on this problem.

Dr Becker raised the question whether the large variation in the number of genome equivalents between different NPC biopsies may reflect differences in the biology of the tumours. One has to be very cautious here because the average genome number per cell can show a spurious variation for entirely trivial reasons, such as the variable admixture of normal cells or necrotic tumour tissue. Apart from the nude mouse study, already mentioned, where correction was made for the mouse stroma (Klein et al., 1974), there is no reason to believe that the genome numbers so far recorded in the literature really reflect the true genome numbers per cell.

[1] See the paper by Menezes et al. (*Part 1*, p.323).

For the same reason, namely, inadequate histological scrutiny, it is not yet possible to answer the question whether EBV genome-negative anaplastic NPC exists. Cases have been recorded but the biopsies were not evaluated with regard to their content of viable tumour tissue.

Dr Simons has discussed the genetic factors that may influence NPC development. Previous epidemiological studies strongly emphasized the importance of genetic factors (Ho, 1972). The intermediate incidence of NPC in the offspring from mixed Chinese-non-Chinese marriages in Macao and in Thailand was particularly suggestive. This is very different from the climate-related geographical correlation, characteristic of African BL. The preliminary results reported by Dr Simons, suggesting an association between HL-A type and NPC risk in Singapore Chinese, would be in line with a genetic risk. It may be pointed out, however, that an HL-A linkage, if confirmed, does not have to mean, *eo ipso*, that the genetic mechanism acts through Ir genes. In the field of experimental carcinogenesis, it is a well-known fact that genes may influence neoplastic development at many different levels, including the probability of an oncogenic change in the target tissue itself. In addition, genetic factors can act at many different host levels, ranging from hormonal factors, through the metabolism of carcinogenic compounds, to the immune response. Genetic factors may also influence the replication of oncogenic viruses, e.g., via amplifying factors, such as Fv-1 in some C-type virus systems. It would be, of course, most interesting if Ir gene-like factors were involved in NPC susceptibility. To approach this question, it would be necessary to design tests to measure NPC-related cell-mediated immunity in the population at risk, not just EBV titres, in parallel with HL-A typing.

Proceeding now to transformation, Dr Miller's term "immortalization" appeals to me very much since it describes the phenomenon quite adequately. Since the Cambridge Symposium, it has been shown that EBV strains from different sources may differ in their immortalizing ability. We heard about this from Dr Menezes and from Dr Miller. Virus derived from the P3HR-1 line is unique in two respects: it is the only known isolate that can induce an abortive viral cycle in superinfected Raji cells, as signalled by the appearance of early antigen (EA). It is also unique with regard to its inability to immortalize normal lymphocytes. All other biologically active virus isolates tested were unable to induce EA but could immortalize lymphocytes. Both Dr Kieff and Dr Roizman have raised the important question of viral defectiveness. Their reasoning starts from the considerable amount of information that has accumulated in relation to HSV and other lytic herpesviruses. Roizman demonstrated an impressive variation in the nucleic acid sequences of different HSV isolates, as judged by the distribution of restriction fragments. Similar evidence has been obtained by Dr Summers, who referred to this approach as "molecular epidemiology". Dr Roizman demonstrated that the DNA composition of laboratory strains differs from strain to strain. This approach will gain in importance if it can be linked with DNA transformation studies. If feasible, this may eventually pinpoint the regions of the genome that are required for transformation or, conversely, the regions that interfere with transformation, e.g., by lytic effects. The remarkable progress that has been made during the last year in the field of the small oncogenic DNA viruses by

restriction analysis, in conjunction with transformation by defined DNA fragments, points the way towards the more difficult but, hopefully, equally fruitful analysis of the large DNA viruses.

Further news on EBV strain differences include Dr Hinuma's report at this meeting, showing a difference between two transforming isolates, judged from the morphological and growth characteristics of the transformed products. There were also differences between membrane-associated versus secretory immunoglobulins and virus producer status. Dr Hinuma pinpointed the dilemma involved in trying to determine whether the differences are due to the presence of different viruses in the same type of cell or to a difference in viral affinity to different lymphocyte subtypes. This is an important question, because the former alternative would imply a direct influence of the transforming virus on the cellular phenotype whereas the latter would be merely a slight variation in viral tropism.

While the question of cellular versus viral variation has to be left open with the Hinuma variants, it can be answered with some conclusiveness with regard to the difference between the non-transforming virus and an immortalizing prototype such as B95-8. In their own home strains, both viruses behave in an approximately equal fashion. Both cell lines proliferate by the multiplication of a virus genome-carrying, non-producer stem line. A small number of cells are switched on from time to time, their proportion is similar and their viral cycle and its antigenic landmarks are indistinguishable in the two lines. The fact that both viruses do equally well in their home strains, but differ in their interaction with a new target cell (normal lymphocyte or EBV genome-negative B lymphoma line) makes it immediately clear that one cannot speak about defectiveness or non-defectiveness in simple terms. The lysogenic and non-lysogenic interaction of temperate phages with different host cells appears a more pertinent parallel. It is most important to stress that, in contrast to the HSV system, viral defectiveness is *not* a prerequisite for EBV transformation. On the contrary, lymphocyte immortalization appears to be part of the normal life cycle of the virus. As shown particularly by Dr Miller's group, EBV has a transforming efficiency per particle that far exceeds the "best" experimental transforming viruses, such as SV40 or polyoma. It therefore appears that EBV has a truly biphasic life cycle with a predominant non-producer and a relatively rare producer phase. Non-producer lines appear to be capable of perpetuating the latent phase of the life cycle indefinitely, *in vitro*.

While B95-8 virus readily induces EBNA in cord-blood lymphocytes, long before an immortalized line emerges, P3HR-1 virus neither immortalizes nor does it induce any detectable EBV antigens in normal lymphocytes. This is in strange contrast to the fact that both viruses induce approximately the same number of EBNA-positive cells in established, EBV genome-negative but EBV-sensitive B lymphoma lines (Klein et al., 1974). The absence of antigen induction in normal lymphocytes may be related to the finding of the Miller group that, unlike B95-8 virus, P3HR-1 virus fails to induce DNA synthesis. EBV antigens are normally observed in blast cells. Conceivably, small lymphocytes may not have the apparatus required for detectable EBV antigen synthesis.

In the genome-negative B lymphoma lines, the P3HR-1 virus infection goes on from EBNA to EA and the cells die.

The B95-8 virus stops at the EBNA-positive stage. Converted cells continue to live and can be isolated as a permanently converted EBV genome-carrying subline (Clements et al.[1]). Since the two viruses thus behave differently in the same target cell, we are obviously dealing with viral variation. P3HR-1 virus is not restricted by the controls of the target cell to the same extent as B95-8 virus and, due to this relative insensitivity, it goes one step too far and kills the cell.

Do cells differ in the degree of restrictive control they can exert on the genome of the same virus strain? The reports of Dr Miller and of Drs Menezes and Leibold showed that the relative frequency of producer and non-producer lines, derived from transformation with the same virus strain, depended on the source of the target cell. Cord-blood lymphocytes immortalized by B95-8 virus were mainly producers. Adult human lymphocytes turned into producer and non-producer lines with an approximately equal frequency. Marmoset and squirrel monkey lymphocytes gave rise to producer lines, as a rule. This suggests that the "leakiness" of the restricting controls is at least slightly different in different lymphocytes and may depend on their exact state of differentiation. In conclusion, there is thus evidence for both viral and cellular variation. Cells vary in the tightness of their restrictive controls whereas virus strains vary in their relative dependence on the controls of the same host cell.

Is there any EBV source that can be regarded as wild type? It seems to me that the virus isolated from the throat washings of acute IM patients is probably the closest approximation. It has been shown at several laboratories that such throat washings have a high transforming ability (Golden et al., 1971; Pereira et al., 1972). This, together with the uniqueness of the non-transforming P3HR-1 virus among all viral isolates tested, suggests that the P3HR-1 virus is the defective mutant of an originally transforming virus. How can this be reconciled with Dr Kieff's demonstration that P3HR-1 virus contains all demonstrable DNA sequences of B95-8 virus and a unique sequence in addition that is not present in B95-8 virus? We do not know where the extra sequence comes from. There is no assurance, furthermore, that *all* B95-8 sequences are present in P3HR-1 virus, since the sensitivity of the method is obviously limited. Although puzzling, the evidence is therefore not necessarily contradictory and more information is badly needed.

In addition to the admirable adaptation of EBV to its highly restricted way of life in the lymphocytes, there is also good evidence of a remarkably well-balanced cell-host relationship. The human host has apparently evolved highly efficient mechanisms capable of restricting the growth of virus-carrying, potentially transformed cells. As in other situations where the host species is selected for resistance against a potentially lethal biological agent, resistance is probably fixed at multiple effector levels. One might even speculate that the presence of such well-controlled transformants may be of some advantage to the host. In IM, numerous "irrelevant" antibodies are formed (e.g., heterophil, anti-vesicular stomatitis virus [VSV], etc.), probably due to the polyclonal proliferation of the affected B cells. Is it conceivable that latent EBV infection boosts the ability of the B-cell system to make antibodies in general?

[1] Unpublished data.

Dr Pope has spoken about an allogeneic effect involved in transformation. Is it really an allogeneic stimulation or is it a syngeneic inhibition? This is unexpected and rather fascinating; it will obviously require more detailed studies. One wonders what other types of syngeneic or allogeneic cells would do in the system.

Before proceeding further with the summary, I would like to turn the rostrum over to some of my colleagues to comment on what has been said so far. Could we start with Dr Epstein?

Epstein: I think that although there is technical difficulty, as you point out, in showing that EBNA is present in peripheral circulating cells in IM or in seropositive normal individuals, we don't have to wait until all the technical problems have been solved in order to get an answer to the question. You quite rightly point out that in IM there is a very dramatic infiltration of lymphoid organs. I think that we ought to have a look at that. If what you believe is correct, one ought to find large quantities of EBNA-positive cells there and this should give you the source of your so-called transformed cells, but the idea that this infiltration is lymphoma-like is rather simplistic. It is only two years ago that it became possible to sort out the B and T cells. We used to say that the abnormal cells circulating in IM patients were like those of acute lymphoblastic leukaemia, yet we now know that the majority of these cells are T cells, which have nothing whatsoever to do with EBV. We can argue around this for a while, but I think we are up against a conceptual difficulty. Dr Klein's difficulty here is very much like the difficulty he had in accepting Dr zur Hausen's finding of EBV in the epithelial cells of NPC, because he felt that the virus should not be there. I wonder whether he feels that the proposal I put forward is unacceptable because in other systems, as in polyoma, it is not like that. But we do not have a herpesvirus model of the kind he cited, whereas we do have herpesvirus models of the type that Dr Deinhardt cited and we can hardly go on arguing about this until we have done more experiments.

I am quite sure that, by the next Symposium, we shall know whether or not there are EBNA-positive cells in the peripheral blood in IM and in the peripheral circulation of normal individuals, and I am quite sure that the phenomena that we are reporting in our experiments cannot just be due to viraemia in IM. If that were all, then the abolishing of the outgrowth of continuous cell lines by neutralizing antisera that we have seen, would not happen. If transformed cells were there originally, they would grow out readily, no matter whether the antiserum was present or not, just as happens when BL grows in culture.

Klein: On the point that we should do more work, I wholeheartedly agree. As far as the conceptual difficulty is concerned, I would return the ball to Dr Epstein. I do have some difficulties in seeing why the virus should transform cells so readily *in vitro* but not *in vivo*. I promise to do more EBNA tests on the peripheral blood of IM patients, but this test is not as good at detecting a small minority of positive cells in a heterogeneous cell population as you seem to think. If I may also suggest some experiments, I would be very grateful if the following two could be performed: (i) direct tests for viraemia, i.e., attempts to transform normal lymphocytes with cell-free serum or plasma from acute IM patients; and (ii) a repeat of your beautiful cocultivation experiment, not with IM donor blood, but with cells explanted from normal, healthy seropositive donors. This would mean spontaneous transformation, for which you would probably need a much larger inoculum than from the IM, with added cord-blood cells of the opposite sex.

Epstein: As far as the second set of experiments is concerned, this is already under way.

Henle: I find it difficult to add anything to this discussion. Dr Klein has expressed our feelings well, and I agree that much work needs to be done. The reason why you, we and others have not yet found EBNA-positive cells in the circulation of IM patients may be purely technical. To judge from the colony formation experiments, we might expect only one in 10 000 cells to be positive. This could be below the level of detectability. There might be a larger proportion of positive cells in lymph-nodes, spleen, bone marrow, etc., but these are not obtainable, as a rule, from IM patients. It might be possible, however, to get the lymph-nodes from viral carriers undergoing surgery for non-EBV-related diseases. If they have relatively high EBV antibody titres, one might find EBNA in some of the lymph-node cells.

Epstein: I would like to add tonsillar cells to Dr Henle's list. I have the feeling that they would be very valuable.

Henle: As far as EBNA staining is concerned, one would face the same problems as with lymphocytes from other sources. Many attempts were made to establish lymphoblast lines from tonsils and adenoids. Quite often, the cultures are lost because of activation of adenoviruses but occasionally EBV-carrying lines come through.

Epstein: What do you actually think about infiltrating cells in the tonsil? I would have thought it would be an interesting source of EBV.

de-Thé: I would now like to turn your attention to the value and unique quality of the epidemiological tool in studying the role of EBV in relation to BL and NPC.

With regard to BL, it seems very unlikely that BL follows primary EBV infection after a short latent period. Two sets of data support this hypothesis. The first relates to the results presented during this Symposium: in Uganda, EBV infection takes place at between one and two years of age, whereas in South-East Asia and, to a greater extent, in Europe such infection occurs much later. It should be appreciated that about 1% of the children aged 3 to 5 have a chance of not being infected by EBV in Uganda. If these children form the cohort at high risk for BL, they would represent the highest cancer-risk group known in natural conditions, since 10% of them would develop BL. This is very unlikely.

The second set of data relates to the "pre-BL" serological profile. In their paper, Drs Magrath and Henle described a child with Hodgkin's disease who later developed BL. This child had antibodies to EBV nine months to a year before the development of BL. A similar situation was found when "pre-BL" sera from three cases from our prospective study in the West Nile, Uganda, were tested simultaneously in Dr Henle's and our laboratories. The "pre-BL" sera were taken at 7, 17 and 18 months respectively and were all found to have EBV antibodies. However, in each case antibodies against EA either appeared or sharply increased after the onset of BL. These three cases, together with the Magrath-Henle case, suggest that the seroconversion that precedes or accompanies BL refers mostly to EA antibodies, which is suggestive of a reactivation of a chronic or latent EBV infection. Such a reactivation might reflect the passive presence of the virus in the lymphomatous cells or result from the oncogenic activity of EBV. No further critical progress can be made until markers for oncogenic transformation by EBV are available.

In view of the very early occurrence of EBV infection in Uganda compared with that in Hong Kong, Singapore or Europe, I should like to suggest that *perinatal* EBV infection might be associated with the risk for BL. Such a relationship would recall that between measles infection at a very early age and the risk of subacute sclerosing panencephalitis. Formation of immune complexes and depression of EBV-specific cell-mediated immunity could follow such perinatal infection. Hyperholoendemic malaria could also prepare the ground for the expression of the oncogenic potential of EBV. The ultimate proof of the role of EBV in BL development might come from intervention experiments.

Such intervention may not necessarily involve a vaccine but simply hygienic measures, once the natural history of the viral infection is better understood. In all events, both animal experiments and population surveys will be required to establish the role of EBV in BL development.

With regard to the relationship between EBV and NPC, again, the epidemiological tool could be of unique value. Immunogenetic and viral factors known to be associated with NPC could be used as markers for an early detection campaign that, if successful, would permit more effective treatment and favour the hypothesis of an etiological involvement of EBV in the development of NPC.

In conclusion, I would like to stress that the establishment of the etiological role of EBV in human malignancies and the possibility of controlling these diseases, require the close integration of epidemiological and experimental approaches.

Klein: Thank you very much. I would like to proceed to transformation.

Pope: It is quite clear that established cells can be transformed from a variety of tissues. There is a certain monotony about the transformed cells, but I would like to state that we never really obtain transformed lines that resemble BL lines.

The second point I would like to make relates to the infection of EBV genome-negative lines, and this may be used to challenge the idea that EBV may be a primary transforming agent in BL. There is, of course, the perfectly reasonable possibility that cells could be transformed by agents other than EBV.

I think that we cannot exclude the possibility that the lymphoma transformation starts in EBV genome-negative cells, even in patients who eventually come to carry EBV genome-positive tumours, and that the tumour cells then meet the EBV genome later, perhaps in the lymph-nodes, and before there is enough neutralizing antibody to prevent interaction. In order to clarify this point, one would have to know that the EBV genome-negative tumours (and the corresponding lines derived from them) did have a fair chance of meeting EBV, comparable to that of the EBV genome-positive tumours. I feel that this process is very likely to occur, but it is difficult to obtain actual proof. The hypothesis, as outlined above, involves essentially a two-step transformation (as far as the EBV genome-positive tumours are concerned), but the crucial question is whether the EBV transformation is the first or the second step.

As to EBV transformation itself, its exact mechanism is not well understood and it is probably a rather complicated interaction, even *in vitro*. As far as the biochemical approach is

concerned, even with the definition of the numbers of EBV genomes and the way in which they are associated with the cell genome, I think that it would be very important to work, not only with long-established lines, such as Raji, but also with early, transformed cells.

Klein: Dr Pope, how would a secondary EBV infection of already transformed neoplastic clones account for the exclusive restriction, at least at this time, of the EBV genome to the African BL cases? What would there be so special about the interaction in the African patients with this particular kind of lymphoma?

Pope: I suppose that one could postulate that there were more EBV-producing cells, whatever they are, in the lymph-nodes of the African, as compared to the non-African patients. To mention another example, the epidemiological picture of EBV infections in the Australian aboriginals is certainly very different from that in the white population.

Miller: I would like to discuss two points: one is the relevance of experimental EBV infections of marmosets to the general question of human *in vivo* diseases, and the second is whether *in vitro* transformation tells us anything about naturally occurring, *in vivo* phenomena.

Concerning the first point, I think that it should be borne in mind that there are considerable differences between the fulminant disease induced by HVS and the tumours induced by EBV, in cotton-topped marmosets. I don't want to list all the differences here, but I want to emphasize that this is by no means the same disease. On the contrary, I would like to say that, as far as EBV is concerned, there are remarkable parallels between what goes on in the marmoset and what goes on in man. Dr Shope and I have together inoculated 25 marmosets with approximately the same virus preparation. Within this group, there is a subgroup of animals with lymphoma, another subgroup with EBV antibodies and hyperplastic lymph-nodes that regress, a third group with inapparent infections that develop antibodies but no lesions, and a fourth, rather small group that do not develop any antibodies. In order to see if there was any parallel with the situation in man, Dr Henle has recently performed serological tests on some of these groups. The tumour-bearing animals had high anti-VCA and anti-EA titres, whereas the animals with hyperplastic nodules or inapparent infections did not. This is in line with the corresponding observations on the human disease. The second observation concerns the presence of EBNA in the tumour *in vivo*. We have had two animals with tumorous lymph-nodes since the EBNA test has become available.

In both cases, the lymph-nodes were replaced by virtually 100% EBNA-positive cells. In contrast, the hyperplastic nodules without neoplastic disease that Dr Shope has described did not contain EBNA-positive cells. A third point concerns the virus-cell relationship. We could regularly grow EBV-carrying cell lines both from the hyperplastic nodules and from the tumours as well. This is in line with the point Dr Epstein discussed earlier today.

I should also like to discuss the efficiency of human lymphoid cell transformation by EBV. These experiments have been carried out by Dr Robinson, working with me. We can achieve only a relatively low multiplicity in the system, always less than ten physical particles per cell, or less than one infectious particle per ten cells. In spite of this, the efficiency of transformation is of the order of 10^{-2}-10^{-3}, i.e., about one cell in 500 becomes transformed. This may be compared with the SV40 human-cell system, where multiplicities of 10^{3} infectious particles per cell are regularly used, and where the efficiency of transformation is 10^{-3}-10^{-4}. We must conclude that EBV provides a particularly efficient transforming system, in comparison with other experimental DNA tumour viruses.

Deinhardt: I can only underline what Dr Miller has said and add to it that, in particular, the oncogenic forms of EBV infection in non-human primates, may provide experimental models that would enable us to test the validity of at least some of the arguments advanced by Drs Klein and Epstein because, in the experimental situation, one can remove each component of the system at will and study the relevance of each at a particular time.

In addition, it is puzzling that virus isolates derived from IM or from BL that have approximately the same transforming ability for primate lymphocytes *in vitro*, have, however, a very different tumour-inducing capacity *in vivo*. It is not clear at this time whether this is really a difference between various virus strains, or is due to a difference in the immune response of the animals. I think it would be very important to isolate various EBV strains and test their antigenic and oncogenic properties in the non-human primate systems without previous long-term passage of the EBV isolates in cell cultures.

Another question arising from the earlier discussions is the following: is it inconceivable that the viral genome can exist in normal cells, even without the expression of EBNA or other virally determined antigens?

Epstein: This is exactly the argument I have been trying to put forward, namely that EBV can exist in cells in a latent form,

without any antigen expression, in an exactly analogous way to the other herpesviruses. This would be a purely non-productive unexpressed infection. Upon explantation *in vitro* and occasionally also *in vivo*, the cell may turn on a productive infection. In the BL and in the *in vitro* transformation system, we have the non-productive infection expressed as transformation. Thus, we have all the three interactions that are observed with other members of the herpesvirus family.

Klein: I should like to warn against taking examples from the rest of the herpesvirus family, particularly if they are taken too literally. HSV sits in the skin and in some ganglion cells. Its cycle is activated by ultraviolet light, fever, emotional stress and other very miscellaneous influences. What happens on activation, we really do not know. The phenomenon has not been studied in detail, and there is no readily accessible system to hand where it could be studied. The life cycle of EBV appears to be quite different. EBV, unlike HSV, has a remarkable transforming efficiency without experimental manipulation, or, to put it in another way, has the natural habit of entering into a non-permissive interaction with the lymphocyte that endows the host cell with an unlimited capacity for proliferation. I don't see why we should look for analogies with other herpesviruses instead of trying to establish the life cycle of EBV as it is. This will be highly interesting in itself; it is no doubt the delicately poised end result of a long and very highly specialized evolutionary adaptation process.

zur Hausen: I have some doubts about Dr Epstein's statement that HSV exists in a completely non-expressed state in persistently infected cells. We know, of course, that EBV persists in the transformed cells in multiple copies. We have now at least some evidence from Dr Schulte-Holthausen's and Dr Baringer's work that suggests that something similar may happen with regard to the latent presence of HSV in human ganglion cells. However, it is highly questionable to my mind, whether all these genomes in the ganglion cells are really unexpressed. If Dr Stevens is right and if IgG antibody can suppress the activation or the expression of the genome in the HSV-carrying ganglion cells, it will be necessary to postulate some kind of viral expression on the membrane of these ganglion cells.

Rapp: I think that biological variation sometimes obscures biological unity. For example, many of the herpesviruses can be activated in a similar way, by a simple temperature shift up or down. Could it be that, where we find EBV, it has been activated as a result of the same process as HSV in a cold store, but we do not see it in the same way, because it does not cause a visually recognizable process?

Thank you very much. I think that we can go on now to the state of the viral genome. We had two very interesting papers, one by Dr Nonoyama, the other by Dr Lindahl. They said quite different things, but each speaker stressed that his or her findings did not exclude those of the other. Dr Lindahl showed that part of the viral genome is covalently integrated with the cell genome. Another, probably larger part, exists in a free, circular form. The relationship between the integrated and the circular form raises many intriguing new questions. It is not clear whether the whole genome is linearly integrated, or only fragments. We heard that this was a remarkable situation, with an integrated and a free form present in the same cell, essentially unparallelled in microbial genetics.

Mechanisms that regulate the interaction between the cellular and viral genomes may turn out to be of crucial importance for the understanding of the way in which the human lymphocyte keeps its large viral genome load under highly efficient control most of the time. Dr Nonoyama mentioned his fascinating, but preliminary finding that he could reduce the total number of genomes carried by non-producer lines by cycloheximide treatment, to only one genome per cell. It would be important to know whether this is now a definite fact. If so, one would like to know whether this genome is covalently integrated, what antigens it determines, etc. Dr Osato mentioned an equally fascinating but equally preliminary finding, namely, reduction of the EBV copy number to a low level (below detectability) with maintained EBNA expression, following Friend virus infection.

It will obviously be very important to pursue these leads, since they may illuminate the puzzling fact that all EBV-transformed cell lines so far studied carry multiple viral genomes. This raises some intriguing questions. Are multiple copies of the viral genome needed to maintain the transformed phenotype or is one genome sufficient, perhaps in a strategic location, and are the rest amplification epiphenomena? What maintains the number of genomes at a relatively constant level from year to year within one line? Is it the amplification mechanism, equilibrated by cellular controls, or is the replication of the viral genomes synchronized with the cell genome? Alternatively, do the free forms arise from the covalently integrated form at a given rate, compensated by a regular elimination mechanism? Does the integrated genome have a compulsory chromosomal localization, or can it be virtually anywhere? We have found (Klein et al., 1974), quite in line with Dr Glaser's report at this meeting, that EBV DNA and EBNA-positive cell hybrids derived from the fusion of an EBV-carrying Burkitt line and a mouse fibroblast lose the viral genome at a time when they still contain a considerable number of human chromosomes. This suggests that the virus is not associated with the majority of the human chromosomes, but is probably limited to a restricted number.

The recent work of Croce & Koprowski (1974) on the SV40 system offers some leads that may facilitate the localization of EBV genomes to specific chromosomes. They fused mouse macrophages that normally lack the ability for continuous multiplication, with an SV40-transformed human cell line. The latter introduced the ability for continuous multiplication into the hybrid. Apparently, unlimited proliferation of the hybrid was dependent on the presence of the SV40-carrying human chromosome. Similarly

to other murine/human hybrids, this hybrid also lost most human chromosomes, but one specific chromosome (C-7) was maintained regularly, together with SV40 DNA and the T antigen.

If mouse-macrophage/human EBV-positive lymphoma cell hybrids could be produced, the EBV DNA-carrying chromosome might be selectively preserved, in a similar way. If so, this will open the way for the comparison of different EBV-carrying *in vitro* and *in vivo* lines, with regard to the exact localization of the viral genome.

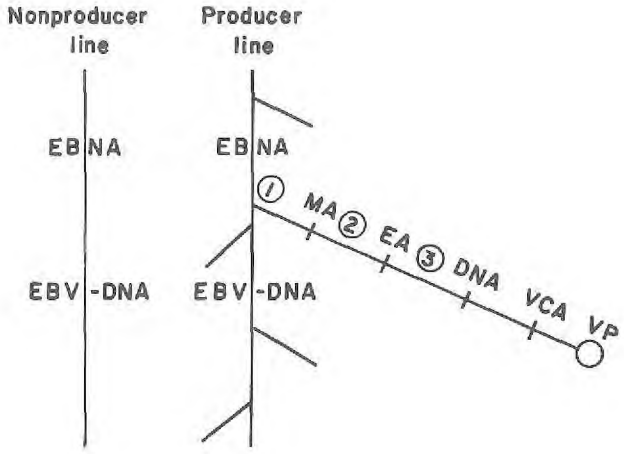
How can the idea that EBV is associated only with a limited number of human chromosomes be reconciled with the earlier, although perhaps somewhat more indirect conclusions of Dr zur Hausen (based on *in situ* hybridization) and of Drs Nonoyama & Pagano (based on a comparison between EBV DNA/cell DNA ratios in whole cells compared to isolated chromatin), suggesting that the EBV genomes of the non-producer Raji line had multiple chromosomal localization? Is it possible that these studies mainly reflected the localization of the free genomes, chromosome-associated but in a loose, non-covalent form, rather than the localization of the integrated genome? Could the free genomes arise by some amplification mechanism from the integrated copy?

Coming now to the expression of the viral genome, Dr Roizman spoke about the cascade of the HSV-determined proteins, arising in an apparently regular sequence. During the lytic infection, one wave of protein triggers off the next and the cascade unfolds without opposition. The EBV system is quite different. I rather liked Dr Hampar's reference to the EBV cycle as proceeding through a series of bottlenecks. We may symbolize the viral cycle roughly as shown in Fig. 1.

At least three distinct blocks can be clearly recognized (designated 1-3 on Fig. 1). Block 1 prevents all cells in non-producer lines and the vast majority of the cells in producer lines, from entering the viral cycle. In cells where the viral cycle has become activated, for unknown reasons, it proceeds to MA. The majority of the MA-positive cells continue to live and divide and only some proceed further to EA (block 2). The EA-positive cells are irreversibly damaged; they may or may not go further; this is the third block. As an example, Hampar and also Glaser and Nonoyama have found that the iododeoxyuridine (IUDR)-induced cycle of, e.g., Raji cells, stops at EA, as a rule, and does not proceed to viral DNA and VCA synthesis. In other cells, e.g., Daudi, induced cells regularly proceed to viral DNA and VCA. Glaser & Nonoyama (1974) have shown that hybridization of Raji with HeLa cells "widens" the pre-VCA bottleneck; IUDR-induced hybrid cells can proceed to viral DNA and VCA without difficulty. There could be a fourth bottleneck, between VCA and viral particles, but this is not entirely clear.

It is very important to understand the nature of this bottleneck regulation. What transcripts are made, at the different stages, what is translated, what polymerases are active, what limiting or repressing factors are in operation? Studies on the IUDR induction and P3HR-1 virus superinfection patterns in somatic-cell hybrids derived from the fusion of two Burkitt lines differing in the "tightness" of block 2 in the scheme above indicated that EA production is under some kind of a positive control, since the properties of the more permissive parent are dominant in the hybrid (Nyormoi et al., 1973).

FIG. 1. DIAGRAM OF VIRAL CYCLE



Both Dr Sugden and Dr Kieff reported that EBV-specific transcription is more limited in a non-producer than in a producer line, as one might expect. Dr Sugden also stated that the virus-specific RNA transcribed would be sufficient, if translated, for approximately 10 virally determined proteins. One, EBNA, has been clearly identified. A second one, tentatively designated as "lymphocyte-defined membrane antigen" or LYDMA, is suggested by the recent cell-mediated immunity studies of Svedmyr & Jondal (1975). It is not known whether more products exist and, if they do, whether they are antigenic.

It was stated that there is more extensive EBV transcription in P3HR-1 virus-superinfected Raji cells than in P3HR-1 itself. This suggests that the artificial superinfection system may be much clumsier than the mutually well-adapted virus-cell interaction within established lines and their endogenous genomes. Probably, the virus "at home" is adapted to the controls of its host cell, to ensure optimal proliferation conditions, whereas when "going abroad", the virus finds itself in an environment to which it has not properly adapted.

Dr Strominger showed that isolated nuclei of EBV-carrying lines continued to make DNA *in vitro*, whereas normal or mitogen-activated lymphocytes stopped after a short active period. Derge and Hampar found that the activation of the virus cycle took place during a specific early part of the S phase (S_1). It was particularly interesting that the incorporation of the inducing IUDR into cellular DNA took place during the same part of the S phase. They also showed that the replication of

viral DNA in the non-producer cell happens at the same time. This suggested that viral DNA synthesis is initiated at some point that is involved in the linkage of the viral to the cellular DNA.

In the area of viral products, we have heard about a number of important technical improvements. Radioimmunometric methods were developed for EBNA by Dr Brown and for EA by Dr Ernberg. They will no doubt facilitate fractionation and purification studies. The first rabbit immune serum that identifies one of the EBV-determined specificities (EA-D) that was previously only detected by human serum reagents was developed by Dr Zajac. Monospecific reagents of this type may be helpful in attempting to isolate and purify the antigens.

Dr Henle viewed the cycle from a mirror, as it were, by analysing the time sequence of the various antibodies during primary infection. It really looks like a mirror image, because VCA antibodies appear first, followed by EA, and EBNA antibodies come last, with a delay of several months. The reasons are not understood, but may be related to an early availability of the virus itself to the immune apparatus, as contrasted with the later availability of virally determined intracellular components.

Among the disease-related antibody patterns, the preferential antibody formation against EA-R in BL patients with progressive or residual disease is perhaps most puzzling, particularly when contrasted to the predominance of anti-EA-D in IM and NPC. This suggests a distinctive difference either in the virus-cell or the cell-host relationship, possibly involving quantitative and/or geometric aspects of antigen presentation and localization.

Cell-mediated immunity has not been adequately studied in the EBV area, but it is clear that the field is now opening up. As suggested by the work of Svedmyr & Jondal (1975) already mentioned, lymphocyte sensitization may define EBV-induced antigens in addition to those recognized by immunofluorescence.

I would now like to invite comments by the members of the panel on these points.

Lindahl: I would like to comment on Dr Pope's statement that long-established lines like Raji may have undergone secondary changes during their long proliferation *in vitro* and may therefore give misleading results. We have actually repeated our Raji experiments with a recently established Burkitt-derived line, Akuba. The results were identical with those obtained for Raji.

The various views on the intracellular state of the viral genome seem to have come closer at this Symposium and we all agree that part of the viral genomes are present in an extrachromosomal form. This situation is without precedent among the oncogenic DNA viruses. We also feel, however, that there are some covalently integrated sequences.

The EBV-system has two interesting features: Firstly, there is not just one viral genome per cell, but typically 20-30 copies. Secondly, the extrachromosomal DNA is maintained in the

form of circles, in contrast to the linear DNA molecule of the virus particle. Some good models can probably be found in the phage field. Even lambda, which is usually regarded as the prototype of the integrated prophage, can exist in the extrachromosomal state in some mutants of the virus and can be maintained in that form serially. P1 would be another model; it is a large phage, usually carried in the extrachromosomal form but can be carried in the integrated form as well. The extrachromosomal state is quite stable. Another lesson of the P1 system concerns the control of the repressed state. This is extremely complex, much more so than the lambda system. There are at least three virally coded proteins that control the repressed state, although the details are not yet understood. Conceivably, several of the 10 EBV-coded proteins postulated in Raji could be related to the control of the repressed state. It may be unrealistic to think of only a single controlling element.

Pagano: Concerning the loss of EBV DNA in the somatic-cell hybridization experiment, it is possible that we keep looking at the bulk of the viral genomes. After the bulk has disappeared, there may be still some portion of the genome left, undetectable by present technology. However, perhaps too much of the emphasis is put on the integrated viral DNA. The free viral DNA is *the* unique feature of the EBV system and could well turn out to be more important. Clarification of the state of the persistent free EBV genomes may also throw light on the nature of herpesvirus latency in general.

Concerning the question of genomes in IM, we reported last summer the existence of highly cytotoxic circulating cells in IM patients that could kill EBV genome-carrying B cell lines with great efficiency.

zur Hausen: If the viral DNA is not integrated with the host-cell DNA, it would be difficult to understand how it is maintained in the same cell line over many years at the same genome-equivalent levels. If the viral genome is covalently integrated, however, it is difficult to understand the high reactivation rate in the producer lines. Obviously, additional data are needed before we can see the picture clearly.

Vonka: I would like to make two comments. The first concerns the complement-fixing soluble (S) antigen of EBV, a substance apparently identical with Klein's nuclear antigen (EBNA) detectable by the immunofluorescence test. Because of the consistent association of this antigen with EBV genome-carrying cell lines, it is reasonable to assume that it plays some role, perhaps the

key one, in the transformation process and/or in the maintainance of the transformation state. This assumption is further substantiated by the demonstration of the association of EBNA with the chromosomes. It would now seem to be of the greatest importance to investigate the nature of the S (EBNA) antigen. For this purpose, biochemical, biophysical and immunological methods should be employed. In the recent past, it has been shown that other EBV-induced antigens are complex in character. It is possible that the so-called S antigen is also a complex of several antigenic substances. This question should be elucidated first. For such studies, S antigens from different lines and S antibody-positive sera from healthy subjects and from persons suffering from various diseases associated with EBV, should be used.

The other point I would like to stress is the importance of further investigation of the biological properties of different EBV strains, with the aim of recognizing different EBV subtypes, if they exist. At this meeting, we heard excellent papers by Dr Miller and Dr Hinuma, who demonstrated beyond doubt that, at least *in vitro*, different EBV isolates behave differently. Drs Hinuma & Katsuki showed differences among cell lines obtained after infection with two different virus strains. It might be useful to use more cell markers in future studies, such as, e.g., susceptibility to various viruses, ability for growth at increased or decreased temperatures, production of interferon, etc. These studies may not only be helpful in discriminating between EBV strains, but may also help to determine which of the cell properties are controlled by the cell genome and which by the virus genome, and may increase our understanding of the regulation processes in the cell. In my opinion, genetic studies on EBV deserve a high priority in future research on this agent.

Henle: You have alluded to differences in anti-EA responses in different disease conditions. We have no real clues except that, in diseases with extensive lymph-node involvement, such as IM and NPC, there is a predominant anti-diffuse (D) response. In chronic lymphatic leukaemia, if there is an anti-EA response, it likewise is anti-D, as a rule. We have no real explanation for the high anti-R titres in BL. In the rare healthy individuals who have low anti-R titres, the anti-VCA titres are invariably high. Perhaps a high anti-R level denotes an unusually extensive viral carrier state.

We shall now proceed to the oncogenic animal herpesviruses. In the field of Marek's disease (MD), there has been remarkable progress since the last meeting. This field was notorious for the lack of established cell lines, to the detriment of many potentially interesting studies.

Now, Dr Kato has succeeded in establishing three permanent cell lines. In the discussion after his paper, he was asked how he had been able to succeed where everybody else had failed. He said that incubation at 41°C, the body temperature of the chicken, was one important difference. I am not sure whether this is the whole explanation but it is clear that the lines have opened many new areas for study.

At the Cambridge Symposium, there was still some discussion about the question whether MD is due to the intrinsic proliferation of neoplastic cells or to an "extrinsic" reaction of normal lymphoid cells to a viral infection in other cells, e.g., the virus-producing feather follicles in the skin lesions. The existence of viral genome-carrying, immortalized and transplantable lines now argues definitely for the existence of a neoplastic Marek cell with intrinsic proliferating capacity.

Dr Nazerian has made the interesting point that multiple tumours tend to carry the same number of genome equivalents in the same chicken. Tumours in different chicks contain very different genome numbers. This is analogous to Dr zur Hausen's and more recently also Dr Lindahl's findings on the numbers of EBV genomes in multiple anatomically distant Burkitt tumours. In view of the proven monoclonality of BL (Fialkow et al., 1970, 1973), this simply means that each monoclonal *in vivo* tumour has a characteristic genome number, like the non-producer *in vitro* lines. In MD, there is no information about the mono- or polyclonal nature of the proliferative lesion, but Dr Nazerian's finding is at least consistent with a monoclonal lesion. This would, in turn, strengthen the argument for an intrinsic neoplastic proliferation.

While the MD-derived lines do not produce virus spontaneously, the cycle can be induced by IUDR, as Dr Nazerian has shown. This opens the way to distinguishing between early and late antigens. This could be very rewarding for comparative studies, in relation to the human EBV system. In the EBV-associated diseases, antibody formation against EA occurs mainly in connection with ongoing proliferation of EBV-carrying neoplastic cells, BL or NPC, or during acute IM. It is relatively rare in other lymphoproliferative or neoplastic diseases or in healthy seropositives with antibodies to the late (VCA) antigen. A similar phenomenon occurs in the HVS system, where marmoset and owl monkeys with malignant lymphoproliferative disease develop antibodies to both early and late viral antigens whereas disease-resistant, healthy squirrel monkeys have antibodies to the late but not the early antigen. The MD system offers a much wider range of experimental variation, including genetically resistant and susceptible birds, the latter untreated or successfully vaccinated with an apathogenic virus strain. In addition to a study of antibody formation against early and late antigens in hosts differing in their disease-resistance status, it would be important to learn more about cell-mediated immunity in the MD system. As pointed out in the thoughtful paper by Dr Biggs, the mechanism of vaccine protection is quite unknown. The established MD-derived lines may be helpful in setting up an appropriate target-cell system for the assessment of host immune status.

Since the Cambridge Symposium it has become clear that MD is mainly a T-cell malignancy. The T-cell characteristics of the Kato lines confirm this further. Does this mean that there is T-cell surveillance against T cells, a sort of civil war? Alternatively, is this a non-T-cell

surveillance system? Dr Biggs mentioned that there is some evidence for both T and non-T cell-mediated reactions, but more work is obviously needed.

Dr Purchase has shown that cyclophosphamide prevents the protective effect of vaccination. This is in line with the idea that vaccination acts through an immune mechanism, since an interferon-dependent mechanism would not be affected in the same way.

It is peculiar that viraemia is a prerequisite for vaccine protection. On the other hand, protection is not directed against infection with the virulent MDV, but against the growth of the neoplastic cells. This is reminiscent of SV40 or polyoma, where one virus inoculation into susceptible animals leads to a certain frequency of tumours whereas a second inoculation of the virus prevents the majority of the tumours. It is as if the immune system had to be alerted antigenically, towards the end of the latency period. Without this alert, the tumours manage to "sneak through". It is interesting to compare these systems with one another. SV40 is *not* a natural oncogenic agent of the hamster. The first, oncogenic virus dose is given to the immunologically immature animal. Without further interference, tumour development apparently proceeds faster in this animal than the development of the immune response. A second dose of *the same* virus is sufficient to shift the balance in favour of the host and to achieve effective tumour prevention. MDV is naturally oncogenic for a considerable proportion of the domestic chick population. In this situation, it is not possible to achieve tumour prevention with a second dose of the same virus. The apathogenic and only partially cross-reactive turkey virus, HVT, can do it, however. The mechanism may be similar to the "immunological reinforcement" that is achieved by a second virus dose in the SV40-polyoma system, i.e., probably an alerting of the immune mechanism, to some antigen or antigens that appear on the virus-transformed, potentially neoplastic cells. It is likely that at least some of the genetic variation in MD susceptibility is mediated by Ir genes. It may be recalled that resistance or susceptibility can be a matter of a single, dominant gene in some strain combinations. It would be important to know which component of the immune system is influenced, how many relevant genes there are, and where they are localized on the genetic map. If the reasoning is correct that multiple-level surveillance results from a long process of evolutionary selection, is it conceivable, furthermore, that MD is of more recent origin than, say, EBV infection in man or polyoma in mice? Have the chicks not yet been selected for an equally efficient surveillance? Alternatively, has the virus not been sufficiently widespread on the global scale to exert sufficient selective pressure?

In the simian herpesvirus field, we heard from Dr Fleckenstein about the heavy and light forms of HSV DNA and the fact that the heavy part was defective. Early and late antigens were defined further by Dr Ablashi and his group. We have also heard that HVS induces a massive polyclonal transformation of the lymphoid system. Here we obviously have an unprotected animal that has not been selected for responsiveness by virus exposure during its evolution. This is very different from BL, where a single clone is creeping through a relatively strong resistance barrier. Nevertheless, HVS-infected susceptible animals can make antibodies, and Dr Laufs has shown that it is possible to protect them against the lymphoma by vaccination.

Dr Falk told us about the curious

T-cell tropism of HVA. Remarkably, both known oncogenic monkey herpesviruses are T-tropic. While this is interesting in itself, it also means that these diseases can hardly be regarded as good models of B-cell neoplasia in man. As Dr Miller said, the induced monkey tumours may represent a better model. T-tropic oncogenic viruses have an intrinsic interest because they provide a model for the study of surveillance mechanisms against T-cell neoplasia in man. I will now ask Dr Biggs and Dr Deinhardt to comment on these points.

Biggs: I have only two points to add. With respect to the MD tumour cell lines, it is interesting to note that, although temperature plays an important part in success in establishing such lines, the proportion of successful to unsuccessful attempts is very low even at optimal temperature. In this way MD tumour cells still apparently differ from those of BL.

Dr Klein pointed out that MD offers a wide range of experimental variation, and he gave as an example the availability of genetically resistant and susceptible birds, both of which can be studied unvaccinated and vaccinated with apathogenic viruses. In addition pathogenic (oncogenic) and naturally occurring apathogenic viruses, as well as attenuated pathogenic viruses, are readily available. This provides a unique opportunity for studying the mechanism of oncogenicity of herpesviruses and the mode of function of successful immunization against them by comparing the behaviour of oncogenic MD viruses with that of their apathogenic and attenuated variants.

Deinhardt: I would like to emphasize that it is now possible to transform T cells at will *in vitro* with HVA and that such transformed cells can be grown as long-term cell lines. This will permit an experimental approach in an autologous system to the important question of how one T cell recognizes its brother T cell that has been changed by a virus, and we may be able to isolate and transform selectively T cells that are sensitized to specific antigens.

The other interesting point is that we now have in the HVS system an attenuated strain of HVS that is no longer oncogenic, a situation that is similar to that of the herpesvirus of turkeys in chickens. This will permit experimentation to determine why one virus variant is oncogenic and the other is not.

I would like to proceed now to transformation by the lytic herpesviruses, and by HVS in particular, and related questions. As Dr Epstein pointed out, there is convincing epidemiological evidence of a relationship between cervical carcinoma and what he, with Victorian euphemism,

called the "life style" of the patient. Perhaps we should call things by their proper name. Papers given by various epidemiologists at the recent Florida conference on the epidemiology of cervical carcinoma emphasized that some relationships are based on solid facts, whereas others are essentially legends and myths. There was a clear relationship between the risk of cervical carcinoma and the age of the first intercourse, and also the number of sexual partners. There was no significant correlation with the number of pregnancies or the circumcision status of the male partner. An important piece of information concerned the contraceptive device: obstructive contraception was clearly of preventive value. The conclusion was drawn that a carcinogenic agent is transmitted from the male to the female during adolescent intercourse that contributes to the risk of cervical carcinoma. Whether the agent is HSV-2 or something else, is not clear. In one particularly interesting epidemiological study, performed by the Melnik group, an effort was made to match the sexual history of the cervical carcinoma cases and the controls and compare their antibody titres against HSV-2. Even after such matching had been performed, there remained a difference between cancer cases and controls, with a higher incidence of antibody positivity and earlier onset of positive antibody titres in the carcinoma patients as compared to the control group. While this is very interesting, one always wonders whether some subtle bias may not creep into such studies. This may concern, in this particular case, the accuracy of the matching, e.g., the possibility that the degree of frankness with which sexual histories are revealed may be different in carcinoma patients and in controls.

At the Florida meeting, we also learned that there is a whole flora of microbial agents that are venereally transmitted and that HSV-2 is neither the only nor the predominant one. In view of these uncertainties of epidemiology, some more solid evidence is obviously required, from the molecular biologists or the immunologists, that can demonstrate the vestiges of the viral genome in the carcinoma cells themselves. Here, we are still seeing a very variegated landscape, to say the least. On the other hand, there is increasing evidence since the last Symposium in the *in vitro* model systems, as Dr Rapp has pointed out, that a fragment of the HSV genome is really present in the transformed cells. It is not as easy to detect it as in the lymphotropic herpesvirus systems where whole, multiple genomes are present, as a rule.

Dr Frankel pointed out that the RNA products of the viral genome are more easily demonstrated than viral information at the DNA level, and she reported some suggestive evidence.

I assume that Dr zur Hausen's statement about meeting continuous frustration, rather than continuous genome persistence in this area was made in relation to the *in vivo*, carcinoma-related studies, rather than the *in vitro* models. The original work of Rapp and his group, showing transformation by UV-inactivated HSV has now been amply confirmed and also extended to temperature-sensitive mutants. We have heard interesting presentations by Dr Boyd and Dr Macnab about this. Dr Macnab induced tumours, transplantable in syngeneic rats, with temperature-sensitive HSV mutants. It was particularly interesting that both DNA+ and DNA- mutants could achieve this effect. Does this imply that viral DNA synthesis is not necessarily deleterious for the transformed cell?

In a way, this would be reminiscent of the EBV system, where a certain degree of viral DNA synthesis can occur, before or in the absence of host-cell killing, apparently leading to amplification of the viral genome number. The viral function that shuts off the macromolecular synthesis of the host cell is obviously the crucial point and cellular controls over this function are presumably more important than control over viral DNA synthesis *per se*.

Dr Duff told us about a more quantitative and more reproducible transformation system; this is certainly badly needed in this field. Another problem concerns the antigenic products of the genomes carried by the transformed cells. It would be very gratifying, for the confused outsider, if the workers in this area could agree on the existence and nomenclature of the virally determined antigen products detected in the transformed cells by different laboratories. You will recall that the development of the EBV field was very much furthered when a number of laboratories agreed that they detected the same antigens and, equally important, arrived at a common nomenclature. It is an axiom of every developing area that an antigen found in two laboratories is not twice as valuable as an antigen found in only one, but some kind of an exponential function. Once several laboratories have agreed about the identity of a reaction, cells and reagents will be provided to others, reactions will be reproduced everywhere, and confidence in the field will grow. I cannot see that this is happening yet in the field of HSV-induced transformation. There are, nevertheless, some encouraging developments; the demonstration by the Rapp group of HSV- and CMV-specific, cell-mediated cytotoxicity is one rather elegant example.

On the human side, we had Dr Nahmias and Dr Aurelian, with their interesting, if preliminary results. Dr Nahmias' finding of a nuclear antigen in cervical carcinoma, possibly of HSV-specificity, and Dr Aurelian's demonstration of tumour-associated antigen, with its IgM antibody, is encouraging, but one would like to see much more of this and, hopefully, confirmation by several laboratories.

Rapp: I agree as to the need for antigenic product identification. On the other hand, one must remember that EBV was discovered in 1964, and yet the first transformation system was described only a few years ago. Moreover, in HSV, there is a tremendous variation in virus subtypes and products, as Dr Roizman has pointed out. While it was clear in the HSV-transformation field from the beginning that different viral types differ in their transforming activities, this type of evidence has only recently emerged for EBV. It would be entirely conceivable that patients who develop cervical carcinoma carry one particular variant of HSV-2, e.g., a transforming variant, whereas HSV-infected persons who do not develop carcinoma carry a non-transforming variant. If and when we develop immunological reagents directed against the transforming viruses, we may get quite a different picture from what we have now.

Many different transformed-cell models have been developed. In the early models, the total viral genome was probably not present,

whereas in some of the more recent models, it may well be, since viral transformation occurs in non-permissive cells. Of course, the models that lack a total genome and contain only a fragment may be better models for what may happen in human cells than more artificial models, even if the latter contain a whole viral genome.

I would also like to comment on the question of immunization. Immunization is very clearly a two-edged sword. At least in the hamster HSV model system, immunization may just as often lead to tumour enhancement and increased metastasis as the opposite. Before immunization attempts are seriously considered in relation to human cancer, much more will have to be known about the immunogenicity of the antigens used, the types of antibodies found and, most importantly, the role of the viral agent in the pathogenesis of the disease. As a final statement, I would like to say that in this case, as in others, the molecular biology is moving very fast, and far exceeds our understanding of the biological system.

Klein: The discussion is now open.

Roizman: I would like to make two comments on the content of the round-table discussion. The first comment concerns EBV itself: I have the suspicion, reinforced as time goes on, that the EBV we have in our laboratories is not the epidemiologically significant virus responsible for IM and for the widespread seroconversion of individuals throughout the world. I suspect that the virus we have in the laboratory is largely defective progeny of the epidemiologically significant virus and that, although it is important that we continue to define its properties, we must not lose sight of the fact that it is not the virus that is transmitted in the human population from one individual to the next. I have two reasons for entertaining this suspicion. The first is the great diversity of EBV gene expression in lymphoblastoid cells. The impression one acquires rather forcefully is that no two lymphoblastoid lines carrying virus express viral genetic information in exactly the same fashion. Let us take for example the fact that very few cell lines make infectious particles in significant amounts and that the two lines that make appreciable amounts of virus - the B95 and HR-1 - differ considerably in the biological properties and genetic complexities of the viruses that they produce. As has been demonstrated at this Symposium, B95 contains less viral genetic information than HR-1 even though it both transforms and replicates whereas HR-1 only replicates and does not transform cells. I should point out that a putative variability in the lymphoblastoid cell function and regulation could explain the observed variability of the viral gene expression in lymphoblastoid cell lines. However, this

hypothesis does not explain the differences between the viruses produced by B95 and HR-1 cell lines. One alternative hypothesis, namely that lymphoblastoid cell lines retain mutants and defective progeny differing in ability to shut off host gene expression and to replicate, could explain most if not all our findings and I shall return to this in a moment. My second reason for suspecting that none of the EBV produced in cell culture is the epidemiologically significant virus arises from the fact that EBV antibody is nearly universally present in adult human population - an indication that the virus is transmitted very efficiently indeed. Yet the EBV produced in culture does not have attributes commonly associated with highly infectious, transmissible agents. Thus it replicates poorly, or not at all. I was struck by Dr Klein's statement at an earlier discussion that some of his associates have remained seronegative even after more than 10 years of exposure to EBV in the laboratory. This hardly conjures up the image of a highly infectious agent. The hypothesis that I think should be thoughtfully considered as possibly explaining the two paradoxes I have just cited is that: (i) in nature the virus multiplies efficiently in the nasopharynx and the viral progeny made in the nasopharyngeal cells are highly infectious; (ii) the epidemiologically significant virus arising from the nasopharynx replicates poorly in cells of lymphoid origin, but does kill them. The only cells that survive are those infected with mutated, "defective" progeny of epidemiologically significant virus; (iii) we can visualize that the virus progeny that remains associated with surviving cells express only functions that are required to cause the cells to replicate but do not kill them, analogous to alpha functions in HSV-1 infected cells, and that the transition from early (alpha) to late (beta or gamma) viral gene expression is permanently or conditionally blocked. A permanent block would lead to non-inducible lines, whereas a conditional block could be overcome by changes in the physiological state of the cell and would lead to the production of inducible lines. Conditional or permanent blocks could result from rearrangements of the DNA sequences, mutations in some genes or deletions.

It should be also pointed out that, if lymphoblastoid cells do not replicate in culture without a viral function, and if the cells are intrinsically restrictive or non-permissive to this virus, the cell that will eventually be selected out following exposure of blood lymphocytes to infectious nasopharyngeal secretions will be precisely the cell infected with a virus that is permanently or conditionally precluded from irreversibly damaging the host. The point I wish to make is that, if this hypothesis is correct, and no data have been presented to show that it is not, the EBV produced in lymphoblastoid cell lines has no single biological identity and may be somewhat remote phenotypically from the epidemiologically significant virus prevalent in nature.

The second comment I wish to make concerns the significance of the finding of BL apparently free of EBV DNA. The hypothesis that this finding enhances the probability that EBV is a causative agent of BL is ingenious but non-exclusive. Among the many possibilities, at least two others should be considered. The first of these is that only a small fraction of the viral genome is present in the tumour cells. Although this hypothesis is inconsistent with the results of analyses of all other tumours that appear to contain numerous copies of intact or nearly intact viral genomes, it cannot be rejected *a priori*. At the same time we must not lose sight of yet another possibility, namely that EBV populates the tumours after they are formed. It is conceivable for example that the persistence of EBV and its ability to transfer from one cell to another is dependent on the age of primary infection. If this were the case, we could expect that lymphoblastoid tumours would become infected at a high frequency in areas in which EBV is endemic early in life and less frequently in areas in which EBV is endemic largely at adolescence. The point I wish to make is that if EBV is a passenger, we might expect that the frequency of its presence would depend on the incidence of the virus in the population as well as on some biological property that could account for its presence at high frequency in tumours in some populations and not in others. The key may well be the ability of the virus to persist and remain transmissible in human cells and particularly in lymphoid cells. I am wondering, therefore, if it would be worthwhile to determine the relationship between the age of primary infection and the ability of the virus to persist. Operationally this would entail attempts to establish continuous lines of lymphoblastoid cells following primary infection of patients of different ages.

The first Symposium in Cambridge was called Herpesviruses and Oncogenesis.

This is the second Symposium and is called Oncogenesis and Herpesviruses. Will the third meeting be called Oncogenesis by Herpesviruses?

Let us hope. Our thanks are due to Drs Epstein, zur Hausen and de-Thé, for having organized this extremely successful Symposium, no doubt a milestone on this road.

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