Frequent Latent Epstein-Barr Virus Infection of Neoplastic T Cells and Bystander B Cells in Human Immunodeficiency Virus-Negative European Peripheral Pleomorphic T-Cell Lymphomas

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We investigated 81 cases of peripheral pleomorphic T-cell lymphoma (PMTCL) occurring in human immunodeficiency virus-negative Europeans for the presence of Epstein-Barr virus (EBV)-DNA through polymerase chain reaction (PCR) for the presence of EBV-encoded small nuclear RNAs (EBER) and immediate early mRNAs (Bam H-fragment, lower strand frame [BHLF]) by in situ hybridization (ISH) and for EBV-encoded latent membrane protein (LMP) and nuclear antigen 2 (EBNA2) by immunohistology (IH). EBER-ISH, which could be applied on all cases, showed an overall incidence of EBV-infected cells in 38 of 81 cases (47%) of PMTCL. These data could be confirmed by PCR, which produced results in the cases with amplifiable DNA. By EBER-ISH, the virus was located in the tumor cells in 30 of the 38 EBV-positive cases, with the proportion of the infected cells ranging from 1% to 100%. In 18 of these cases and in the 8 cases without EBV-infected tumor cells, the virus was, respectively, either additionally or exclusively detectable in occasional nonmalignant lymphoid bystander cells. An LMP expression was observed in several of the EBER-expressing tumor cells in 18 cases, whereas EBNA2 was detectable only in one case, which also displayed signs of viral replication. Some nonmalignant EBV-infected B immunoblasts also expressed LMP in several cases. Primary cutaneous and enteropathy-associated PMTCL displayed less frequent EBV infection when compared with other extranodal or nodal manifestations.

The Epstein-Barr virus (EBV) is a DNA gamma herpes virus capable of transforming B cells in vitro and has been found to be the causative agent of infectious mononucleosis. Since its first isolation from cell lines established from African Burkitt's lymphoma, EBV has been linked with a variety of neoplasms, including nasopharyngeal carcinoma, Hodgkin's disease, polymorphic B-cell lymphomas and atypical B-cell lymphoproliferations in immunocompromised individuals, and some nasopharyngeal T-cell lymphomas occurring most frequently in Asian patients. To determine the incidence of EBV infection in non-Hodgkin's lymphomas (NHL) from Northern European patients without congenital or acquired immunodeficiency we screened more than 600 NHLs collected from former West and East Germany for the presence of EBV-DNA using the polymerase chain reaction (PCR). We noticed, to our surprise, that, apart from T-cell lymphoma of angioimmunoblastic lymphadenopathy type (AILD-TCL), EBV-DNA was more frequently detected in peripheral pleomorphic T-cell lymphomas (PMTCL) than in any other European NHL type. This finding prompted us to purposely collect a large series of 81 PMTCL cases from two German lymphoma reference centers (Berlin and Kiel) and to further analyze them in addition to the presence of EBV-DNA for cellular localization of the EBV-encoded transcripts EBER-1, EBER-2, and BHLF, and the expression of latent infection gene products, such as the latent membrane protein (LMP) and the nuclear antigen 2 (EBNA2).

Materials and Methods

Biopptic material. Paraffin-embedded specimens with PMTCL from lymph nodes (44), skin (15), nasopharyngeal/oral cavity (6), gastrointestinal tract (15), and spleen (1) obtained from 81 patients were selected from the files of the Institute of Pathology, Klinikum Steglitz, Free University, Berlin (Berlin, Germany) and from the Institute of Hematopathology, University of Kiel (Kiel, Germany). None of the patients was of Asian descent or evidenced a history or other signs of acquired or congenital immunodeficiency. PMTCL was diagnosed according to the criteria of the Updated Kiel Classification. These lymphomas consisted of sheets of T cells with medium-sized to large and irregular nuclei, small to moderately prominent nucleoli, and a varying amount of usually pale cytoplasm. Mitotic figures were frequent but varied from area to area. The architecture of the infiltrated lymph nodes was completely effaced, with the exception that, in some instances, B-cell zones were partially spared. The pleomorphic T cells in the skin frequently showed a marked epidermotropism with infiltration of the basal cell layer often accompanied by ulceration. The manifestations of these lymphomas in nasopharyngeal and oral areas exhibited prominent vascular invasion leading to extensive necrosis, corresponding to the clinical diagnosis of lethal midline granuloma. The collection of PMTCL also included 10 neoplasms from the jejunum showing villous atrophy and dense infiltration of the mucosa by the neoplastic intraepithelial T cells. Thus, these lymphomas fulfilled the criteria of enteropathy-associated T-cell lymphoma. As a control group, multiple, small-sized axillary lymph nodes removed from 12 patients during the course of mastectomy were used. Histologically, they did not display any signs of activation and are therefore referred to as normal lymph nodes.

Histology and immunohistology. Four-micron sections were cut and stained with hematoxylin and eosin (H & E), Giemsa, perin...
odic acid (PAS), and Gomori silver impregnation. Immunohistological analysis involved the use of monoclonal antibodies (MoAbs) and polyclonal antibodies for the detection of lineage-specific or lineage-characteristic antigens in paraffin sections. The anti-T-cell antibodies CD3 polyclonal, CD4 (MT310), CD45RB (G-B3), Ki-M4p (FDC), CD43 antifollicular dendritic cell antibody CD21 (1F8); the anti-EBV-latent membrane protein (LMP) antibody CS1-4; and the anti-EBNA2 antibody PE2 were obtained from Dako (Glostrup, Denmark). The anti-CD30 antibody Ber-H2; the anti-C3d receptor/antifollicular dendritic cell antibody CD21 (1F8); the anti-EBV-LMP antibody CS1-4; and the anti-EBNA2 antibody PE2 were obtained from Dako (Glostrup, Denmark). The anti-T-cell receptor β chain antibody βF1 was purchased from T Cell Sciences (Cambridge, MA). The antibodies CD45RB (Ki-B3), Ki-M4p (FDC), and CD68 (Ki-M1p) were provided by the laboratory of Dr Parwaresch (Kiel, Germany). The bound antibodies were made visible by using the alkaline phosphatase antialkaline phosphatase (APAAP) method in association with the New Fuchsin development according to Cordell et al.27 or with the streptavidin-biotin complex method (ABC) described elsewhere.23

**PCR**. PCR for the detection of EBV- and β-globin–specific sequences in paraffin-embedded tissue sections was performed as described previously.14

**In situ hybridization (ISH)**. EBER-ISH was performed with in vitro-transcribed digoxigenin (DIG)-labeled sense (negative control) and antisense RNA probes on paraffin sections (3 to 5 μm) for the detection of EBER-positive cells. After dewaxing, rehydration, and proteinase K digestion, the sections were hybridized overnight at 42°C in a solution of 50% formamide containing 5 ng DIG-labeled probes. After washing in Tris buffered saline (TBS) buffer and RNease treatment, detection of the bound labeled probes was achieved by incubation with a monoclonal DIG-specific antibody conjugated with alkaline phosphatase. The EBV-positive cells became visible after development involving Naphthol-As-Biphosphate and New Fuchsin (Merck, Darmstadt, Germany). The sensitivity of our approach and the suitability of the tissue sections used were assessed by a former parallel hybridization of Hodgkin’s disease and AILD-TCL14 tissue sections with radioactively and DIG-labeled in vitro-transcribed probes and by hybridization with a β-actin probe, respectively.

For the detection of BHLF-mRNA, a mixture of three oligonucleotides complementary to two abundant immediate early mRNAs encoding proteins belonging to the subgroup of EA-D of EBV-encoded antigens12,13 was used for ISH. The procedure was performed according to the manufacturer’s protocol (Dako; code no. Y 018) with the exception that we used Naphthol-As-Biphosphate/ New Fuchsin instead of nitro-blue-tetrazolium/brom-chlorindoxylphosphate (NBT/BCIP) for the detection of BHLF-expressing cells.

**Double labeling**. To determine the B- or T-cell nature of EBV-infected cells, we performed a double-labeling procedure using immunohistology (IH) and EBER-ISH. The immunohistologic demonstration of CD20 and CD45RO antigens involved the ABC method (DAKO) that was applied before EBER-ISH. EBER-ISH was performed as described above using a development either involving Naphthol-As-Biphosphate and New Fuchsin or alternatively NBT/BCIP (BioRad, Munich, Germany). All steps were performed under RNease-free conditions. After development, the slides were washed in phosphate-buffered saline (PBS) buffer and submitted to EBER-ISH as described above. As a result of this procedure, EBV-infected B or T cells became visible by their purple-red or deep blue, respectively, nuclei and brown membrane staining.

**RESULTS**

**EBV-DNA detection by PCR**. In 30 of the 81 PMTCL cases and 2 of the 12 normal lymphoid tissue samples, the extracted DNA was not amplifiable, as shown by the nonappearance of the β-globin–specific band after two-step nested primer PCR with the appropriate primers. In an additional 19 PMTCL cases, the scarcity of embedded tissue did not allow the application of PCR. PCR analysis of the remaining 32 PMTCL cases detected EBV-specific sequences in 20 cases. EBV-specific DNA was only present in 2 of the remaining 10 normal lymphoid tissue samples with amplifiable DNA.

**Correlation of PCR and EBER-ISH results**. The comparison of the EBER-ISH data with the PCR results obtained from the 32 cases in which extracted DNA was suitable for amplification showed a complete congruence of the findings, except for one case. In that instance, EBV-specific sequences were shown by PCR, whereas no positive cells could be identified after EBER-ISH. This difference implies that the positive PCR result was due to the presence of one single EBV-infected cell in the section used for DNA extraction and that the section used for EBER-ISH was devoid of such a cell.

**Detection of EBER and BHLF by ISH**. By application of DIG-labeled antisense probes specific for EBER-1 and EBER-2, EBV-infected cells could be shown in 38 of the 81 PMTCL cases as well as in 3 of the 12 normal lymphoid tissue samples. Only one case was found to be positive when a mixture of three BHLF-specific oligonucleotides was used for ISH.

The preservation of morphology achieved by application of DIG-labeled probes for ISH allowed precise morphologic evaluation of the EBV-infected cells. It could be clearly shown in each case that only lymphoid cells were EBV-positive. All other cell types, including macrophages, granulocytes, endothelial cells, fibrocytes, plasma cells, and epithelial cells, were EBV-negative. For lineage allocation of the EBV-infected cells, double labeling was performed on all cases containing EBER-positive tumor cells. This procedure led to satisfactory results in 20 of 30 cases. The lineage of the EBV-infected cells in the remaining cases was established after comparing the EBER-ISH with immunohistologic staining reactions performed on serial sections. The following groups of EBV presence could be distinguished (see also Table 1).

Group I displayed a diffuse and focal distribution of many EBV-infected tumor cell nuclei in 8 of 81 cases (Fig 1A). The labeled cells corresponded in size and cytology to the neoplastic blasts expressing T-cell antigens. The amount of the labeled cells made up 20% to 100% of the whole tumor cell population (Fig 1B). Additional EBER-positive small B lymphocytes and B immunoblasts could be identified in 5 cases (Fig 1C), whereas coexistent infected small T cells occurred only in 1 case. In addition to the cases with latent EBV infection, only 1 case displayed signs of a lytic infection in single neoplastic T cells as proved by BHLF-ISH (Fig 1E).

Group II showed an EBV infection of scattered tumor cells, often with focal accumulation, in 22 of 81 cases (Fig 2A). The amount of EBV-infected tumor cells accounted for 1% to 20% of the tumor cell population (Fig 2B). In 13 cases of this group, a coexistent labeling of several B im-
munoblasts and small-sized B cells was observed, lying in the remnants of B-cell zones, whereas a labeling of small T lymphocytes was observed in one specimen.

Group III presented with an occasional occurrence of EBER-positive cells in 8 of 81 TCL. These cells possessed a round or slightly irregular nucleus that resembled that of the rare EBER-positive lymphoid cells encountered in 3 of 12 normal lymphoid tissue samples.

Group IV consisted of 43 cases without any EBER-labeled cells.

Detection of EBV-encoded LMP and EBNA2. The cocktail of four MoAbs against different epitopes on the LMP led to the staining of lymphoid cells in 20 of the 81 T-cell lymphomas (Table 1). The number of LMP-positive cells was in all instances much less than the number of EBER-positive cells. The intensity of the LMP staining varied considerably within the same case and between the cases. The comparison with the infection patterns seen with EBER-ISH led to the following findings: Of the eight cases of infection pattern group I displaying a high proportion of EBV-infected tumor cells, six contained LMP-expressing tumor cells (Fig 1D). In addition to LMP expression, one of these cases displayed EBNA2 expression in single tumor cells, as shown by application of the MoAb PE2. This case also showed an expression of BHLF mRNA. Furthermore, two of these cases also contained LMP-positive B immunoblasts. The remaining 14 cases with LMP expression belonged to group II. Twelve of these cases showed LMP staining in neoplastic T blasts with additional occurrence of LMP-positive B immunoblasts in 2 cases. In 2 further cases, the LMP expression among the B cells was restricted to B immunoblasts. No LMP expression could be detected in the third group with occasional EBER-positive small lymphoid cells, similar to the EBER-positive normal lymphoid tissue samples.

Correlations of EBV infection patterns with patients' age and tumor localization. The male/female ratio of the lymphoma patients was 2.2:1. The groups with EBV-positive tumor cells (I and II) and the EBV-negative group (IV) displayed similar age distribution, with an increased incidence in those of greater age (61 to 90 years), whereas an even distribution was observed in the group with EBV-harboring bystander cells (III). The correlation with the tumor localization showed preferential EBV infection of PMTCL that primarily occurred in the nasopharynx, lymph nodes, and gastrointestinal tract, with a trend of higher incidence in the nasopharynx. In contrast, EBV infection in primary cutaneous and enteropathy-associated PMTCL was significantly lower (Table 2).

DISCUSSION

The present study shows in a large series of cases (n = 81) an unexpected high incidence of EBV infection in European cases of PMTCL. All patients of our study were HIV-negative and did not display any history or signs of congenital or acquired immunodeficiency. By using two-step PCR, EBV-DNA was detected in 20 of 32 cases of PMTCL in which amplifiable DNA could be extracted from paraffin-embedded biopsies. In contrast, EBV-DNA was detectable in only 2 of 10 normal lymph node samples with amplifiable DNA. These results could be confirmed by EBER-ISH. The nearly 100% congruence between the results obtained by PCR and EBER-ISH is in line with findings indicating that EBER-ISH allows the detection of all latently EBV-infected cells. The EBER-ISH could also be applied to those cases that proved to be unsuitable for PCR due to the absence of amplifiable DNA, resulting in the fact that all PMTCL cases collected could be used for our study. With this approach, EBV-harboring cells were detected in 38 of 81 (47%) of the PMTCL and in 3 of 12 (25%) of the normal lymphoid tissue samples. The application of nonisotopic, (DIG)-labeled EBER probes on routinely processed paraffin sections allowed, thanks to their superb morphologic preservation, subtle evaluation of both the morphology and the amount of infected cells. When combined with immunohistologic analysis of adjacent sections and/or the same sections (double labeling), EBER-ISH could identify most EBV-harboring cells as being neoplastic T cells in 30 of 81 PMTCL cases, with their percentage ranging from 1% to nearly 100%. Abundant EBV-harboring neoplastic T cells (more than 20%) occurred in 8 cases. In addition to infected neoplastic T cells, 18 cases displayed EBV-infected small bystander B lymphocytes. In 6 cases, some of the EBV-positive B cells had the morphology of immunoblasts. Of the remaining 51 PMTCL cases without EBV-positive tumor cells, 8 cases contained occasional EBV-positive small lymphocytes was observed in one specimen.
phocytes similar to the findings observed in 3 of 12 normal lymph nodes. It should be stressed that, in all instances, EBV expression was restricted to cells of lymphoid immunophenotype and/or morphology. As EB expression may be downregulated in the case of a lytic infection, the presence of nuclear immediate early mRNAs was investigated by applying ISH for BHLF detection. BHLF-mRNAs were present only in one case, which additionally showed abundant EBER-expressing tumor cells.

To clarify whether the functional state of EBV genomes differs between the latently infected tumor and bystander cells, the expression of EBV-encoded LMP and of EBNA2 was studied by IH. Small EBV-infected bystander cells proved to be constantly LMP-negative, in contrast to the neoplastic T cells, which were LMP-positive in 18 of the 30 cases of PMTCL containing EBER-positive tumor cells. Interestingly, LMP was also detectable in some EBER-positive B immunoblasts. In contrast to LMP, EBNA2 was detectable only in one case that also contained EBV in its replicative form, as described above. In all instances, the number of LMP- and EBNA2-positive cells was considerably lower than the number of EBER-positive cells. Previous studies on EBV gene expression during latency have found concordant expression of LMP and EBNA2 in most cases of posttransplant lymphomas\(^3\) and nasal T-cell lymphoma\(^3\) similar to the EBV phenotype observed in lymphoblastoid cell lines. The lack of EBNA2 expression in the vast majority of our LMP-positive cases is similar to the discordant patterns of expression described in Hodgkin's disease,\(^2\) in most EBV-positive acquired immunodeficiency syndrome (AIDS)-related lymphomas,\(^2\) and in nasopharyngeal carcinoma.\(^9\) Because LMP and EBNA2 detection by the MoAbs used is slightly reduced in paraffin sections, it is likely that the expression of these proteins may be more extensive than what we were able to document in this study. Our findings show that EBV is transcriptionally active in the infected neoplastic T cells. Because LMP has transforming potential,\(^2\) and can prolong cell survival,\(^3\) it might well be that EBV is involved as a cofactor in the genesis of EBV-positive PMTCL. Our results are principally in accordance with observations made in two recent studies\(^5,14\) that report on a relatively frequent presence of EBV-DNA and its gene products in PMTCL. However, due to their inherent insensitivity, the techniques applied in these studies (ie, Southern blot and/or ISH for EBV-DNA detection; IH for LMP and EBNA2 expression) could not detect the entire amount of EBV-harboring cases nor were they capable of providing precise information regarding the amount, morphology, and lineage allocation of the infected cells.

However, the role of EBV in PMTCL cannot be clearly understood unless the reason for the presence of EBV in only a proportion of and not in all neoplastic cells in the majority of PMTCL studied is clarified. It is also necessary to explain the frequent coinfection of B cells in addition to the EBV infection of the tumor cells. EBER-ISH has shown that in other EBV-associated neoplasms, such as Burkitt's lymphoma (from our own unpublished data) and Hodgkin's disease,\(^2\) all tumor cells harbor the virus, implicating that such neoplasms are derived from a single EBV-infected cell. Such a mechanism is also likely that the expression of these proteins may be more extensive than what we were able to document in this study. Our findings show that EBV is transcriptionally active in the infected neoplastic T cells. Because LMP has transforming potential,\(^2\) and can prolong cell survival,\(^3\) it might well be that EBV is involved as a cofactor in the genesis of EBV-positive PMTCL. Our results are principally in accordance with observations made in two recent studies\(^5,14\) that report on a relatively frequent presence of EBV-DNA and its gene products in PMTCL. However, due to their inherent insensitivity, the techniques applied in these studies (ie, Southern blot and/or ISH for EBV-DNA detection; IH for LMP and EBNA2 expression) could not detect the entire amount of EBV-harboring cases nor were they capable of providing precise information regarding the amount, morphology, and lineage allocation of the infected cells.
The proliferation rate of the infected tumor cells may increase, resulting in an eventual predominance of EBV-positive tumor cells. The high frequency of EBV-infected neoplastic T cells in PMTCL indicates that the differentiation stage represented by the tumor cells in these cases is particularly susceptible to EBV infection. Although EBV receptor (CD21) of low density occurs on up to 40% of circulating T cells, the mechanism by which the neoplastic T cells were infected could not be clarified because the CD21 molecule was not detectable on the lymphoid cells in our material. However, this may be due to the staining of paraffin sections in which a low expression of these molecules escapes detection.

Correlation of the presence of EBV in tumor cells with the site of the primary lymphomatous manifestation disclosed that EBV infection of PMTCL located in the nasopharyngeal region is frequent not only in Asian countries but also in Northern Europe. However, our report is the first that describes, based on a large number of cases and the application of two highly sensitive EBV detection systems, a high frequency of latent EBV infection in nodal and gastrointestinal PMTCL and a much rarer EBV infection of cutaneous and enteropathy-associated PMTCL (Table 2). The reason for this difference in the incidence of EBV infection between PMTCL of different localizations requires further clarification. It may very well be that EBV has less tropism towards skin and gut mucosa-affine T cells or the tissue-specific homing of the skin or gut mucosa-affine T cells has been reduced after EBV infection. These issues should be addressed by a prospective study that includes experimental investigations and the comparison of the clinical course of EBV-positive and EBV-negative cases.

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EBV IN PLEOMORPHIC T-CELL LYMPHOMAS


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