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The Expression of Epstein-Barr Virus Latent Proteins Is Related to the Pathological Features of Post-Transplant Lymphoproliferative Disorders

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Transplant recipients are at increased risk for the development of post-transplant lymphoproliferative disorders (PTLDs). PTLDs harbor genomes of the Epstein-Barr virus, a herpesvirus that immortalizes B cells in vitro. At least five viral proteins are required for immortalization. Two of them are particularly important. Latent membrane protein (LMP) has transforming activity in fibroblasts, and Epstein-Barr antigen (EBNA)2 transactivates the expression of numerous cellular and viral genes. To determine whether the expression of EBNA2 and LMP is related to the histological and clinical presentation of PTLD, we tested their expression in 14 Epstein-Barr virus-positive cases. Using monoclonal antibodies to EBNA2 and LMP on paraffin sections, we found an expression of both proteins in 2 of 3 polymorphic PTLD and in 7 of 8 cases of monomorphic, large cell PTLD, without plasmacytic differentiation. One polymorphic and one large cell PTLD expressed LMP only. LMP and EBNA2 were found particularly in immunoblasts. The number of positive cells was extremely variable in the different cases as well as within the same biopsy. Three cases of PTLD had morphological and phenotypical features of plasmacytomas and did not stain for EBNA2 or LMP. This suggests that the expression of EBNA2 and LMP is related to the differentiation stage of the infected cells and that other viral or cellular proteins may contribute to tumor growth. (Am J Pathol 1995, 146:1113–1120)

Post-transplant lymphoproliferative disorders (PTLDs) represent potentially fatal complications of organ and bone marrow transplantations.[1] The detection of the Epstein-Barr virus (EBV) and of some of its proteins in PTLD cells has made a critical contribution to the elucidation of the pathogenesis of these diseases.[2] EBV has the ability to infect and immortalize human B lymphocytes *in vitro*.[3] At least six nuclear Epstein-Barr antigens (EBNAs), two nuclear EBV-encoded small RNAs (EBERs), and three membrane proteins (latent membrane protein (LMP) and terminal proteins (TP) 1 and 2) are expressed in these continuously growing cell lines referred to as lymphoblastoid cell lines.[3] The EBNAs and the membrane proteins are termed latent, because their expression does not lead to viral production.[4] Two of them, EBNA2 and LMP, have been proved to be central in virus-induced cell growth.[4] A link between latent protein expression and B cell immortalization has been provided by the demonstration that EBNA2 transactivates the expression of cellular genes involved in the control of proliferation (CD23, CD21, and c-*fgr*).[5–7]

The detection of the EBNA2 and LMP viral proteins, as well as CD23, in EBV-infected PTLDs developed by bone marrow transplant recipients indicates that PTLDs represent EBV-induced proliferations, as do lymphoblastoid cell lines.[8] However, other groups reported a marked heterogeneity at the level of expression of latent proteins in PTLD after bone marrow and solid organ transplantation with rare cases that did not express these proteins at all.[9,10] Finally, a recent study of PTLD in solid organ transplant recipients failed to demonstrate any EBNA2 protein production in the majority of these tumors.[11] A difference that might conceivably account for these discrepancies is that PTLDs encompass different entities and that

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EBNA2 and LMP expression is restricted to a given fraction of these diseases. It has been firmly established that the clinical and histological features of PTLDs are heterogeneous. Clinically, two forms of the disease have been clearly isolated.[12] Some PTLDs mimic infectious mononucleosis. They occur in young patients or children who were heavily immunosuppressed shortly after the graft. Other cases present with a tumor syndrome in older patients several years after the graft. Similarly, PTLDs exhibit a broad spectrum of morphological expression. They have been divided into monomorphic and polymorphic proliferations, according to their cellular composition.[13] Monomorphic proliferations are composed of large or small cells, whereas polymorphic proliferations contain cells at all stages of lymphoid differentiation. Finally, the degree of plasmacytic differentiation is taken into account in the classification of these proliferations.

We wondered whether the expression of EBNA2 and LMP is related to particular histological and clinical features. Fourteen cases of PTLD were therefore evaluated for clinical and histological features, phenotype, association with EBV, and expression of the viral latent proteins EBNA2 and LMP. The detection of viral EBERs is possible in formalin-fixed paraffin-embedded tissues by *in situ* hybridization. Moreover, antibodies to EBNA2 and LMP that work well on paraffin sections are now available. The use of these techniques enabled a precise identification of the morphology of the EBV-infected cells as well as those of the cells that expressed the viral latent antigens. We conclude from these findings that the level of expression of EBNA2 and LMP varies greatly between the different tumors and is highly dependent on the stage of differentiation of the infected cells. In particular, we have identified a distinct subtype of PTLD consisting of very large cells with an unusually pronounced plasmacytic differentiation as seen in plasmacytomas that expressed neither EBNA2 nor LMP.

Materials and Methods

Clinical, Histological, and Phenotypical Features

This study is based on a review of 14 cases of PTLD diagnosed in the pathology department of E. Herriot Hospital between 1985 and 1994. The relevant clinical features of the patients are summarized in Table 1. At least one biopsy specimen from each patient was available. In all cases, part of the tumor samples were fixed in Bouin solution and tissue blocks were either used for immunophenotyping in suspension or snap-frozen in liquid nitrogen. The histological evaluation was reviewed by two of the authors (FB and HJD) on H&E- and Giemsa-stained sections of routinely processed paraffin-embedded tissues. Immunophenotyping was performed by immunofluorescence on cells in suspension and/or on frozen sections fixed in cold acetone for 10 minutes, according to a standard double peroxidase method. The expression of the following molecules was investigated: CD3 (Leu 4, Beckton Dickinson, San Jose, CA), CD19 (B4, Coulter, Hialeah, FL), CD20 (B1, Coulter), CD21 (BL13, Immunotech, Marseille, France), CD22 (Leu14, Beckton Dickinson), CD23 (IOB8, Immunotech), CD38 (Leu 17, Beckton Dickinson), CD45 (Dako, Carpinteria, CA), and epithelial membrane antigen (EMA; Dako).

Table 1. *Relevant Clinical Findings of 14 Cases of PTLD*

Case	Transplanted organ	Delay after grafting (months)	Clinical presentation	Tumor site	Evolution in months
1	BM	3	IM	LN	Dead 1
2	K+P	1	IM	LN	Alive >24
3	K	6	IM	Tongue	Alive >24
4	K	1	IM	LN	Alive >24
5	L	2	Tum	Allograft	Dead 2
6	BM	5	IM	LN	Dead 2
7	K+P	4	IM	LN	Dead*
8	K	2	IM	LN	Dead 2
9	K	12	Tum	Allograft	Alive >24
10	K	3	IM	LN	Dead 3
11	K+P	6	Tum	Lung	Dead*
12	H	30	Tum	Intra-abdominal LN	Dead 2
13	L	20	Tum	Stomach	Dead 6
14	K	14	Tum	Bladder	Dead 6

BM, bone marrow; K, kidney; K+P, kidney and pancreas; H, heart; L, liver; IM, infectious mononucleosis-like syndrome; Tum, tumor syndrome; LN, lymph node. *Death is unrelated to the PTLD.

Association with EBV and Viral Latent Protein Expression

All cases were examined for the presence of EBV by RNA *in situ* hybridization with EBER1-specific probes (provided by G. W. Bornkamm). This probe is cloned in an expression vector in front of the T4 and T7 promotors. Sense and antisense RNAs were produced *in vitro* with T4 or T7 RNA polymerase in the presence of digoxigenin-11-UTP (Boehringer, Mannheim, Germany). Three-micron sections were cut with a sterile razor blade, fixed on silanized slides, and incubated overnight at 60 C. Sections were then dewaxed, soaked in HCl, 0.2 N, for 12 minutes, and incubated with proteinase K for 10 minutes at 37 C (10 μg/ml; Boehringer). Hybridization mixture (50% deionized formamide, 2X standard saline citrate (SSC), 10% dextran sulfate Denhardt's reagent (1X), and salmon sperm DNA (10 μg/ml)) containing 5 ng of the *in vitro* transcription products were then applied on sections and incubated overnight at 42 C. Slides were then extensively washed in 4X SSC followed by 0.1X SSC at 42 C. The staining consisted of a first stage incubation with a monoclonal mouse antibody to digoxigenin (Boehringer). After washing, a biotin-conjugated sheep antimouse antibody (Boehringer) was applied before a third stage of streptavidin-conjugated alkaline phosphatase (Bethesda Research Laboratories, Gaithersburg, MD). The sections were developed with naphthol AS-BI phosphate and fast red, counterstained with hematoxylin, and mounted with glycergel.

The pattern of EBV latent expression was further studied by using human sera with high titers against the total EBNA proteins, one monoclonal antibody to EBNA2 (EBNA2/R3),[14,15] and a cocktail of four monoclonal antibodies to LMP (CS1–4) (Dako). The total EBNAs were detected by the anticomplement immunofluorescent technique on frozen sections or on cytospins fixed in methanol/acetone (1/1) for 15 minutes at room temperature. Formalin-fixed and frozen sections were incubated with monoclonal antibodies to EBNA2 and LMP. After incubation with EBNA2/R3 slides were stained by using the avidin-biotin system described above. A standard alkaline phosphatase, antialkaline phosphatase (Dako) procedure followed the first stage incubation of formalin-fixed sections with the antibodies to LMP. The efficacy of a microwave-based antigen unmasking technique for the staining with the EBNA2 and LMP antigen on paraffin-embedded sections was also tested (immersion of the sections in 0.01 mol/L citrate buffer, three times for 5 minutes in a microwave oven at 750 watts).

Determination of Clonality

Extraction of DNA from frozen tumor tissues and Southern blot hybridization followed standard protocols.[16] Briefly, after digestion with the *Hin*dIII, *Bam*HI, and *Xba*I restriction enzymes, the extracted DNAs were electrophoresed in an agarose gel and blotted onto a nylon membrane. After hybridization to a probe specific for the heavy chain immunoglobulin gene (JH), membranes were washed and autoradiographed.

Results

Clinical Features

The main clinical notes of the patients are listed in Table 1. Six patients presented with a tumor syndrome. In two cases, the tumor localized to the allograft. Four of them developed PTLD more than 1 year after grafting. The other eight patients experienced symptoms of infectious mononucleosis that included fever and multiple lymphadenopathies at a short interval after grafting (1 to 6 months).

Histological and Immunological Features

The histological and immunological data are compiled in Table 2. The 14 cases of PTLD were classified according to the guidelines proposed by Nalesnik et al.[13] All were diffuse proliferations that completely effaced the normal architecture of the involved organ. Necrosis was a feature of all cases, destroying large areas of tissues in some proliferations. The morphological features of the PTLDs greatly varied with regard to the cellular composition.

Three cases were polymorphic in appearance and were composed of cells encompassing the whole B cell differentiation from the small lymphocyte to the plasma cell, including immunoblasts and large noncleaved cells. No atypical blast-appearing cells were present. A high amount of small T cells was mixed with the B cells, representing up to 60% of the cellular population. These PTLD cells strongly expressed the CD19 and the CD20 antigens but were less positive and in one case negative with the CD21-specific antibody. The tissue samples stained strongly with the antibody to CD38, an antigen expressed by T cells and plasma cells. CD23-positive cells were found in the only tested case. The staining with the EMA antibody was restricted to mature plasma cells present in the three cases.

Table 2. *Immunophenotyping and Expression of EBV Latent Proteins in 14 Cases of PTLD*

Case	Histology	Clonality	EBER	Total EBNA	LMP	EBNA 2	T/B cells	CD19 /20/22	CD21	CD23	CD38	EMA
1	Pol	M	pos	pos	++	++	50/50	++++	++	++	++++	–
2	Pol	M	pos	NT	++++	–	60/40	++++	++	NT	++++	*
3	Pol	P	pos	pos	++++	++	40/60	+++	–	NT	NT	–
4	Sl. Pol	P	pos	pos	+	++	10/90	++++	NT	NT	NT	*
5	Mon/L/PD–	M	pos	NT	+++	+	20/80	++++	++	++	++++	–
6	Mon/L/PD–	M	pos	NT	+++	++++	20/80	++++	NT	NT	NT	–
7	Mon/L/PD–	M	pos	NT	+	++	40/60	++++	NT	NT	NT	–
8	Mon/L/DP–	M	pos	pos	+++	+++	40/60	++++	++++	NT	NT	–
9	Mon/L/DP–	M	pos	pos	+++	–	15/85	++++	++++	NT	++++	–
10	Mon/L/DP–	P	pos	pos	+	++++	5/95	+++	++	++	++++	–
11	Mon/L/DP–	M	pos	pos	+++	++++	10/90	+++	+++	NT	NT	–
12	Mon/L/DP+	M	pos	pos	–	–	5/95	–	–	–	++++	+++
13	Mon/L/DP+	M	pos	pos	–	–	5/95	–	–	–	+++	–
14	Mon/L/DP+	M	pos	pos	–	–	5/95	–	NT	NT	++++	+++

Pol, polymorphic PTLD; Sl. Pol, Slightly polymorphic; Mon/L/PD–, monomorphic large cell PTLD without plasmacytic differentiation; Mon/L/PD+, monomorphic large cell PTLD with marked plasmacytic differentiation; M, monoclonal; P, polyclonal; pos, positive; NT, not tested.
+, rare positive cells; ++, 5 to 10% of tumoral cells are stained; +++, 10 to 30% of tumoral cells are stained; ++++, more than 30% of tumoral cells are stained. *Staining limited to normal plasma cells.

Eight cases were monomorphic and devoid of plasmacytic differentiation. In seven of these, the infiltrate consisted of large cells without plasmacytic differentiation that resembled immunoblasts or large noncleaved cells. In the last case, the pathological sample was composed of small lymphoplasmocytoid cells, mixed with rare mature appearing plasma cells and was therefore classified as slightly polymorphic. T cells were detected but to a lesser extent than in the polymorphic cases described above (less than 20% in 6 of the 8 cases). B cells showed the same phenotype as in the first group, with an expression of CD19, CD20, CD21, CD22, and CD38. PTLDs stained positively for CD23 in the two tested cases. None of the samples were reactive for the EMA antigen.

The last three cases displayed distinctive histological features and immunophenotypes. These PTLDs consisted of very large cells with a marked plasmacytic differentiation (Figure 1). The nucleocytoplasmic ratio was rather small, the nuclei were frequently eccentric, and a paranuclear hof was often present. Bi- or multinucleated cells were common. The nuclei of these tumors were frequently irregular in shape and size and often harbored a clumped chromatin associated with a unique, centrally placed prominent nucleolus. Mitotic figures were numerous. Only a few T cells were identified (less than 5%). These morphological features, resembling plasmacytomas, were confirmed by the immunophenotypic studies that demonstrated a strong expression of CD38 and EMA (in two of the three cases) with a concomitant negative staining with the CD19-, CD20-, CD21-, CD23-, and CD45-specific antibodies.

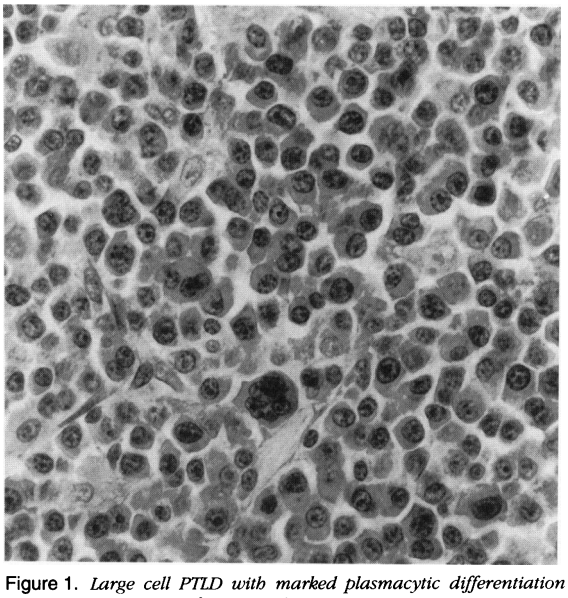

Figure 1. *Large cell PTLD with marked plasmacytic differentiation (case 13). H&E; magnification ×400.*

Association with EBV and Latent Protein Expression

After *in situ* hybridization, EBV-specific EBER1 was detected in all cases of PTLD reported in this work. The percentage of positive cells varied from case to case, being roughly similar to the number of B cells present in the proliferation. The results of the immunohistochemical studies dealing with the EBV latent proteins are listed in Table 2.

The recently produced antibody to EBNA2 (EBNA2/R3) has been found to work efficiently on fro-

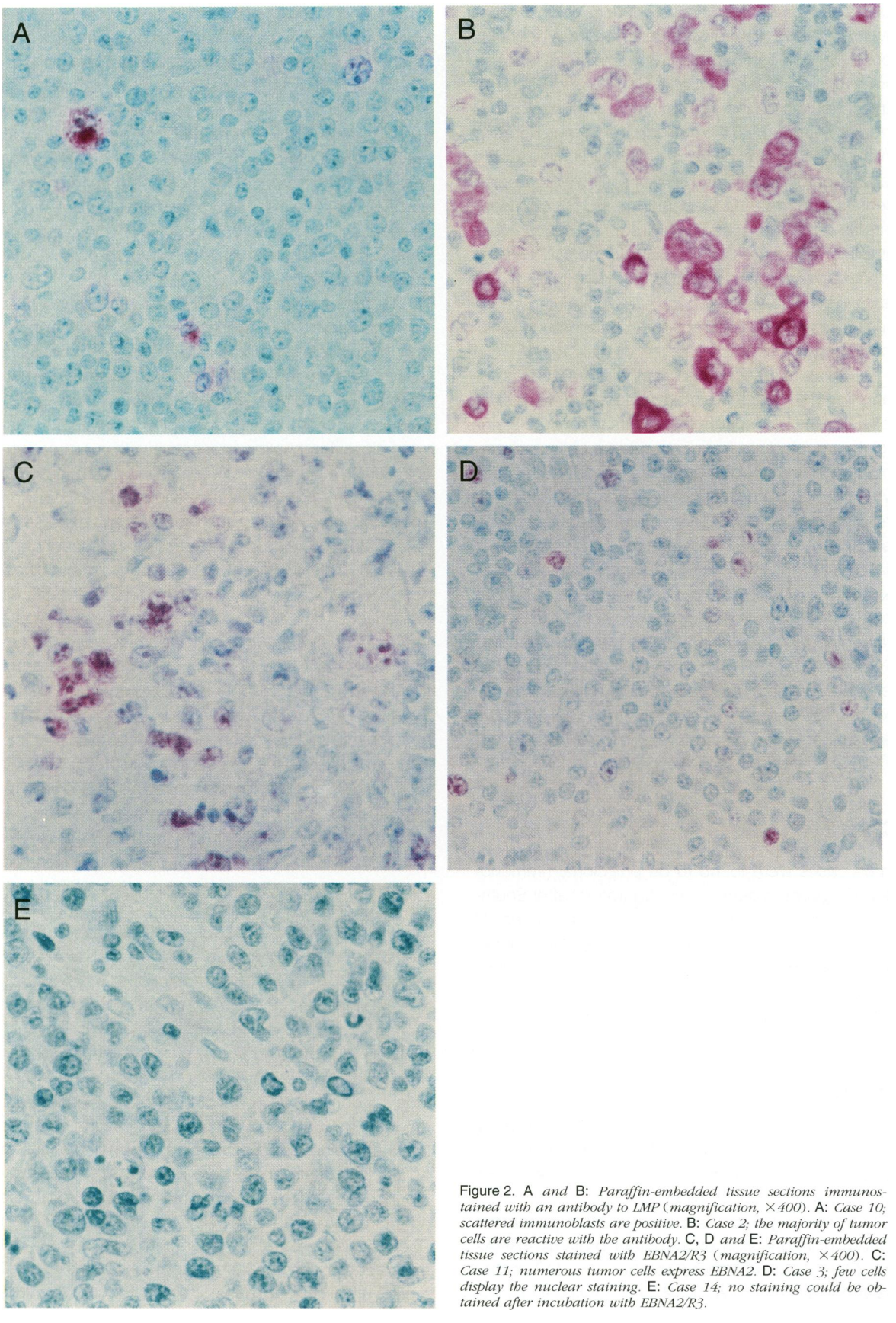

Figure 2. A *and* B: *Paraffin-embedded tissue sections immunostained with an antibody to LMP (magnification, ×400).* A: *Case 10; scattered immunoblasts are positive.* B: *Case 2; the majority of tumor cells are reactive with the antibody.* C, D *and* E: *Paraffin-embedded tissue sections stained with EBNA2/R3 (magnification, ×400).* C: *Case 11; numerous tumor cells express EBNA2.* D: *Case 3; few cells display the nuclear staining.* E: *Case 14; no staining could be obtained after incubation with EBNA2/R3.*

zen as well as on fixed tissue sections, albeit with a lower efficacy.[15] In line with these previous observations, we got comparable results with frozen and formalin-fixed tissue sections from two positive and three negative cases after staining with the EBNA2 and LMP antibodies, the staining being more intense on frozen sections in cases of positivity. However, only paraffin-embedded tissue sections allowed a precise identification of the labeled cells. Bouin-fixed, paraffin-embedded sections from all cases were tested for the expression of EBNA2 and LMP, with or without the antigen unmasking procedure. In all cases, results were comparable, the staining being more intense but with a higher background after antigen unmasking.

LMP was expressed in all but the three cases resembling malignant plasmacytomas. The number of positive cells varied from case to case, but the staining was much more frequent on large cells and particularly on immunoblasts (Figure 2A, B). Cells reactive for LMP were preferentially found around the areas of necrosis. With EBV-immune human serum, all cases were found to express the total EBNA. Cells that stained positively for EBNA2 were identified in all except two LMP-positive cases (Figure 2C, D). Frozen and fixed sections from the plasmocytoma-like PTLDs stained negatively after incubation with the antibodies to EBNA2 and LMP (Figure 2E). Monomorphic large cell PTLDs without plasmacytic differentiation tend to express EBNA2 more strongly than polymorphic cases. There was no correlation between the number of EBNA2- and LMP-positive cells.

Clonality

Three cases were found to be polyclonal (immunoglobulin genes in germline configuration) after Southern blot hybridization with a probe specific for the JH segment of the immunoglobulin heavy chain gene, the remaining cases being monoclonal (one or two discrete bands apart from the germline genes after hybridization, data not shown and Table 2).

Discussion

This report details the pattern of expression of the viral latent proteins EBNA2 and LMP in biopsies from 14 cases of EBV-associated PTLD and compares it with their histological and clinical features.

Two distinct groups were defined with regard to the pattern of expression of the latent proteins EBNA2 and LMP. All cases found to stain for LMP and/or EBNA2 were mature B cell PTLDs, as shown by the positivity of pan-B cell markers. It is possible that the level of expression of these latent proteins in some cells was too low to be detected by immunohistochemical methods and that we underestimated the number of EBNA2- and LMP-positive cells. However, the number of EBNA2- and LMP-positive cells, as well as the proportion of cells staining for CD23, was highly variable from case to case or even in different parts of the same tissue sample. This subset of cases comprised the three polymorphic PTLDs and the eight monomorphic cases without plasmacytic differentiation.

The three polymorphic PTLDs as well as five of the monomorphic PTLDs were revealed by an infectious mononucleosis syndrome. It is interesting to note that case 2, characterized by a strong plasmacytic differentiation, did not express EBNA2. Three other cases of monomorphic large cell PTLD without plasmacytic differentiation presented with a tumor syndrome, localized in the transplanted organ in two of them. There was no clear relationship between the morphological features and the pattern of expression of the latent proteins in these cases. Interestingly, one case of PTLD arising in the allograft exhibited few EBNA2-positive cells, whereas the other stained negatively. The staining for LMP was positive in both cases. The allogeneic stimulation caused by the foreign cells has long been proposed as a cofactor for PTLD, but its precise role is still unknown.

In the PTLDs described above, there was no plain relationship between the number of cells that expressed LMP and the number of those that expressed EBNA2. Moreover, two cases expressed LMP alone. *In vitro*, EBNA2 has been shown to induce the expression of LMP, but our observation as well as those of others in PTLD and in other EBV-associated tumors, such as human immunodeficiency virus-related lymphomas or Hodgkin's lymphomas, cast some doubt on whether EBNA2 is absolutely required for induction of the LMP expression.[17,18] LMP and, to a lesser extent, EBNA2 were particularly found in large cells resembling immunoblasts, a stage of differentiation commonly observed in lymphoblastoid cells, suggesting that the expression of these latent proteins leads to a cellular activation. Cells bordering the areas of necrosis reacted more intensively with the antibodies to EBNA2 and LMP. This raises the question of whether the expression of these proteins can lead to cellular destruction, perhaps via an immune response. The percentage of T cells differed from case to case. Polymorphic PTLDs contained up to 60% of T cells, whereas most monomorphic PTLDs without plasmacytic differentiation tend to possess few T cells, with six of eight cases containing only 10 to 20%

of T cells. These T cells can account for the cytotoxic response against viral proteins. However, it is of interest to note that *in vitro* immortalization of B cells by EBV is greatly reduced when T cells are removed before the infection.[19] The development of tumors in severe combined immune deficient mice after injection of human B cells infected by EBV is inhibited if T cells are absent.[20] The possibility that the T cells somehow contribute to the generation of some of these PTLDs, at least initially, should therefore be considered.

The other group comprised three cases of PTLD that developed in patients who presented with a tumor syndrome more than 14 months after grafting. These cases did not express EBNA2 and LMP and possessed particular morphological features. They were monotonous large cells resembling plasmacytomas. The immunophenotyping confirmed the morphological data (strongly reactive for CD38 and EMA (two of three) and negative for CD19, CD20, CD21, CD22, CD23, CD24, and CD45). The tumoral tissues contained only very few T lymphocytes. Studies with lymphoblastoid cell lines cultured *in vitro* or after injection into severe combined immune deficient mice have clearly established that the plasmacytic differentiation is followed by a down-regulation of EBNA2 and LMP.[21,22] In these observations, however, the plasmacytic differentiation was followed by a decrease in the cellular growth rate, which is in contrast to the clinical aggressive behavior of the three cases reported in this work. Interestingly, these three cases stained positively with an antibody to total EBNAs, whereas EBNA2 was not expressed. This raises the question of whether or not other members of the EBNA family contributed to the tumor phenotype.

Monoclonality is considered to be highly suggestive or if not specific for malignancy. In line with this assertion, polyclonal cases were associated with a better prognosis than the monoclonal ones, confirming numerous reports in the literature. No direct relationship between monoclonality or polyclonality and pattern of expression of viral latent proteins could be established.

It is widely accepted that the development of cancer results from the accumulation of genetic abnormalities. Even if the association between EBV and PTLD has been strongly documented, the observation of chromosomal abnormalities in some cases of PTLD raised the question of whether they contributed to the acquisition of the tumor phenotype.[23] In particular, rearrangements of the oncogene c-*myc* have been described in PTLD.[24] We also observed such rearrangements in two cases of PTLD, one case belonging to the series reported here.[25] Interestingly, this case was one of the EBNA2- and LMP-negative plasma cell-like PTLDs. However, the remaining cases, including the two other plasma cell PTLDs, carried only germline c-*myc* genes (data not published). No direct parallel between the pattern of expression of the latent proteins and the existence of c-*myc* rearrangements can therefore be drawn. Whether genetic abnormalities other than c-*myc* rearrangements are present in the two plasma cell-like EBNA2- and LMP-negative tumors remains to be determined.

In summary, it is clear that even if some PTLDs resemble lymphoblastoid cell lines cultured *in vitro*, this model does not account for all of the features of these proliferations. Whether viral proteins other than EBNA2 and LMP are expressed or other cellular events are responsible for the tumor proliferation remains to be investigated. Finally, the expression of EBNA2 and LMP seems to be highly dependent on the stage of differentiation of the infected cells (expression in immunoblasts and absence of expression in cells with a marked plasmacytic differentiation).

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