

3. Studies of Cancer in Animals

3.1 EBV in non-human species

Considerable efforts have been made to develop suitable animal models to study EBV infection and pathogenesis; however, such attempts have had little success, because Old World primates carry their own EBV-like virus and cross-reacting antibody (Kieff, 1996). New World primates do not carry EBV homologues, however, and at least five species appear to be infectable by EBV, i.e. the cotton-topped tamarin (*Saguinus oedipus oedipus*), white-lipped marmoset (*Saguinus fuscicollis*) and common marmoset (*Callithrix jacchus*), owl monkey (*Aotus trivirgatus*) and squirrel monkey (*Saimiri sciureus*). The most susceptible non-human primate for the carcinogenicity of EBV is the cotton-topped tamarin. This is, however, an endangered species and not widely available for use in laboratories. When common marmosets are infected with EBV, only some animals develop an infectious mononucleosis-like syndrome (Wedderburn *et al.*, 1984).

3.1.1 Infection of non-human primates with EBV

3.1.1.1 New World primates

The special susceptibility of cotton-topped tamarins to EBV is not well understood. The response to EBV infection varies from occult infection to benign lymphoproliferation to malignant lymphoma (Shope *et al.*, 1973; Deinhardt *et al.*, 1975; Werner *et al.*, 1975; Miller *et al.*, 1977; Rabin *et al.*, 1977; Johnson *et al.*, 1983; Cleary *et al.*, 1985;

Niedobitek *et al.*, 1994). The lymphomas induced in cotton-topped tamarins are morphologically similar to EBV-associated lymphomas in humans (Rabin *et al.*, 1977; Cleary *et al.*, 1985) and are frequently observed in the mesenteric nodes and small intestine. Each tumour nodule in an animal has a dominant clonotype, but many different clones are found in different locations.

Shope *et al.* (1973) induced malignant lymphomas in four of eight cotton-topped tamarins inoculated with cell-free EBV extracted from a lymphoblastoid cell line (B95-8) originally transformed *in vitro*. The first tumour developed after 222 days.

Epstein *et al.* (1973) reported that one of three owl monkeys inoculated with EB3 cells from a Burkitt's lymphoma (frozen and thawed) developed lymphoproliferative disease without evidence of true lymphoma. Antibodies to VCA were detected in the serum obtained from this animal.

Leibold *et al.* (1976) inoculated EBV-transformed squirrel monkey lymphocytes ($6-12 \times 10^8$ cells) by intraperitoneal or subcutaneous injection into three squirrel monkeys. All three animals developed multiple undifferentiated malignant lymphomas and died 8-10 days after inoculation. Necropsy specimens showed the presence of EBV DNA and contained 7-21 genomes per cell. Lymphoblastoid cell lines were established from the tumour tissue and were found to express EBNA and EA.

Werner *et al.* (1975) showed that inoculation of concentrated EBV from a transforming (Kaplan) mononucleosis cell line resulted in malignant lymphoproliferation in one of three cotton-topped tamarins six weeks after inoculation, whereas inoculation of non-transforming EBV (P3HR-1) did not result in lymphoproliferation. The tumour induced by the Kaplan cell line was a lymphosarcoma and contained EBV DNA. The tumour-bearing animals produced anti-VCA antibodies.

Sundar *et al.* (1981) reported that one of six white-lipped marmosets inoculated with a transforming EBV (B95-8) developed diffuse malignant lymphoma within 18 weeks. EBV-DNA was detected in the pathologically enlarged mandibular lymph nodes, and the animal had an anti-VCA antibody titre of 1:320.

In a study in which eight cotton-topped tamarins were inoculated with sufficient quantities of transforming EBV (B95-8), multiple tumours were induced in each animal within 14-21 days which were identified histologically as large-cell lymphomas. The tumour cells contained multiple copies of the EBV genome. Cell lines derived from the tumours showed no chromosomal abnormalities. Hybridization of tumour DNA with immunoglobulin gene probes revealed that the tumours were oligo- or monoclonal in origin; in each animal, individual tumours arose from different B-cell clones (Cleary *et al.*, 1985).

Horizontal transmission of EBV in common marmosets was demonstrated when two animals experimentally infected with EBV (from M81-transformed cells) were paired with two animals seronegative to EBV. The seronegative animals seroconverted after four to six weeks, and the presence of EBV DNA was demonstrated in their peripheral blood lymphocytes (Cox *et al.*, 1996).

3.1.1.2 *Old World primates*

Levine *et al.* (1980a) inoculated 42 rhesus monkeys (*Macaca mulatta*), four chimpanzees (*Pan troglodytes*) and one cynomolgus monkey (*Macaca fascicularis*) with non-transforming EBV (P3HR-1). None of the animals developed any clinical manifestation or tumour within three to eight years. [The Working Group noted that the lack of response in this study could be due either to the use of a non-transforming EBV strain or the presence in these primates of their own EBV-like herpesvirus (see section 3.2).]

3.1.2 *Transformation of monkey cells by EBV in vitro*

Desgranges *et al.* (1976) transformed peripheral blood mononuclear cells of common marmosets with concentrated EBV (HKLY-28 virus) obtained from a nasopharyngeal carcinoma lymphoblastoid cell line. The monkey cells produced more EBV than the original human cell line, and the transforming activity of the EBV obtained from monkey cells was similar to that of EBV from B95-8 cell lines.

Ablashi *et al.* (1977) showed that squirrel monkey peripheral blood mononuclear cells infected with transforming EBV (B95-8) were immortalized and that these cells contained EBV DNA.

Griffin and Karran (1984) showed that specific EBV DNA fragments could immortalize a subpopulation of epithelial cells of the African green monkey (*Cercopithecus aethiops*). EBV-related sequences were observed in all the established cell lines.

3.1.3 *Rodent models for EBV infection and pathogenesis*

3.1.3.1 *Severe combined immunodeficiency (SCID) mouse model*

The SCID mouse model has been widely used to study lymphomas induced by EBV *in vivo*, EBV protein expression and specific therapies against EBV-induced lymphoproliferation (e.g. anti-CD40 and anti-CD23 monoclonal antibodies) (Garnier *et al.*, 1993; Funakoshi *et al.*, 1995; Katano *et al.*, 1996). SCID mice lack mature B and T cells but can be immunologically reconstituted to a certain extent by xenografts of human leukocytes. If the lymphocytes are derived from human EBV-seropositive donors, the animals frequently develop multiple foci of proliferating EBV-positive human B cells within 10–16 weeks. The SCID mouse lesions are composed of immunoblasts and plasmacytoid cells.

In SCID mice grafted with EBV-positive human peripheral blood mononuclear cells, Okano *et al.* (1990) detected EBV-positive lymphoproliferative lesions that expressed LMP and B-lymphocyte activation antigen (CD23) and adhesion molecules (ICAM-1 and CD18). The authors suggested that these lymphoid lesions in SCID mice were comparable to EBV-immortalized lymphoblastoid cells and not to malignant lymphomas like Burkitt's lymphoma.

SCID mice were grafted with peripheral blood lymphocytes from immunodeficient patients with X-linked lymphoproliferative syndrome. Mice receiving lymphocytes from EBV-seronegative patients were then infected with B95-8 EBV. The mice developed EBV-induced oligoclonal or polyclonal disease comparable to that occurring in both

immunodeficient patients and normal immunocompetent donors. The lesions were diffuse, monomorphic and human B-cell-related, and activated associated antigens were present. Analysis of the sera revealed that the B-cell populations were oligoclonal or polyclonal; however, phenotypic and cytogenetic analysis did not reveal the monoclonality generally observed in Burkitt's lymphoma (Nakamine *et al.*, 1991; Purtilo *et al.*, 1991).

Walter *et al.* (1992) compared the local growth of a Burkitt's lymphoma with the disseminated, invasive growth of EBV-immortalized lymphoblastoid cells in SCID mice. The lymphoma grew progressively but was locally restricted, whereas the lymphoblastoid cells were both locally invasive and disseminated into lymphoid tissue.

Katano *et al.* (1996) studied the lymphoid tumours induced in SCID mice after grafting of human EBV-infected B cells. Molecular analysis of the tumours revealed expression of EBV LMP-1 molecules at quantitatively different levels in the individual tumours.

[The Working Group noted that the SCID mouse model is limited to the study of lymphoproliferative disorders.]

3.1.3.2 *Nude mouse model*

Ablashi *et al.* (1978) inoculated EBV-positive P3HR1 cells (1.0×10^6 cells in 0.2 mL medium) into 30 athymic nude mice. All animals developed lymphoid tumours after an average latent period of 36 days. The tumours were poorly differentiated lymphomas of varied cell size and shape. The nuclei were variably hyperchromatic and were reminiscent of those of Burkitt's lymphoma. EBNA was detected in 80% of these cells and in 90% of the cells used for the inoculum, but VCA was detected in only 2% of the cell lines obtained from the tumour and in 13% of cells in the inoculum.

Yang *et al.* (1982) inoculated cells from 36 biopsy samples of nasopharyngeal carcinoma into BALB/c nude mice. In three mice, the inoculum grew to detectable tumours with metastases within 20–43 days. The tumours were then passaged 6–15 times over a period of 12–22 months into other groups of nude mice, with an average take of 67–73%. Touch smears from the tumours showed the presence of EBNA. Only two of four of the cell cultures derived from tumours expressed VCA after induction with 5-iodo-2'-deoxyuridine. Co-cultivation of epithelial cells with human cord blood mononuclear cells provoked transformation of the latter, and the transformed cells produced IgM.

3.2 EBV-like viruses isolated from non-human primates

The *Gammaherpesvirinae* subfamily of Herpesviridae is subdivided into two genera: *Lymphocryptovirus* (e.g. HHV4 or EBV) and *Rhadinovirus* (e.g. ateline herpesvirus-2) (Roizman *et al.*, 1992). For further details of the classification of herpesviruses that infect animals, see the Introduction.

Lymphocryptoviruses (so-called gamma-1) include EBV-like viruses isolated from Old World primates such as chimpanzees (*Pan troglodytes*), baboons (*Papio hamadryas*) and orangutans (*Pongo pygmaeus*). The gamma-1 lymphocryptoviruses are B lympho-

tropic, transforming, generally restricted in their host range and similar to each other in genomic organization (Kalter, 1994). Their genomes are collinearly homologous to that of EBV and are more closely related to that of the saimiri herpesvirus-1, a member of the *Rhadinovirus* genus, than to genomes of the beta- or alphaherpesviruses.

Non-human primate rhadinoviruses (gamma-2 herpesviruses) are discussed in the monograph on KSHV/HHV8.

Lymphocryptoviruses cross-react antigenically with EBV and can transform or immortalize cells. These viruses are transmitted horizontally and infect their host at the juvenile stage, inducing the production of specific antibodies (Deinhardt & Deinhardt, 1979). The first isolation of a lymphocryptovirus in non-human primates was reported by Landon *et al.* (1968), who established a permanent lymphoblastoid cell line from a healthy chimpanzee in which they detected a herpesvirus. A similar virus was reported in chimpanzees (Gerber *et al.*, 1976b), which was B-lymphotropic and transformed human and other primate cells. Since the first isolation of *Herpesvirus pan* (pongine herpesvirus 1) from chimpanzees (Gerber *et al.*, 1977), the following primate lymphocryptoviruses have been reported: *Herpesvirus gorilla* (pongine herpesvirus 3; Neubauer *et al.*, 1979a), *Herpesvirus pongo* (pongine herpesvirus 2) from orangutans (Rasheed *et al.*, 1997), *Herpesvirus papio* (cercopithecine herpesvirus 12) from baboons (Falk *et al.*, 1976), *Herpesvirus cercopithecus* (cercopithecine herpesvirus 14) from African green monkeys (Böcker *et al.*, 1980) and herpesviruses from rhesus monkeys (*Macaca mulata*; Rangan *et al.*, 1986), cynomolgus monkeys (*M. fascicularis*; Feichtinger *et al.*, 1992; Li *et al.*, 1994) and *M. arctoides* (Lapin *et al.*, 1985).

The double-stranded DNA of these viruses has a molecular mass of about 110×10^6 , and the genome of cercopithecine herpesvirus 12 (baboons) and pongine herpesvirus 1 (chimpanzees) is about 170 kb. The virion has a diameter of 118–220 nm. The viral morphogenesis is similar to that of EBV. The natural host range is limited to the family or order of its host. DNA sequence homology among primate gamma-1 herpesviruses is approximately 40%. The DNAs of cercopithecine herpesvirus 12 and pongine herpesvirus 1 show extensive collinear homology (Lee *et al.*, 1980, 1981; Kieff, 1996).

3.2.1 *Herpesvirus papio* (cercopithecine herpesvirus 12)

Agrba *et al.* (1975) reported the establishment of two lymphoblastoid cell lines (SPG-1, KMPG-1) from bone marrow and spleen of hamadryas baboons with malignant lymphomas in the Sukhumi colony. Herpesvirus particles were detected in these cell lines by electron microscopy. Upon further investigation, other herpesvirus types (HSV-1, HSV-2, cytomegalovirus) were ruled out. The virus was later named *Herpesvirus papio* (HVP). Increased antibody titres to this virus were demonstrated in baboons which developed lymphoma (Voevodin *et al.*, 1983).

A similar virus was isolated in the United States from several baboon colonies (*Papio hamadryas*, *P. ambis*, *P. cynocephalus*) by Falk *et al.* (1976). Antibodies that reacted with viral antigens in both HVP and EBV cell lines (P3HR1, B95-8) were demonstrated. All of the established cell lines were B cell-derived, and 5–15% contained antigens (see also Falk, 1979).

HVP is indigenous to Africa and is restricted to that continent; however, animals bearing the virus have been transported throughout the world. Although no specific seasonal variation in infection is known in the wild, peak infection in the Sukhumi colony occurred in the autumn and spring (Kalter, 1994).

3.2.1.1 *Cell lines, persistence and transformation*

To date, the cell lines established from peripheral blood mononuclear cells or other tissues of HVP-infected tumour-bearing animals have been B lymphocytes (Falk, 1979), and infected monkey and human cord blood mononuclear cells exhibit B-cell markers; however, the markers are not uniform (Deinhardt & Deinhardt, 1979). HVP transforms lymphocytes of several species of non-human primates (*Macaca mulatta*, *M. arctoides*, *Hylobates lar*, *Saguinus* sp., *Callithrix jacchus*, *Saimiri sciureus*) as well as human cord blood mononuclear cells *in vitro*. The presence of the virus can be demonstrated by the presence of antigens (Ohno *et al.*, 1978; Falk, 1979). The nuclear antigen of HVP is similar to EBNA. In a comparative study, all EBNA-positive sera reacted with HVP nuclear antigen, but none of the sera containing this antigen reacted with EBNA.

3.2.1.2 *Prevalence of infection with Herpesvirus papio*

Voevodin *et al.* (1985), using an assay to detect anti-VCA to HVP, showed that the prevalence of antibody in two groups of baboons, one with a high prevalence of lymphoma and the other lymphoma-free, was 80–90% in both groups, in contrast to the results of previous studies in which complement fixation was used (Voevodin *et al.*, 1979). Newly imported baboons had a significantly lower prevalence (35%). Moreover, the prevalence of infection was age-dependent, increasing during the first years of life, reaching the highest rate (100%) at five years of age, staying stable up to 18 years of age and decreasing subsequently. Infection in newly imported animals increased with age up to 71%. Nevertheless, when antibodies to early, capsid and nuclear antigens were compared in baboons in the Sukhumi colony, animals with lymphoid disease had higher frequencies and higher titres than did control animals (Neubauer *et al.*, 1979b).

3.2.1.3 *Molecular biology*

The organization of the genome of HVP shows similar unique features to that of EBV DNA (see section 1.1) and shows 40% homology with EBV DNA (Lee *et al.*, 1980). Replication of HVP involves both *cis*- and *trans*-acting functions, analogous to those found in EBV (Pesano & Pagano, 1986). For more information, see Lee *et al.* (1980, 1981) and Kieff (1996).

It has been shown that non-producer HVP cell lines have two or three viral genome equivalents per cell in an apparently integrated form, whereas in HVP producer cell lines the HVP-free DNA fraction is composed of both linear and circular DNA molecules that are similar in length to the circular DNA of EBV in human cell lines, as shown by electron microscopy (Falk *et al.*, 1979).

Howe and Shu (1988) showed that two small RNAs of HVP hybridize to EBERs, and two genes of HVP RNAs, *HVP-1* and *HVP-2*, are located together in a small unique

region at the left end of the viral genome and are transcribed by RNA polymerase III in a rightward direction, like the *EBERs*. There is considerable similarity between *EBER-1* and *HVP-1* RNA, except for an insert of 22 nucleotides which increases the length of *HPV-1* RNA to 190 nucleotides. *HVP-2* RNA and *EBER-2* are less similar, but both are about 170 nucleotides long. Like *EBERs*, *HVP* RNAs are abundant in HVP-transformed cells and are efficiently bound to cellular proteins. *EBNA-1* and *EBNA-2* of EBV do not cross-react well with HPV nuclear antigen, and cloning of *EBNA-2* of the HVP homologue showed significant nucleotide and amino acid divergence, as predicted by serological studies. The HVP *EBNA-2* homologue does not retain the acidic transactivating domain of EBV *EBNA-2*. This gene, as in EBV, is important in B-cell immortalization and the resultant alterations in B-cell growth pattern which regulate expression from specific viral and cellular promoters. Ling *et al.* (1993b) showed that, in comparison with the *EBNA-2* of EBV, all of which are either type 1 or type 2, the HVP *EBNA-2* gene falls into neither type 1 or type 2. The divergence of *EBNA-2* of HVP from that of EBV is thus ideally suited for comparative studies. Similarities between the EBV and the HVP *LMP-2* gene indicate that the latter is important in infection *in vivo* (Franken *et al.*, 1995).

These molecular biological studies with HVP provide not only an understanding of the action and function of various viral and cellular genes but also a unique opportunity to study their role in pathogenesis, because they can infect, replicate and cause B-cell lymphoproliferation in various non-human primates.

3.2.1.4 Pathogenesis and immune response to Herpesvirus papio

HVP is a potent transforming agent, since it can efficiently transform human and non-human primate peripheral blood mononuclear cells. Falk *et al.* (1976) established three lymphoblastoid cell lines from the splenic lymphocytes of a lymphomatous baboon (*Papio hamadryas*) in which they could identify the presence of HVP. Adult or newborn marmosets of three species (*Saguinus oedipus oedipus*, *S. fasciollis*, *Callithrix jacchus*) were inoculated with lymphoblastoid cells derived from this baboon (Deinhardt *et al.*, 1978). All six adult animals developed moderate leukocytosis, relative lymphocytosis beginning 7–16 days after inoculation. Three of the animals, two of which also had developed large tumours at the site of inoculation, developed marked generalized lymphadenopathy and died 13–22 days after inoculation. None of the eight newborn marmosets died after inoculation; they developed only minimal lymph-node swelling during the first two to three weeks after inoculation, without haematological abnormalities.

Gerber *et al.* (1977) inoculated two HVP-seronegative baboons (*Papio cynocephalus*), two rhesus monkeys (*Macaca mulatta*) and two cotton-topped tamarins (*Saguinus oedipus oedipus*) either intraperitoneally, intravenously or subcutaneously, with a total of 3.0 ml of HVP with a transforming titre of 10^{3-5} units derived from a spontaneously transformed baboon lymphoid cell line. The animals were kept for six months. Both baboons seroconverted, and HVP was excreted by the oropharyngeal route. Seroconversion was seen in the rhesus monkeys but not the tamarins, and neither species excreted detectable

levels of HVP from the oropharynx. None of the inoculated animals had detectable disease or palpable tumours.

3.2.2 *Gamma-1 herpesvirus from cynomolgus monkey (Macaca fascicularis)*

Fujimoto *et al.* (1990) established a B-lymphoblastoid cell line from lymph nodes of an apparently healthy cynomolgus monkey (*Macaca fascicularis*) in which EBV-like herpesvirus was demonstrated. The cell-free supernatant from the cell line transformed the lymphocytes of another cynomolgus monkey.

Feichtinger *et al.* (1992) inoculated cynomolgus monkeys with simian immunodeficiency virus type 1, and about 40% of the animals developed B-cell lymphoma. A herpesvirus termed HVMF1 was detected in all tumours tested, and lymphoma tissue and two established cell lines reacted with EBV-specific DNA probes (Li *et al.*, 1993, 1994). Homology to EBV was found in *EBNA-2*, *EBNA-5*, *EBERs* and *oriP*, and proteins that cross-reacted with EBNA-1 and EBNA-2 were identified by western blotting. On the basis of the close homology with EBV, the investigators proposed that malignant B-cell lymphoma in cynomolgus monkeys experimentally infected with simian immunodeficiency virus could be used as a model for EBV-associated lymphomagenesis in immunodepressed persons.

3.2.3 *Rabbit model of malignant lymphoma induced by EBV-like virus from Macaca arctoïdes*

An EBV-like herpesvirus isolated from *Macaca arctoïdes* was successfully used to induce malignant lymphoma in rabbits (*Oryctolagus cuniculus*). Sixteen out of 32 infected rabbits became ill between days 21 and 143 after inoculation, and in 15/16 animals with clinical signs, lymphoproliferative disease was shown by histological examination. The disease consisted either of malignant lymphoma or lymphoid hyperplasia of the spleen and parenchymal organs (Wutzler *et al.*, 1995).

3.3 Other models of relevance to EBV

3.3.1 *Murid herpesvirus 4*

Murid herpesvirus 4, or MHV-68, is a B-lymphotropic gammaherpesvirus which on phylogenetic grounds was classified as a gamma-2 herpesvirus. It induces B-lymphoproliferative disease in mice and has therefore been proposed as a model for EBV infection. As it is a gamma-2 herpesvirus, it is discussed in the monograph on KSHV/HHV8.

3.3.2 *Marek's disease*

Marek's disease virus (MDV), first described by Josef Marek in 1907, has clearly played a major role in comparative herpesvirology and oncology (Calnek, 1986). MDV is a lymphotropic herpesvirus which, after an early cytolytic infection, induces T-cell lymphomas in chickens, its natural host. The lymphoma cells are latently infected with MDV, but the viral contribution to the transformed phenotype is not fully understood.

The integration pattern of MDV DNA suggests the clonal nature of tumour formation, and MDV-transformed cell lines established *in vitro* maintain the integration pattern of primary lymphomas (Delecluse *et al.*, 1993b).

Malignant lymphomas occur in a variety of organs and tissues, including the spleen, gonads, liver, lungs, heart, mesentery, bursa of Fabricius, thymus, adrenal gland, pancreas, proventriculus, intestine, skeletal muscle and skin. The various strains of MDV have different organ distributions. Marek's disease is also characterized by the formation of lesions in peripheral nerves, the spinal roots and/or the root ganglia. The affected peripheral nerves show loss of cross-striations, enlargement, discolouration and sometimes oedema. Affected spinal root ganglia become enlarged and appear translucent and somewhat discoloured (Calnek & Witter, 1991).

MDV is transmitted through direct or indirect contact between chickens. The fully infectious virus becomes airborne when it is shed from the feather follicle epithelium during skin keratinization (Calnek *et al.*, 1970). Both diseased and apparently unaffected chickens can continually shed MDV continuously for many weeks (Kenzy & Cho, 1969).

Propagation of the causative agent in cell culture (Churchill & Biggs, 1967) was followed by transmission of the disease with the cell culture-propagated virus (Churchill & Biggs, 1968). Eventual transmission of the disease with cell-free infectious herpesvirus particles isolated from the feather follicles of infected chickens was the definitive study that dispelled doubt that MDV is the causative agent of Marek's disease (Calnek *et al.*, 1970).

Marek's disease is the first, most important system in which a herpetic affliction and/or a neoplastic condition has been successfully controlled by vaccination. Vaccination against Marek's disease was first demonstrated in 1969 with a serotype-1 strain attenuated by passage in cell culture (Churchill *et al.*, 1969). Herpesvirus of turkeys, isolated in 1969 (Kawamura *et al.*, 1969), provides good protection against Marek's disease (Okazaki *et al.*, 1970).

Three avian herpesviruses share common antigen determinants: Marek's disease herpesvirus 1 (gallid herpesvirus 2), Marek's disease herpesvirus 2 (gallid herpesvirus 3) and turkey herpesvirus 1 (maleagrid herpesvirus 1) (Witter *et al.*, 1970).

A major step in MDV virology was made by Buckmaster *et al.* (1988), who showed that MDV and turkey herpesvirus sequences bear greater similarity to varicella-zoster virus (human herpesvirus 3), an alphaherpesvirus, than to EBV sequences, and that the MDV and turkey herpesvirus genomes are collinear with that of varicella-zoster virus. Therefore, even if some biological aspects of Marek's disease resemble EBV infection in humans, it cannot be considered virologically to be a true model for human EBV infection because MDV is clearly an alphaherpesvirus.