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Epstein–Barr virus latent membrane protein expression in Hodgkin and Reed–Sternberg cells

(Hodgkin disease/malignant lymphomas/oncogenes/PCR/immunohistology)

HERMANN HERBST*†, FRIEDERIKE DALLENBACH*, MICHAEL HUMMEL*, GERALD NIEDOBITEK*, STEFANO PILERI‡, NIKOLAUS MÜLLER-LANTZSCH§, AND HARALD STEIN*

*Institute of Pathology, Klinikum Steglitz, Free University of Berlin, 1000 Berlin 45, Federal Republic of Germany; ‡Institute of Hematology, University of Bologna, 40138 Bologna, Italy; and §Division of Virology, Institute of Medical Microbiology and Hygiene, University Hospital, University of Saarland, 6650 Homburg/Saar, Federal Republic of Germany

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ABSTRACT **Cryostat sections from lymph nodes of 47 Hodgkin disease patients were examined by immunohistology for the Epstein–Barr virus (EBV)-encoded latent membrane protein (LMP), nuclear antigen 2, and late viral glycoprotein gp350/250. A distinct LMP-specific membrane and cytoplasmic staining was detected exclusively in Hodgkin and Reed–Sternberg cells in 18 patients (38%); EBV nuclear antigen 2 and gp350/250 immunoreactivity was absent in all instances. Thirty-two of 47 (68%) cases contained EBV-specific DNA sequences as detected by PCR, all LMP-positive cases being in this category. Our results confirm previous studies establishing the presence of EBV genomes in Hodgkin and Reed–Sternberg cells by demonstrating expression of an EBV-encoded protein in the tumor-cell population. The finding of LMP expression in the absence of EBV nuclear antigen 2 suggests a pattern of EBV gene expression different from that of B-lymphoblastoid cell lines and Burkitt lymphoma, whereas this finding shows similarities with that seen in undifferentiated nasopharyngeal carcinoma. Because the LMP gene has transforming potential, our findings support the concept of a pathoetiological role of EBV in many cases of Hodgkin disease.**

The association of Epstein–Barr virus (EBV), the etiologic agent of infectious mononucleosis, with endemic Burkitt lymphoma, nasopharyngeal carcinoma, and secondary B-cell proliferations in immunosuppressed individuals is well established (1, 2). More recently, EBV was also found in high proportions of other entities, such as nasal T-cell lymphomas (3) and angioimmunoblastic lymphadenopathy (4). In particular, EBV genomes have been detected in up to 60% of Hodgkin disease (HD) cases (5). In EBV-positive HD cases studied in more detail, the viral genomes were present as episomes of monoclonal origin and confined to Hodgkin and Reed–Sternberg (HRS) cells, which constitute the malignant cell population of HD (6, 7). Although associating EBV with a considerable proportion of HD cases, these experiments could not provide information about the functional state of the viral genomes—i.e., which EB viral genes were transcribed and translated to protein. We, therefore, extended our studies to the *in situ* detection of the EBV-encoded proteins latent membrane protein (LMP), EBV nuclear antigen 2 (EBNA2), and the late viral glycoprotein gp350/250 by immunohistology on tissue sections of lymph nodes from HD patients.

Although gp350/250 indicates productive viral infection, EBNA2 and LMP genes may be expressed during latent EBV infection (8, 9). LMP has transforming properties when introduced into rodent fibroblastoid cell lines and renders these cells tumorigenic in nude mice (10). LMP is also expressed in the majority of undifferentiated nasopharyngeal carcinomas, the human malignancy most consistently associated with EBV (11, 12). Using a polyclonal rabbit antiserum raised by immunization with an *Escherichia coli* trpE fusion protein with the 155 carboxyl-terminal amino acids of the LMP-encoding BNLF-1 open reading frame (13), we found LMP expression in more than one-third of the HD cases investigated, thus supporting the view that EBV plays an etiologic role in HD.

MATERIALS AND METHODS

Tissues. Lymph node biopsy tissue specimens from 37 HD patients submitted to the Institute of Pathology at the Klinikum Steglitz (Berlin), and from 10 HD cases from the Institute of Hematology, University of Bologna, were routinely processed into paraffin sections. In addition, cryostat sections were cut from tissue aliquots that were snap-frozen and stored at −80°C. The histological typing of HD followed the Rye Classification.

Immunohistology. The production and specificity profiles of the rabbit antisera directed against LMP (no. 749) (13), EBNA2 (14), and gp350/250 (N.M.-L., unpublished data) are described elsewhere. These sera were used on cryostat sections only. Specificities of the monoclonal antibodies used are listed in Table 1. Four-micron sections of either formol-fixed/paraffin-embedded or snap-frozen tissue blocks were stained by the alkaline phosphatase–anti-alkaline phosphatase method (15). Affinity-purified mouse anti-rabbit immunoglobulin, rabbit anti-mouse immunoglobulin antibodies, and alkaline phosphatase–anti-alkaline phosphatase complex (diluted 1:20) were obtained from Dakopatts (Glostrup, Denmark). Cryostat sections were fixed in acetone and chloroform (30 min each at room temperature). Fixation of cryostat sections in cold methanol was also tested for detection of EBNA2-specific staining. Formol-fixed sections required a proteolytic treatment with *Streptomyces griseus* protease at 1 mg/ml (Sigma) for 10 min before incubation with monoclonal antibodies βF1 and Ber-H2. In cytospins of the EBV-producing cell line B95-8, provided by E. Kieff (Harvard Medical School, Boston), large proportions of the cells were labeled by the EBNA2, LMP, and gp350 antisera. Control stainings on normal lymphoid tissues showed no labeling with these antisera and labeling patterns typical for the monoclonal reagents listed in Table 1, confirming specificity of reagents and staining method. The preimmune rabbit sera did not produce staining in any of the normal and HD tissues.

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Abbreviations: EBNA, Epstein–Barr viral nuclear antigen; EBV, Epstein–Barr virus; gp, glycoprotein; HD, Hodgkin disease; HRS cells, Hodgkin and Reed–Sternberg cells; LMP, latent membrane protein; ns, nodular sclerosing; mc, mixed cellularity; lp, lymphocyte-predominant; ld, lymphocyte-depleted.

†To whom reprint requests should be addressed.

Table 1. Monoclonal antibodies

Antibody designation	Cluster of differentiation	Specificity	Source
T9-10	CD2	T cells	Dakopatts
T3-10	CD4	Helper/inducer T cells	Dakopatts
Tü71	CD5	T cells	A. Ziegler
S-HCL3	CD11c	Macrophages, granulocytes	Becton–Dickinson
B4	CD19	B cells	Coulter
L26	CD20	B cells	Dakopatts
S-HCL1	CD22	B cells	Becton–Dickinson
Tü-1	CD23	B cells	A. Ziegler
ACT-1	CD25	IL-2-receptor/activated cells	Dakopatts
Ber-H2	CD30	Activated cells	Dakopatts
Ki-24	CDw70	Activated cells	Authors' laboratory
Tü35		HLA-DR/activated cells, macrophages	A. Ziegler
βF1		T-cell receptor β chain	T-Cell-Sciences
V9		Intermediate filament vimentin	Dakopatts
Ki-67		Proliferation-associated nuclear antigen	Dakopatts

DNA Amplification. DNA was extracted from frozen tissue, carefully avoiding cross-contamination of samples. Aliquots of 0.5 μg of DNA were made up to 0.1 ml containing 40 mM KCl; 10 mM Tris·HCl (pH 8.4), 2.5 mM $MgCl_2$; 0.25 mM each of dATP, dCTP, dGTP, and dTTP; 20 μg of gelatin; 0.2 μM of primer; and 2 units of *Taq* DNA polymerase (Perkin–Elmer/Cetus), and subjected to 30 amplification cycles (16) (1 min at 94°C, 30 sec at 59°C, 30 sec at 72°C; complete cycle, 3.5 min) in a Thermocycler 60 (Bio-Med, Theres, F.R.G.) water bath as outlined (5). The primer and probe oligonucleotides corresponded to sequences in the *Bam*HI W fragment of the EBV genome (5) (primers: 5'-GCAGTAACAGGTAATCTCTG-3', nucleotide positions 20124–20143, and 5'-ACCAGAAATAGCTGCAGGAC-3', positions 20523–20504; probe: 5'-TATCTTTAGAGGGGAAAAGAGGAATAAG-3', positions 20314–20341). PCR products were visualized by ethidium bromide staining on 2% agarose gels. The sensitivity threshold of this approach was estimated by diluting DNA of plasmid pBa-W, which harbors one copy of the large internal *Bam*HI W repeat of the EBV genome, in EBV-negative human DNA equivalent to 1–5 × 10^5 cellular genomes. The plasmid target sequence (10^4 copies) was amplified to a clearly visible band in the expected range of 400 base pairs (bp) upon ethidium bromide staining of 2% agarose gels. Because the *Bam*HI W fragment is reiterated ≈10 times in the EBV genome, this number corresponds to ≈10^3 viral genomes. Thus, a substantial number of cells harboring EBV genomes had to be present to generate a positive PCR signal. For control, 20-μl aliquots of PCR product and a 250,000 cpm-labeled oligonucleotide probe were hybridized in 150 mM NaCl/2.5 mM EDTA for 30 min at 55°C and screened on 6% acrylamide gels for band shift. Negative EBV-screening results were considered only when human genomic sequences could be amplified in parallel experiments.

Table 2. EBV DNA in HD biopsies and EBV proteins in HRS cells

HD histology	EBV DNA	LMP	EBNA2	gp350/250
lp	0/1	0/1	0/1	0/1
ns	19/27 (70%)	9/27 (33%)	0/27	0/27
mc	13/18 (72%)	9/18 (50%)	0/18	0/18
ld	0/1	0/1	0/1	0/1
All types	32/47 (68%)	18/47 (38%)	0/47	0/47

lp, lymphocyte-predominant; ns, nodular sclerosing; mc, mixed cellularity; ld, lymphocyte-depleted types of HD.

RESULTS

Table 2 summarizes the results of routine histological typing, PCR analysis for EBV-specific DNA sequences, and immunostaining of cryostat sections for three EBV-encoded proteins on lymph nodes of 47 HD patients. One of these cases was diagnosed as lymphocyte-predominant (lp), 27 as nodular sclerosing (ns), 18 as mixed cellularity (mc), and one as lymphocyte-depleted (ld) types of HD. Expression of T- or B-cell lineage-specific markers was displayed by HRS cells in a total of 23 cases: among 27 ns-type HD cases, tumor cells of six and eight biopsies were related to B or T cells, respectively; among 18 mc-type HD cases four cases with B-cell markers and four cases with T-cell marker expression were found. Tumor cells of the one lp-type HD case carried B-cell markers. The labeling index of HRS cells with antibody Ki-67 (17) was high in all instances; HRS cells of all cases expressed activation markers CD25 (interleukin 2 receptor), CD30 (Ki-1 antigen), CDw70 (Ki-24 antigen), and HLA-DR. DNA sequences specific for the internal repetitive region of the EBV genome were detected in 32 of 47 cases—i.e., 68% (Table 2, Fig. 1). Application of the EBNA2 and gp350/250-specific sera revealed absence of detectable levels of these proteins in all tissue sections. With the LMP-specific

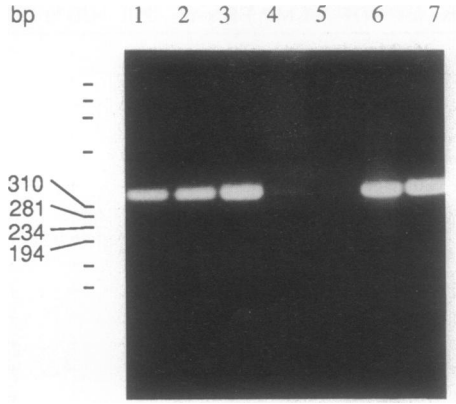

FIG. 1. PCR amplification products of EBV-specific DNA sequences in lymph node tissues with HD. DNA was extracted from seven different HD cases, subjected to PCR, and analyzed on ethidium bromide-stained agarose gels. Products are visible as bands of ≈400 bp in various quantities due to either different numbers of EBV-infected cells in the tissue or variable EBV genome copy numbers in infected cells.

antiserum, 15 cases from Berlin (eight ns- and seven mc-type HD biopsies) and three specimens from Bologna (one ns- and two mc-type HD cases) showed staining exclusively on HRS cells (Table 3, Fig. 2). The immunoreactive protein was present mainly on the cell membrane, usually in a patchy distribution, but additional cytoplasmic staining was also seen. The intensity of the LMP staining varied from intense to weak, with most multinuclear tumor cells (Reed–Sternberg cells) being moderately stained. Selective visualization of all HRS cells with the monoclonal antibody Ber-H2 (CD30) on adjacent sections revealed that the proportion of LMP-positive cells was in the range between 30 and 90%. All LMP-positive cases contained EBV DNA as shown by PCR analysis. Application of several B- and T-cell lineage-specific markers identified the HRS cells of some patients as related to B- or T-lymphoid cells. HRS cells of all 47 cases studied expressed the intermediate filament vimentin, which is known to interact with LMP (18).

DISCUSSION

HD has been associated with EBV infection by various lines of evidence, including serological demonstration of elevated antibody titers before disease onset (19, 20) and detection of EBV genomes in the DNA of a proportion of cases (5–7). The demonstration of monoclonal EBV genomes confined to HRS cells indicated that infection must have occurred before clonal expansion of the tumor-cell population (6, 7). However, whether EBV merely constitutes a "silent passenger" within the malignant cell population or contributes significantly to the malignant phenotype could not be decided. Here we demonstrate LMP-specific immunostaining in the absence of EBNA2 and gp250/350 expression in HRS cells of 18 of 47 HD cases. PCR analysis revealed the EBV DNA in over 60% of the biopsies, most of which were of mc- and ns-type HD. This number is well in line with our previously reported association of EBV with ≈60% of a large, random series of HD cases (5). The selective expression of LMP protein in HRS cells confirms that EBV infection in HD is restricted to HRS cells and shows that EBV-encoded genes may be transcribed and translated into protein in this cell type. The absence of the late viral glycoprotein gp350/250 from the LMP-positive cases indicates latent infection by EBV in HD (8). The observation of LMP expression appears relevant for the following reasons: Transfection studies have shown that this gene has transforming properties in rodent fibroblastoid cells; LMP-transformed Rat-1 cells produce tumors in nude mice (10). In transgenic mice, LMP induces epidermal hyperplasia and aberrant keratin expression (21). In epithelial cells, LMP transformation inhibits differentiation and induces a malignant phenotype—e.g., as evidenced by loss of contact inhibition (22, 23). LMP transfection deregulates a number of genes in EBV-negative Burkitt lymphoma cell lines and induces morphological changes (24). Some of these details are reminiscent of HRS cell characteristics—atypical cell morphology, expression of activation markers, and large growth fraction. In respect to CD21 or CD23 expression, however, HRS cells differ from EBV-infected B lymphocytes, as these antigens are detectable on HRS cells of only a few HD cases but are clearly expressed or even up-regulated in EBV-transformed B cells (8). Induction of CD23 expression appears to require a cooperation of LMP with EBNA2 (25), which is expressed in EBV-immortalized lymphoblastoid B cells but which was not found on HRS cells. Absence of EBNA2 may, therefore, explain the lack of CD23 expression. It also has to be noted that our LMP-positive HD biopsies included not only five cases with expression of B-cell specific markers but also cases without expression of B- or T-cell markers, as well as four cases with a T-cell phenotype. As compared to the situation *in vitro*, the spectrum of EBV target cells *in vivo* appears less restricted, as EBV-related benign and malignant epithelial lesions are known to exist and as EBV has also been found in T cells (3, 4). The observation of EBV-infected HRS cells expressing the T-cell antigen receptor β chain and other T-cell markers is, thus, well in line with these reports.

Interestingly, LMP is not detectable in some EBV-DNA-positive HD cases. Also, in LMP-positive cases variable proportions of CD30-positive HRS cells do not express LMP levels detectable by immunohistology. Although one reason for the failure to detect LMP in such cases may be related to the sensitivity of the method, the absence of EBNA2 in all cases is unlikely to be due to technical reasons because the antigen was consistently demonstrated in lymphoblastoid cell lines or cases of infectious mononucleosis (data not shown).

Table 3. Summary of immunohistological and PCR results

	HD			EBV protein			Activation marker				B-cell marker				T-cell marker						
Case	diag-nosis	Age, sex*	EBV DNA	LMP	EBNA2	gp350/250	CD30	CD25	CDw70	HLA-DR	CD19	CD20	CD22	CD23	CD2	CD4	CD5	TCRβ	CD11c	V9	Ki-67†
1	ns	26, M	+	+	−	−	+	+	+	+	−	−	+	−	−	−	−	−	−	+	60
2	ns	7, M	+	+	−	−	+	+	+	+	−	−	+	+	−	−	−	−	−	+	40
3	ns	6, F	+	+	−	−	+	+	+	+	−	−	−	−	+	+	−	+	−	+	50
4	ns	11, M	+	+	−	−	+	+	+	+	−	−	−	−	+	−	−	+	−	+	50
5	ns	80, F	+	+	−	−	+	+	+	+	−	−	−	−	+	+	+	+	−	+	70
6	ns	20, F	+	+	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	+	70
7	ns	5, M	+	+	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	+	70
8	ns	48, M	+	+	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	+	70
9	ns	64, M	+	+	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	+	60
10	mc	51, M	+	+	−	−	+	+	+	+	−	+	−	−	−	−	−	−	−	+	60
11	mc	53, M	+	+	−	−	+	+	+	+	−	−	+	−	−	−	−	−	−	+	70
12	mc	50, M	+	+	−	−	+	+	+	+	−	−	+	−	−	−	−	−	−	+	70
13	mc	27, M	+	+	−	−	+	+	+	+	−	−	−	−	+	+	−	−	−	+	50
14	mc	6, M	+	+	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	+	60
15	mc	39, M	+	+	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	+	40
16	mc	24, M	+	+	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	+	50
17	mc	33, M	+	+	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	+	50
18	mc	64, M	+	+	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	+	60

For monoclonal antibodies, see Table 1; + and − refer to reactivity with HRS cells; TCRβ, T-cell antigen receptor β chain.
*Age in yr; M, male; F, female.
†% of HRS cells labeled.

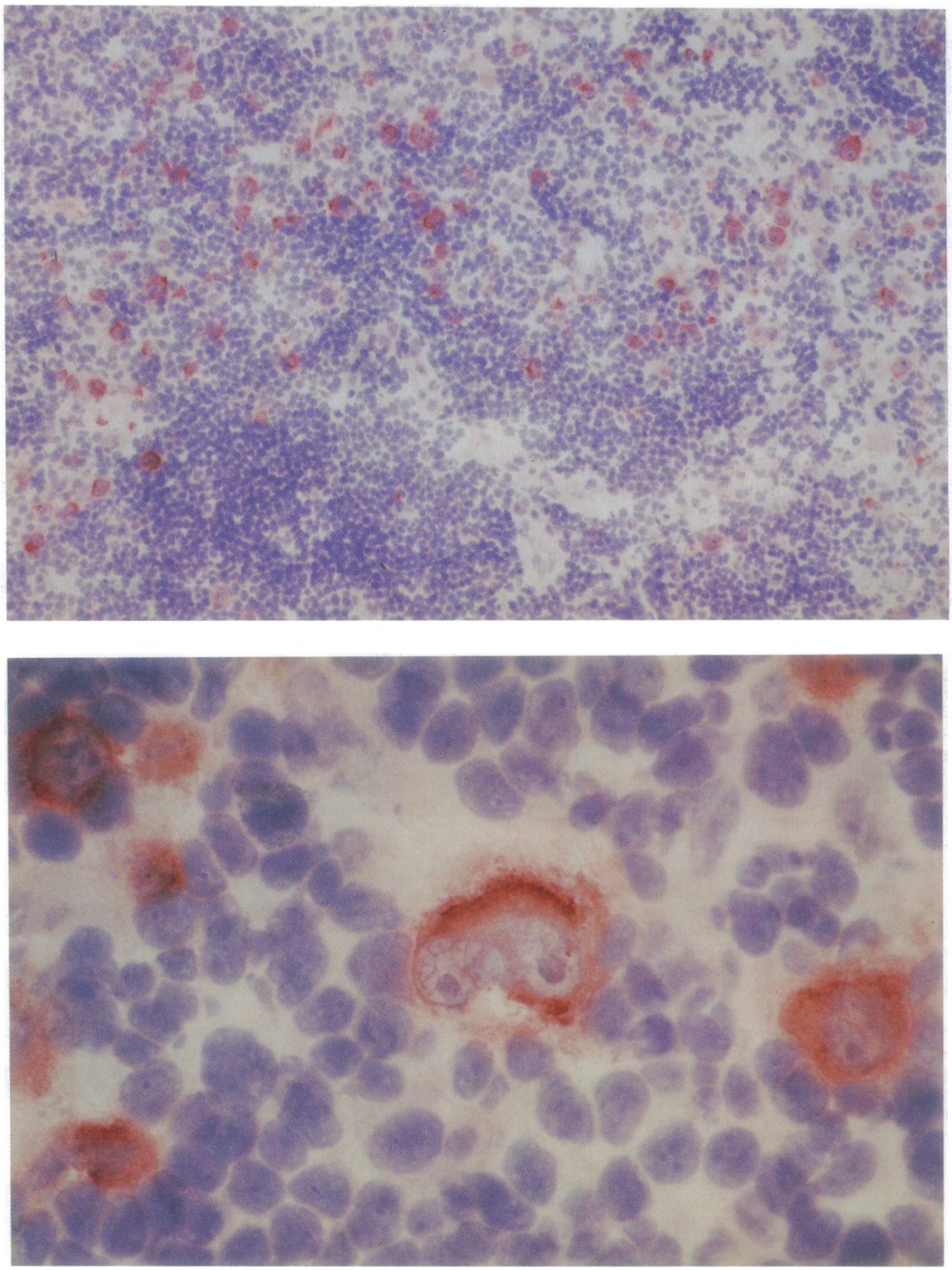

FIG. 2. Immunohistological detection of EBV-encoded LMP expression confined to mononuclear Hodgkin and multinuclear Reed–Sternberg cells (lymph node, mc-type HD). [Cryostat sections, alkaline phosphatase–anti-alkaline phosphatase technique; ×180 (*Upper*); ×1120 (*Lower*).]

The finding of variable numbers of LMP-positive tumor cells in some HD cases is in keeping with the observations on lymphoblastoid cell lines, which also express this antigen in only a proportion of cells (26).

The immunohistochemical demonstration of LMP provides a simple approach for analysis of EBV gene expression in HD, which has otherwise proven difficult for several reasons. Extractive techniques as applied, e.g., for the investigation of nasopharyngeal carcinoma, is not suitable for HD because the relative sparsity of tumor cells when compared to the admixture of reactive cells would make interpretation of any result difficult. The standard anticomplement immunofluorescence technique for detection of EBNA with human antisera (27) is usually applied to cytological preparations of single-cell suspensions or imprint preparations. When applied to tissue sections, this technique usually results in excessive background labeling due to cross-reacting antibodies within the human sera. The analysis

of EBV gene expression in human lesions by standard immunohistological techniques is also hampered by the lack of a broad spectrum of well-characterized antibodies of immunohistological quality. Future investigations using *in situ* hybridization detection of EBV RNA gene transcripts may help to overcome this problem. However, the interpretation of results may be complicated due to the unusual splicing pattern of EBNA mRNAs (28).

Although only limited data on expression of EBV-encoded antigens in HD are available, comparison of HD with other EBV-associated human malignancies reveals some similarities with undifferentiated nasopharyngeal carcinomas. In these carcinomas, the tumor cells invariably carry the EBV genome, and LMP expression is observed in up to 65% of the cases, whereas EBNA2 is absent (11). At present, whether lack of LMP-specific immunostaining in some EBV-DNA-positive HD cases is from low expression levels or from absence of LMP gene expression is unclear. The demonstration of EBV-carrying and EBV-negative cases of HD points to a parallel situation in Burkitt lymphomas, where morphologically undistinguishable EBV-positive (mainly endemic) and EBV-negative (mainly nonendemic) lesions exist. The important difference between HD and Burkitt lymphoma, however, is the observation of LMP expression in HRS cells of HD, whereas Burkitt lymphomas and corresponding cell lines do not express this EBV gene product. Because the LMP gene product has transforming potential, our findings support the concept of a pathoetiological role of EBV in many cases of HD.

In vivo, EBV-transformed cells are apparently controlled by specific T-cell subsets, and the development of EBV-associated neoplasia has been related to deficient T-cell surveillance. As functional deficiencies in the T-cell system of HD patients have been reported (29), it is tempting to speculate that HD patients with LMP-positive HRS cells may suffer from deficiencies in T cells with this specificity. It may, therefore, prove worthwhile to follow the clinical course of LMP-positive HD cases under the aspect of a, perhaps, more aggressive behavior of the disease.

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