A Monkey Model for Epstein Barr Virus-associated Lymphomagenesis in Human Acquired Immunodeficiency Syndrome

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Summary

High-grade malignant nonHodgkin's lymphomas — five lymphoblastic, three pleomorphic, and two immunoblastic — developed in 10/25 cynomolgus monkeys (*Macaca fascicularis*) followed for up to 746 d after infection with simian immunodeficiency virus, strain SIVsm. These lymphomas were shown to be associated with an Epstein-Barr (EB)-like cynomolgus B-lymphotropic herpesvirus (CBLV) by electron microscopy, by Southern blot hybridization with probes against human EBV, and by the expression of antigens corresponding to EBV-associated nuclear antigens (EBNAs) involved in human B cell transformation. Southern blot demonstration of immunoglobulin gene rearrangements and homogeneous EBV episomes indicated that all the lymphomas were CBLV-associated monoclonal B cell proliferations. Our findings suggest that these tumors correspond to the EBV-associated malignant lymphomas in acquired immunodeficiency syndrome with respect to clinical, morphological, phenotypic, and genotypic characteristics. The particular susceptibility of SIVsm immunodeficient cynomolgus monkeys for CBLV-associated lymphomagenesis appears therefore a useful model for EBV-associated lymphomas in humans.

Patients with AIDS are at high risk to develop malignant lymphoma (1). The majority of these AIDS-related lymphomas (ARL) are high-grade malignant, B cell-derived, with extranodal growth at uncommon anatomical sites, and with a restricted range of morphologies (2–4). Molecular characterization has revealed subtypes with respect to clonality, association of EBV, and involvement of the c-myc oncogene (2–6). In those ARL associated with EBV, the virus is suggested to play an important pathogenic role (7, 8).

Infection of macaques with SIV has recently been established as a primate model for human AIDS (9). In these monkeys infected with various strains of SIV, development of lymphoproliferations or lymphomas associated with immunodeficiency have been reported in some animals (10). When examined, these lymphomas were of B cell origin, and association with an EB-like virus was occasionally inferred (10, 11). Recently, we reported a high incidence of malignant B cell lymphomas in a cohort of cynomolgus monkeys experimentally infected with SIVsm (12). Here we demonstrate that all these lymphomas are monoclonal by Ig gene rearrangement, and are associated with an EBV-like cyno-

molgus B lymphotropic herpesvirus (CBLV). We provide evidence for the expression in the tumor cells of a nuclear molecule similar to the EBV-associated nuclear antigen type 2 (EBNA-2), suggesting an important role of the CBLV in the pathogenesis of the malignant lymphomas. The high incidence of CBLV-associated B cell malignancies in cynomolgus monkeys infected with SIVsm, and their close resemblance to EBV-associated lymphomas in HIV patients provides a model for studies on the relative importance of immunosuppressive and oncogenic factors in AIDS lymphomagenesis.

Materials and Methods

SIV Inoculation and Serology. 25 wild caught cynomolgus monkeys (*Macaca fascicularis*) were inoculated with SIVsm (strain SMM-3) as previously described (13). Screening of monkey sera for IgG antibodies to viral capsid antigen (VCA) of human EBV was performed according to previously published methods (14). All animals used in this study were maintained and handled according to the Guidelines of the Swedish Ethics Committee of Animal Protection. Animals with tumors were killed within one week after tumor presentation.

Histopathology, Immunocyto/Histochemistry, and Electron Microscopy. Tissue and tumor biopsies were partly frozen in liquid nitrogen for DNA extraction and immunohistochemistry, partly fixed in 10% buffered formalin or Carnoy's fixative, and processed for conventional histopathology.

Nuclear antigens (EBNAs) were studied by an anticomplement immunofluorescence assay (ACIF) on frozen sections and cytospins of cultured tumor cells fixed with methanol/acetone (1:1; −20°C; 5 min) using six positive (HK, FF, LH, SP, IA, 107) and five negative (IE, GD, PB, ER, SN) human reference sera, as well as sera from tumor-bearing animals before and after SIV infection (15). Reactivity of lymphoma cells was evaluated by fluorescence microscopy (Zeiss, Obercochen, Germany), and compared with that of the EBNA-positive RAJI clone NC37.

Immunostaining for an EBNA-2-like molecule (mAb PE-2) (16) was performed on acetone-fixed frozen lymphoma sections with a double indirect immunoperoxidase method as previously described (12).

For electron microscopy (EM), cultured cells (see below) from tumor nos. 6 and 9 were spun down, and the pellets fixed in 2% glutaraldehyde/2% paraformaldehyde, and in OsO4, and embedded in Spurr resin (Polysciences Inc., Warrington, PA). Ultrathin sections were stained with uranylacetate and lead and examined in a JEOL 100 TEM.

Cell Cultures. Lymphoma cell cultures were established from minced tumor tissues and single cell suspensions were grown in RPMI, supplemented with 15% FCS, penicillin, and streptomycin.

DNA Isolation and Southern Blotting. DNA was isolated from lymphoma and nonlymphomatous tissue of the same animal, as well as from cultured cells of one of the tumors (no. 9; A4-27) according to standard procedures (17), electrophoresed on 0.6% agarose gels, and transferred to nylon membranes (17). Human placental DNA was included as a control.

Hybridization for Ig gene rearrangement was done with a probe specific for human J_H (2.5 kb) (18). Presence of DNA hybridizing with sequences of EBV was probed by hybridization with a cloned 3.1-kb BamHI W fragment directed against the long internal repeat sequence of human EBV (19) on BamHI-digested DNA. In addition, eight tumors and one cell line were tested on BamHI-digested DNA by hybridization with a 1.9-kb XhoI fragment against a sequence right to the terminal repeats of EBV, which are intracellularly joined to form closed episomal viral DNA and are useful for determination of clonality (20). DNA isolated from B95-8 and RAJI cells was included as a positive control in all EBV hybridizations. c-myc was detected with a 1.8-kb probe against the 3rd exon (21) (Oncor Inc., Gaithersburg, MD) on EcoRI and HindIII-digested tumor and nontumor DNA. All DNA probes were labeled with P^{32} (17) (random primed labeling kit; Boehringer Mannheim, Mannheim, Germany).
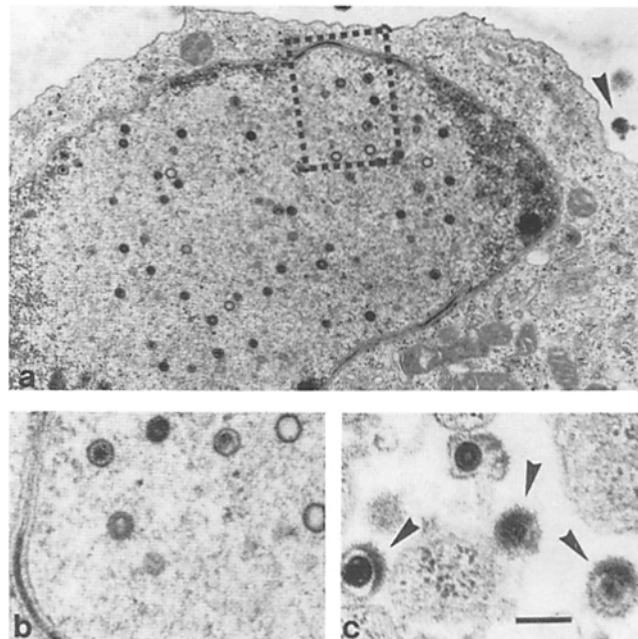
Results and Discussion

The previously described morphology and B cell origin of the examined monkey lymphomas suggested similarities with the EBV-associated subtype of AIDS-related lymphomas in humans (12). Sera from the tumor-bearing and other monkeys were therefore screened for EBV-specific antibodies. IgG antibodies reactive with VCA in titers ranging from 1/40–1/320 were detected in multiple serum samples taken before, and at various stages after SIV infection from 13/25 monkeys tested, including eight with lymphomas. No significant difference was observed in antibody titers of tumor-bearing and other SIV-infected monkeys. Also, eight HIV-

2-infected animals without any sign of immunodeficiency, as well as one uninfected animal, had anti-VCA titers up to 1/640 in three serum samples from each animal. As these serological findings strongly suggested that the cynomolgus monkeys were infected with an EB-like virus before SIV infection, we looked for the presence of such a virus in lymphoma cells by EM, DNA and antigen analyses.

Characteristic EBV-like particles (22) were found ultrastructurally in a small number of cells in two cell lines established from different lymphoma biopsies (Fig. 1). Furthermore, a nuclear antigen reactivity could be demonstrated in most cells of these two cell lines, and in frozen sections of 8/10 tumor biopsies by ACIF with four reference EBNA-positive human sera (Table 1) (Fig. 2 a). Five EBNA-negative human sera did not show any reactivity with the same lymphoma sections and cultured cells. Serum samples of tumor-bearing animals taken before or in early and late stages during SIV infection showed a typical EBNA-like reactivity with cells of autologous lymphomas (Fig. 2 b), as well as in NC37 (Raji) cells (not shown). This reactivity was usually seen in most of the lymphoma cells, strongly suggesting the expression of EBNA-like antigens in these lymphomas.

Similarly, immunostaining with the EBNA-2-specific mAb PE-2 also showed a nuclear reactivity in a variable number of the cells of all tumors (Fig. 2 c and Table 1) and of cytospins from the cultured lymphoma cells. Taken together, 8/10 tumors showed an EBNA-like reactivity with the polyvalent human sera in the ACIF assay, and all including the two ACIF-negative cases reacted with the anti-EBNA-2 mAb PE-2 (Table 1). The reactivity of all tumors with the anti-EBNA-2 mAb,

Table 1. *Histopathology, Genetic Configuration and EBV Status of Malignant Lymphomas in SIVsm-infected Monkeys and Cultured Lymphoma Cells*

| Tumor | Histology | Southern blot analysis | | | | Immunostaining | |
| | | G-R* | c-myc‡ | CBLV§ | | ACIF¶ (human sera) | EBNA-2** (mAb PE-2) |
				BamHI W	XhoI‖		
1	Lymphoblastic	1, 2	G	Positive	13; 6	+ + / + + +	+ +
2	Immunoblastic/ plasmacytic	1	G	Positive	13; 6	+ / + + +	+ + +
3	Lymphoblastic	1	G	Positive	13; 9	+ / + + +	+ + +
4	Lymphoblastic	1, 2	G	Positive	13; 6	+ / + + +	+ + +
5	Pleomorphic	ND	ND	ND	ND	+ / + + +	+ + +
6	Lymphoblastic	1, 2	G	Positive	13; 9	+ + / + + +	+ +
7	Lymphoblastic	1, 2	G	Positive	13; 6	+ / + +	+ +
8	Pleomorphic	1	G	Positive	13; 9	n.n.	+ +
9	Immunoblastic	1, 2	G	Positive	13; 6	+ / + + +	+ + +
10	Pleomorphic	1, 2	G	Positive	13; 9	n.n	+ + +
Cell line							
A1(no. 6)	Lymphoblastic	ND	ND	ND	ND	+ / + +	ND
A4(no. 9)	Immunoblastic	1, 2	G	Positive	13	+ / + +	+ +

* G–R, Ig gene rearrangement numbers specify clonal bands.
‡ G, germine configuration.
§ Cynomolgus B lymphotrophic virus.
‖ Numbers specify molecular weight of observed bands.
¶ Range of minimum and maximum positivity with five different EBNA-positive human reference sera, all diluted 1:10, when compared with their reactivity with NC37 control cells. n.n., non nuclear staining.
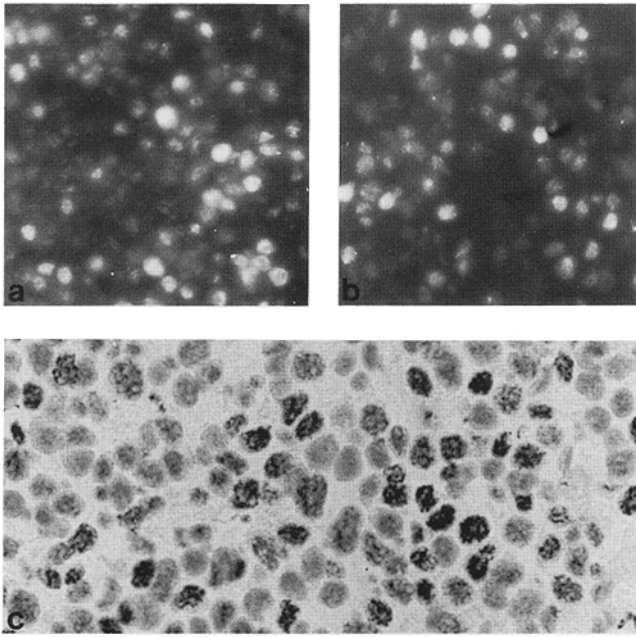** Positivity score: +, 0–10%; + +, 10–30%; + + +, >30% positively stained cells.

but not with two EBNA-1–specific human sera (JA, 107) (23) indicates that the demonstrated crossreacting human antibodies are predominantly directed against a cyno-EBNA-2–like antigen. This is in accordance with observations made by Dillner et al. (24) in a comparison of human and simian EBNAs. Considering the important role of EBNA-2 in the EBV-induced growth transformation of human B cells (16), our findings suggest a similar role for the CBLV in the lymphoma-genic process in the SIV-immunosuppressed cynomolgus monkeys.

In Southern blot studies of nine tumors and one cultured cell line (A4; no. 9), all were shown to contain DNA sequences crosshybridizing with a 3.1-kb BamHI W probe against the long internal repeat of EBV, indicating the presence of EBV-like DNA sequences (Fig. 3 a) (Table 1). Hybridization of the same tumors with a 1.9-kb XhoI probe covering the exons coding for the latent membrane protein (LMP) in human EBV gave no signal at the same hybridization conditions, but a weak signal at lower stringency (Fig. 3 b) (Table 1). This may indicate that the CBLV has a relatively low homology to the human EBV sequence in this

region, and/or that this sequence is in a different context, since the B-95-8 control cells give smaller fragments (Fig. 3 b). As the XhoI fragment in human EBV is localized close to the terminal repeats generating covalently closed episomal DNA in all virus-carrying cells, the observed single band (13 kb) (Fig. 3 b) could represent a clonal episomal form of the CBLV (20), while the appearance of a second band (~6 or 9 kb, respectively) can be due to integration or a more complex arrangement. Crosshybridization to cellular monkey sequences is excluded by the negative findings in the control tissues (Fig. 3 b).
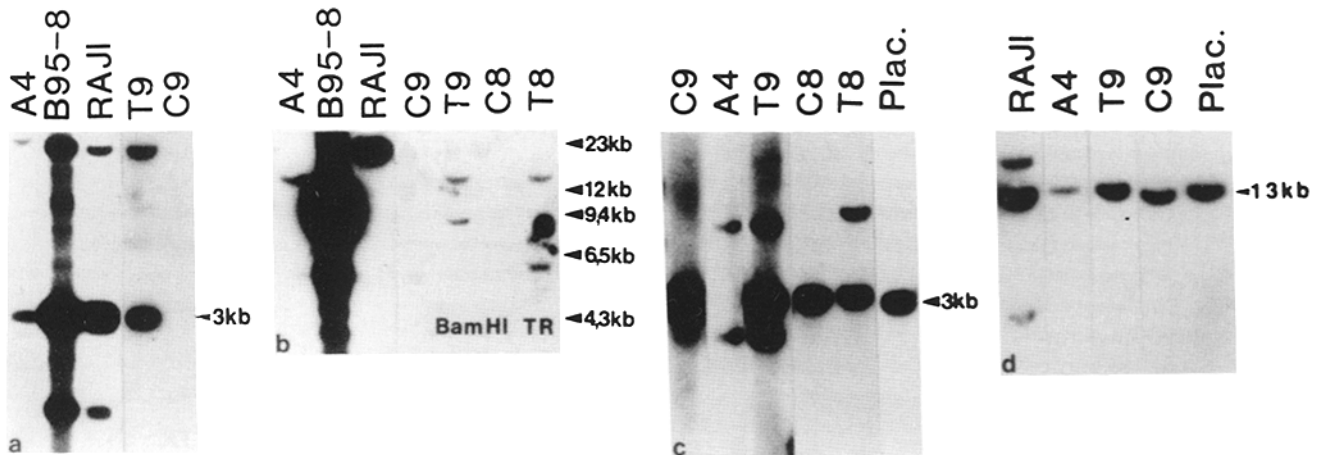
Clonality of the lymphoma cell populations was evident from Southern blots showing clonal Ig gene rearrangements of the H chain locus with a human J_H-specific probe in nine lymphomas tested (Fig. 3 c and Table 1). The cultured cell line (A4; no. 9) had an identical Ig gene rearrangement pattern to that seen in the primary lymphoma biopsy, besides a loss of the germline fragment (3 kb) (Fig. 3 c), indicating that the in vitro growing cells indeed were derived from the primary tumor clone. Southern blotting with a probe specific for the 3rd exon of the myc oncogene after EcoRI and HindIII

Figure 2. Demonstration in cryosections of EBNA-like reactivity. (*a*) By ACIF staining in tumor cell nuclei with a human EBNA-positive reference serum; (*b*) With autologous monkey serum on a lymphoma biopsy (no. 9; ×400); (*c*) Immunohistochemical staining for EBNA-2 in the majority of tumor cell nuclei of a lymphoma biopsy (no. 9; mAb PE-2; double indirect peroxidase method, ×600).

(data not shown) digestion of DNA from nine tumors and the A4-cells revealed a germline configuration (Fig. 3 *d* and Table 1).

The studied malignant lymphomas in the present series of SIVsm-immunosuppressed monkeys seem to be homogeneous with respect to clonality, the presence of an EBV-like B lymphotropic cynomolgus herpesvirus (CBLV), and lack of detectable *myc* rearrangement. The expression by the lymphoma cells of an EBNA-2–like protein in the lymphoma cells indeed suggests that this CBLV may have an important role in the pathogenesis of the examined malignant lymphomas. The apparent clonal episomal virus expression and the demonstration of EBNA-like viral antigens in most tumor cells indicates that the viral infection preceded the clonal B cell proliferation (8, 20). In vitro studies are needed to further elucidate the functional characteristics of the CBLV with regard to polyclonal B cell activation, transformation, and other pathogenic mechanisms of importance for the outgrowth of malignant B cell clones. Furthermore, the high frequency of CBLV-associated lymphomas observed in our monkeys may relate to an indirect transforming effect of SIV by CBLV up-regulation and c-*myc* activation, as recently suggested for HIV and EBV (25). The monkey model described may therefore become an important tool for studies of the interactions between EBV and immunodeficiency viruses in the pathogenesis of malignant lymphomas in AIDS.

Figure 3. Southern blot results from two (nos. 8 and 9) lymphomas showing: (*a*) DNA sequences of tumor no. 9 and corresponding cultured cells (*A4*) crosshybridizing with a 3.1 kb BamHI W probe against the long internal repeat of the EBV. Raji cells and the B95-8 lymphoblastoid cell line are included as positive controls (BamHI digest). (*T*) Tumor CNA. (*C*) Nonlymphomatous tissue from the same animal. (*b*) Hybridization with a 1.9-kb XhoI probe to the right terminal repeats of EBV. One band in the lymphoma DNA is seen at ~13 kb, strongly suggesting the presence of a clonal episomal population of the EBV-like virus. A second signal is of variable molecular mass (6 and 9 kb, respectively). The signals in the tumor DNA were detected under low stringency (filters were washed twice with 3× SSC for 20 min, and once with 1× SSC for 10 min). The cells cultured from tumor no. 9 (A4) exhibit a single signal at 13 kb, RAJI cells show a single band at 21 kb, and multiple bands are seen in the lymphoblastoid cell line B95-8 under high stringency conditions (BamHI digest). (*c*) Rearrangement of Ig H chain genes with a human J$_H$-specific probe. Cultured cells from one tumor (A4; no. 9) show a rearrangement pattern identical to that seen in the primary lymphoma biopsy, accompanied by a loss of the germline (at ~3 kb). Human placenta was included as a control (BglII digest). (*d*) Hybridization for c-*myc* with a 1.8-kb probe covering the 3rd exon showing a germline band at 13 kb without any evidence of rearrangements of the c-*myc* gene. Human placenta was included as a control (EcoRI digest).

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