Identification and Characterization of an Epstein-Barr Virus-specific T-Cell Response in the Pathologic Tissue of a Patient with Hodgkin’s Disease

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ABSTRACT

Several lines of evidence indicate that an impairment of EBV-specific immune responses may contribute to the pathogenesis of Hodgkin’s disease (HD). At present, however, it is not clear whether a defective immunity to EBV is a characteristic restricted to EBV-associated HD cases or a more generalized phenomenon, part of the inherent immune deficiency of HD patients. In this study, we have addressed this issue by analyzing EBV-specific responses in infiltrating T lymphocytes (TILs) from one HD biopsy, where the virus was confined to a small proportion of apparently normal lymphocytes. TIL cultures were established using low amounts of recombinant interleukin 2 and in the absence of specific stimulation, conditions that preferentially induce the proliferation of in vivo activated T cells. An EBV-specific cytotoxic component was revealed by the capacity of these TILs to lyse autologous EBV-positive lymphoblastoid cell lines (LCLs) obtained by spontaneous transformation from the lesion but not HLA-mismatched LCLs and autologous phytohemagglutinin blasts. This cytotoxic activity closely resembled that of EBV-specific memory T cells, which may be reactivated from the blood lymphocytes of healthy donors by in vitro stimulation with autologous LCLs. The use of a panel of appropriately HLA-matched B95.8-transformed LCLs as targets in standard 51Cr release assays revealed EBV-specific cytotoxic responses to be restricted mainly through the A2 and B44 HLA alleles with a minor HLA-A26-restricted component. Using autologous fibroblasts infected with recombinant vaccinia viruses expressing the EBV latent antigens, the TIL culture was shown to recognize latent membrane protein 2 and, to a lesser extent, EBV-encoded nuclear antigen 6. In addition, a strong proliferative response was induced by coculture of TILs with autologous but not with allogeneic LCLs or autologous phytohemagglutinin blasts. Six CD4-positive, EBV-specific T-cell clones were isolated by limiting dilution. The study of cytokine mRNA expression, carried out by reverse transcriptase-assisted PCR, revealed that three of these T-cell clones expressed a Th0 phenotype, whereas 1 had a Th2 phenotype. These findings are consistent with the presence in this HD lesion of an ongoing immune response against EBV-carrying cells and suggest that the complex immune deficiency that characterizes HD patients probably does not include a generalized, constitutional defect of EBV-specific T-cell responses.

INTRODUCTION

Extensive in vivo and in vitro studies carried out in patients with HD have revealed abnormalities of T-cell-mediated immune functions (reviewed in Ref. 1). Patients with untreated HD frequently exhibit impaired delayed hypersensitivity responses (2, 3), lymphocytopenia (4), reduced T-lymphocyte proliferation in response to lectins or soluble antigens (4–7), and decreased resistance to certain types of infection (8). Unlike patients with non-Hodgkin’s lymphoma receiving similar treatment regimens, these immune defects may persist in radiotherapy- or polychemotherapy-cured HD patients (9, 10). Moreover, abnormal T-cell responses have been demonstrated in healthy twin siblings and in first-degree relatives of patients with HD (11–13). These findings have suggested that the cellular immune defect may precede the development of HD, as a constitutional characteristic that may contribute to the development of the disease.

Like other persistent herpesviruses, EBV establishes lifelong infections that are asymptomatic in immunocompetent healthy individuals. HLA class I-restricted CTLs are believed to play a major role in controlling the level of EBV-carrying B cells in healthy virus carriers (14). The importance of EBV-specific memory CTLs in the long-term control of EBV infection in vivo is highlighted by the frequent occurrence of EBV reactivation and development of lymphoproliferative disorders in patients whose CTL responses are suppressed (15–17). Memory CTLs specific for EBV can be reactivated readily from the blood of healthy virus-carrying individuals by in vitro stimulation with autologous EBV-transformed B-LCLs (18). Recent evidence indicates that EBV-specific CTLs recognize a definite set of processed peptide fragments derived from the EBV latent proteins and presented at the cell surface in association with HLA class I molecules (19–21).

On the basis of epidemiological and serological findings, EBV has long been suspected to be implicated in the pathogenesis of HD (reviewed in Ref. 22). This hypothesis has been recently supported by the observation that Hodgkin’s cells and RSCs of approximately 40% of HD cases carry EBV genomes (23–26) and express some EBV-encoded antigens such as LMP-1 and LMP-2 (27, 28). These findings, however, are intriguing because LMP-1 and LMP-2 can provide target epitopes for EBV-specific CTLs and suggest that, at least in EBV-associated cases, an underlying impairment of cell-mediated immunity to EBV is present. Currently, only limited information is available with regard to EBV-specific immune responses in HD patients. Nevertheless, elucidation of this issue appears to be of relevance to better understand the complex HD-related immune suppression and its possible role in the pathogenesis of the disease.

This study was carried out with the aim to assess whether a general impairment of EBV-specific cell-mediated immunity is a common feature of HD. To this end, we have investigated EBV-specific responses in an EBV-immune patient who carried an HD lesion in which the virus was confined to a small proportion of apparently normal lymphocytes.

MATERIALS AND METHODS

Patient Characteristics and Tissue Samples. G. S., a 44-year-old Caucasian woman, presented at the Centro di Riferimento Oncologico (Aviano, Italy) with an enlargement of mediastinal and left supraclavicular lymph nodes. A biopsy of the supraclavicular lymph node performed for diagnostic purposes revealed the presence of an HD, nodular sclerosis subtype, according to the Rye modification of the Lukes and Butler classification (29). Representative
portions of the involved tissue were collected under sterile conditions and used for virological studies and for the establishment of TIL cultures, spontaneous EBV-infected LCLs, and fibroblast cultures. At the time of the diagnosis, the antibody titers against EBV-encoded antigens were as follows: anti-viral capsid antigen IgG, 1:256; anti-viral capsid antigen IgM, <1:16; anti-early antigen, 1:10; and anti-EBNA, 1:40. The HLA class I typing performed by standard microcytotoxicity tests using at least three monospecific alloantisera for the definition of each antigen revealed that the LCL established from the patient was HLA-A11, HLA-A26, HLA-B44, and HLA-B52.

**Immunohistochemistry.** Deparaffinized and cryostat sections were used for immunohistochemistry with mAbs specific for the following determinants: CD1, CD3, CD4, CD5, CD8, CD15, CD20, CD30, CD43, CD45RO, CD45, CD45R, CD74, CDw75, LN3, and epithelial membrane antigen. Immunohistochemistry was performed with the avidin-biotin-peroxidase complex or alkaline phosphatase anti-alkaline phosphatase method as described previously (30).

**Detection of EBV.** Genomic DNA was purified from 3–5-mm³ biopsy material according to conventional methods. Twenty µg of DNA were digested with the appropriate restriction endonucleases. Size fractionation of DNA fragments, Southern blotting, molecular hybridization, and autoradiography were performed as described previously (31). The following probes were used: a 3.1-kb BamHI W fragment of B95.8 EBV corresponding to the large internal repeat of the virus (32); a 1.9-kb XhoI fragment corresponding to the EcoRI D fragment adjacent to EBV right terminal repeats; and a 4.1-kb EcoRI I fragment corresponding to the EcoRI region adjacent to EBV left terminal repeats (33).

To amplify EBV sequences, PCR was performed in a reaction mixture, as described by Saiki et al. (34). A 20-mer sense oligonucleotide spanning between bases 107,963 and 107,982 and a 28-mer antisense oligonucleotide spanning between bases 108,175 and 108,202 of the EBV IE3 sequence (35) were used as primers. After incubation at 95°C for 7 min, the reaction mixture was subjected for 35 cycles to denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 1 min. For a final extension, samples were kept at 72°C for 10 min. After electrophoresis in 3.5% agarose gels, the amplified products were transferred to nylon membrane and hybridized at 55°C with a 32P-labeled antisense oligonucleotide spanning between bases 108,062 and 108,102 of the EBV IE3 sequence (35). After overnight hybridization, the membranes were washed at 50°C in 6X SSC and 0.05% sodium pyrophosphate for 15 min. The sensitivity of this PCR protocol was assessed on serial dilutions of DNA from a recombinant plasmid containing EBV IE3 sequences. After hybridization of Southern blots and overnight exposure of the autoradiograms, the assay allows to detect between 10 and 20 target molecules.

The characterization of the EBV subtypes was accomplished by a PCR amplification of the EBNA-2 region using primers specific for type 1 and type 2 EBV (36). A common 5’ primer 5’-AGGGATCCGGACGTCAGAAG-3’, a type 1-specific 3’ primer 5’-TTTGGTACAGGAGTTGAGAAA-3’ and a type 2-specific 3’ primer 5’-TTTGAAGAGGTTGTCCAAAG-3’ were used to amplify products of 249 and 300 bp, respectively. The amplification was preceded by an initial denaturation step at 95°C for 7 min and followed by a primer extension step at 72°C for 10 min. The PCR was performed for 10 cycles of 1 min at 94°C, 2 min at 55°C, and 1 min at 70°C; followed by 40 additional cycles of 1 min at 90°C, 1 min at 55°C, and 1 min at 70°C. PCR products were subsequently analyzed by electrophoresis in a 3.5% agarose gel stained with ethidium bromide and optically visualized by UV transillumination. The specificity of the amplified fragments was confirmed by Southern blot hybridization with type-specific 32P-labeled oligonucleotide probes: EBV type 1, 5’-TCCAGCCCATGGTCCTCCCTCTACGGCCGACA-3’; and EBV type 2, 5’-AACGTCGACTGCCAAGACCGGAG-3’.

In situ hybridization was performed on Bouin-fixed, paraffin-embedded tissue sections by using a mixture of FITC-conjugated oligonucleotides complementary to the two EBV-encoded nuclear mRNAs, EBER-1 and EBER-2 (Dakopatts, Glostrup, Denmark), according to the instructions of the supplier, with minor modifications. In brief, after deparaffinization, the slides were treated with 1–3 µg/ml Proteinase K (Sigma Chemical Co., St. Louis, MO), dehydrated in alcohol, air dried, and incubated for 2 h at 37°C with 10–15 µl hybridization buffer containing EBER-1/2 probes. After washings in Tris-buffered saline-0.1% Triton X-100, slides were treated with RNase A (Boehringer Mannheim, Germany; 100 µg/ml in 2× SSC) for 1 h. Hybridization products were detected by using an alkaline phosphatase-conjugated anti-FITC polyclonal antibody (Dakopatts) and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium as a chromogen. The specificity of RNA signals was ascertained by abolition of hybridization after pretreatment of sections with RNase. Slides were then counterstained with nuclear fast red, dehydrated, and mounted with a permanent mounting medium.

For the immunohistochemical detection of the EBV-encoded LMP-1 antigen, a pool of four specific mAbs (CS.1–4; Dakopatts) was used. Immunostaining was performed on Bouin-fixed, paraffin-embedded tissue sections by the alkaline phosphatase anti-alkaline phosphatase method (30). The study of EBNA-2 expression was accomplished on cryostat sections using PE-2 mAb (Dakopatts), specific for both EBNA-2A and EBNA-2B antigens (37). Positive controls consisted of smears of LMP-1- and EBNA-2-expressing EBV-infected cell lines. Negative controls consisted of consecutive test sections in which the primary antibody was replaced by nonimmune serum of the same IgG subclass (Dakopatts).

**Isolation of TILs and Cell Cultures.** Lymph node biopsy from patient G. S. was collected into sterile RPMI 1640 immediately after surgery. Necrotic tissue was removed, and the pathological tissue was gently minced into pieces smaller than 2 mm with sterile surgical blades. For TIL isolation, mononuclear cells were purified from the single cell suspension by centrifugation on Ficoll/Hypaque density gradients. After three washings in PBS, cells were seeded in 24-well plates (Costar, Cambridge, MA) at a density of 1–2 × 10⁶ cells/ml and cultured in RPMI 1640 supplemented with 2 mM l-glutamine, 10 IU/ml recombinant IL-2, 30% (v/v) filtered culture supernatant from the gibbon lymphoma line MLA144 (38), 100 IU/ml penicillin, 100 IU/ml streptomycin, and 10% heat-inactivated FCS ( Gibco-BRL, Grand Island, NY).

**Vaccinia Virus Recombinants and Fibroblast Infection.** Generation of vaccinia virus recombinants carrying the coding sequence for EBNA-2A, EBNA-5, and EBNA-6 from B95.8 virus has been described previously (39). The viruses were expressed and titrated in CV-1 cells as described (39). Subconfluent monolayers of autologous fibroblasts were exposed to the recombinant viruses at a multiplicity of infection of 0.5 for 12 h at 37°C in 96-flat well plates (Costar). Cells infected with the Vacc-EBNA-1 recombinant were coinfected with a vaccinia recombinant carrying the 77 polymerase gene to induce EBNA-1 expression (40).

**Cytotoxicity Assays.** The cytotoxic activity was assayed in standard 4-h ¹⁵¹Cr release assays (41). Appropriate panels of EBV-transformed LCLs and recombinant vaccinia virus-infected fibroblasts were labeled with Na₂¹⁵¹CrO₄ (0.1 µCi/10⁶ cells) for 1 h at 37°C and used as targets. The cytotoxicity tests were run routinely in triplicate. Under the conditions of infection described above, the spontaneous release of uninfected and recombinant vaccinia virus-infected fibroblasts did not, as a rule, exceed 15% of the total incorporation.

**Proliferation Tests.** Polyclonal TILs (10⁵ cells) and individual T-cell clones (5 × 10⁴ cells) were cocultivated with irradiated (50 Gy) LCLs or PHA blasts at a 1:1 responder:stimulator ratio. Triplicate samples were cultured in 200 µl AIM-V medium (Gibco-BRL) supplemented with 5 IU/ml recombinant IL-2 in 96-well round-bottomed microtiter plates. After a 3-day incubation at 37°C in a 5% CO₂ atmosphere, 1 µCi [³¹P]orthophosphate was added to each well during the last 6 h. The cells were harvested on a fiberglass filter, and their radioactivity was measured in a liquid scintillation counter. Results were expressed as RI, calculated from the mean [³¹P]orthophosphate incorporation of triplicate cultures using the following formula: RI = mean cpm induced by stimulator cells/mean cpm induced by medium alone.

**Isolation of T-cell Clones.** Single cell cloning was performed by limiting dilution as described previously (41). In brief, cells were seeded in 96-well, round-bottomed plates in 200 µl of RPMI 1640 supplemented with 10% FCS and containing 30% MLA144 culture supernatant (38), 10 IU/ml recombinant IL-2, and 10⁵ irradiated (30 Gy) allogeneic PHA-activated PBLs as a feeder. Growing cultures were transferred in 48-well plates and fed twice a week by replacing one-half of the medium.

**Surface Marker Analysis.** The surface antigen expression of EBV-carrying LCLs, IL-2-responsive polyclonal TIL culture and individual T-cell clones was investigated by indirect immunofluorescence staining with anti-Leu 12 (CD19), anti-Leu 16 (CD20), anti-CR2 (CD41), anti-Leu 14 (CD22), anti-Leu 20 (CD23), anti-IL-2 receptor (CD25), and anti-HLA-DR from Becton Dickinson (Mountain View, CA), OKT3 (anti-CD3), OKT4 (anti-CD4), and OKT8 (anti-CD8) from Ortho Pharmaceutical Corp. (Raritan, NJ); BL-αβTCR (anti-TCR-αβ); Immunotech, Marseille, France); and a FITC-conjugated rabbit anti-mouse IgG (Dakopatts). Aliquots of 5 × 10⁵ cells were incubated at 4°C
for 30 min with a saturating concentration of the primary mAb. After two washings in PBS, the cells were resuspended in 100 μl of the FITC-conjugated antibody and, after a 30-min incubation at 4°C, washed twice in PBS. As a control, cells were incubated with PBS instead of the first step antibody before staining with second step reagent. The percentage of positive cells was determined with a FACSort analyzer (Becton Dickinson).

Cytokine Analysis. Cytokine-specific mRNAs were detected by reverse transcriptase-PCR amplification as described (42). PCR products were separated on 1.6% agarose gel. As a control for the reverse transcription and PCR amplification steps, β2-microglobulin mRNA was amplified. The sensitivity of the reverse transcriptase-PCR for each cytokine was calibrated to give negative results in freshly separated blood lymphocytes from healthy controls. Lymphokine-specific primers were synthesized on a DNA synthesizer (Applied Biosystem). The following oligonucleotide 5' and 3' primer sequences were used: IL-1α, 5'-GCAATGACTCAGAAGACA-3' and 5'-TCTCAGGATCTCCACGCTC-3'; IL-2, 5'-TGTCAGGATCCAAGACTCTG-3' and 5'-CAATGTTGTGCTTCTCATCAG-3'; IL-3, 5'-TCTCAGGATCCAAGACTCTG-3' and 5'-AGGCTTTCTCAGGATGTTG-3'; IL-4, 5'-CGTTCAAGGATGTCGACCA-3' and 5'-CCGTTCAAGGATGTCGACCA-3'; IL-5, 5'-TGGCAAAGGCAAAGGCAGA-3' and 5'-CTCCTCGCTTCTTCTTCACCA-3'; IL-6, 5'-TGAACTCCTTCTCCCTGAAG-3' and 5'-ATGCTCAGCTTCGATCA-3'; IL-7, 5'-AGTTGATTGCTGTTCTCCAGG-3' and 5'-TGCAGACTCCATAGGAGGCGG-3'; IL-8, 5'-TCAATGACTGACTCCACGCTC-3' and 5'-AGTTGATTGCTGTTCTCCAGG-3'; and IFN-γ, 5'-TCTCAGGATCCAAGACTCTG-3' and 5'-CAATGTTGTGCTTCTCATCAG-3'.

RESULTS

Histopathological, Immunophenotypic, and Virological Characterization. The involved node showed the typical features of nodular sclerosing HD. The capsule was thickened with bands of fibrous tissue passing into the node from the capsule. Concentric periarterial fibrosis was also present. Lacunar cells were distinctly seen within cellular nodules in which the background cell population exhibited a preponderance of lymphocytes, among which scattered eosinophils and histiocytes were seen; necrosis was absent. The immunohistological staining results on frozen and paraffin sections indicated that RSCs displayed the classic (CD30+, CD15−, and epithelial membrane antigen+) phenotype. A large proportion of the lymphocytes in involved areas were CD3+, CD4+, CD5+, CD7+, CD10, CD19+, CD45RO+ (UCHL-1+), and CD45RO− (MT2+). The CD4+:CD8− ratio was 5:1 (Fig. 1, A and B).

Hybridization of BamHI-digested DNA with the BamHI W probe, specific for a reiterated sequence of EBV large internal repeat, gave

![Image](https://cancerres.aacrjournals.org/article-figures/10.1158/0008-5472.CAN-94-0819_F1A.png)

**Fig. 1.** A and B, alkaline phosphatase anti-alkaline phosphatase immunostaining on frozen tissue sections. In A, Leu3/CD4 mAb gives strong staining of most small lymphocytes surrounding RSCs, whereas in B, Leu2/CD8 mAb reacts with a smaller number of lymphocytes (hematoxylin counterstain, × 250). C and D, in situ hybridization on Bouin-fixed, paraffin-embedded tissue sections. C, an intense nuclear hybridization signal for EBV EBER-1/2 is localized to a small lymphocyte; D, RSCs are negative for EBV EBER-1/2 (nuclear fast red counterstain; C, × 250; D, × 400).
Evidence of LMP-1 or EBNA-2 expression (data not shown). On the basis of these virological findings, the HD was classified as EBV negative; the presence of the EBV genome was restricted to normal-looking lymphocytes.

Establishment and Characterization of Spontaneous EBV-transformed LCLs. From the pathological tissue of patient G. S., 11 different EBV+ LCLs have been established in vitro and subsequently expanded in long-term cultures. All of these cell lines expressed B-lineage-specific surface markers (CD19, CD20, and CD21) together with several activation markers (CD23, CD25, and HLA-DR; data not shown). The overall surface phenotype and the pattern of in vitro growth of these cell lines are comparable with those of classical LCLs obtained by EBV infection of normal B lymphocytes. Southern blot analysis confirmed the EBV positivity of these LCLs and demonstrated that each cell line carried a monoclonal EBV episome (data not shown). The presence of viral episomes of different sizes in the various LCLs indicates that the cell lines obtained were probably derived from different EBV-infected cells. As is consistent with the results of the virological analyses performed on the pathological tissue of this HD, all of the LCLs obtained were shown to carry type 1 virus (data not shown).

TIL Culture Characteristics and Detection of an EBV-specific Cytotoxic Response. Mononuclear cells isolated from the lesion were cultured in medium containing low concentrations of recombinant IL-2, in the absence of any other stimulus, to allow the expansion of in vivo activated lymphocytes (43). TILs were cultured for 15 days and subsequently cryopreserved until the autologous EBV-infected LCLs became available for functional studies. Phenotypic characterization of IL-2-responsive cells revealed a prevalence of CD3+ (94%) and CD4+ (70%) lymphocytes. CD8+ cells constituted only a minority (17%) of the culture. The observed CD4+:CD8+ ratio of 4.1:1 was similar to that observed in the fresh pathological tissue and consistent with the average ratio reported for HD (44). More than 96% of the cells stained positively with a mAb specific for TCR-αβ. The increase of CD8+ cells from 17 to 38% and a concomitant decrease of CD4+ lymphocytes from 70 to 45% was observed after 1 month of culture.

The cytotoxic activity of IL-2-responsive cells was tested against a panel of targets including autologous and allogeneic PHA blasts, several autologous LCLs (obtained by spontaneous outgrowth) carrying the endogenous EBV strain, and a large panel of appropriately HLA-matched and HLA-mismatched B95.8 virus transformed LCLs. Representative results of this analysis are reported in Fig. 2. The negative results. No evidence of monoclonal EBV episomes was obtained after the hybridization of HindIII-, BamHI-, and EcoRI-digested DNAs with the probes specific for the right and left unique sequences flanking the terminal repeats of EBV. However, the presence of EBV genomes was revealed by PCR amplification of a fragment corresponding to the EBV IR3 region (data not shown). Subsequent characterization of the EBV subtype, performed by PCR amplification of EBNA-2 sequences, revealed that, in pathological tissue of this HD, type 1 EBV only was present. In situ hybridization analysis failed to demonstrate the presence of EBV DNA or RNA in RSCs and their mononucleated precursors (Fig. 1D). EBV positivity was detected only sporadically in normal-looking bystander lymphocytes scattered throughout the tissue sections (Fig. 1C). In addition, immunohistochemical analysis gave no evidence of LMP-1 or EBNA-2 expression (data not shown). On the
presence of an EBV-specific cytotoxic component was revealed by the lysis of autologous LCLs but not of HLA-mismatched LCLs and autologous PHA blasts. Three different autologous LCLs used as targets in these experiments were all recognized efficiently with equivalent levels of lysis (data not shown). The cytotoxic response was mainly HLA-A11 and HLA-B44 restricted, but a minor HLA-A26-restricted component was also present (Fig. 2).

The EBV antigen specificity of the polyclonal TIL culture was investigated using as targets autologous fibroblasts infected with recombinant vaccinia viruses expressing individual EBV-latent antigens. Vacc-LMP-2 recombinants and, to a lesser extent, Vacc-EBNA-6 sensitized the target cells to lysis, whereas infection with the other recombinants and with Vacc-TK− controls yielded the same low background lysis (Fig. 3). These results were reproducibly obtained in four different experiments.

Proliferative Responses. Proliferative responses to autologous or allogeneic LCLs were evaluated by [3H]thymidine incorporation of triplicate cultures using the following formula: RI = mean cpm induced by stimulator cells/mean cpm induced by medium alone. Allogeneic proliferation was tested using two different HLA-mismatched LCLs. The counts of control cultures ranged from 500 to 1500 cpm. ++, Auto/Ri/Allo RI >5; +, Auto/Ri/Allo RI between 2 and 5; +/−, Auto Ri/Allo RI between 1 and 2; −, Auto Ri/Allo RI −1.

Proliferative responses to autologous and allogeneic EBV+ LCLs. A summary of the results is shown in Table 1. Two of the 18 T-cell clones investigated (clones 1 and 2) exerted an EBV-specific cytotoxic activity with a significant lytic activity of autologous EBV+ LCLs and no cytotoxicity against allogeneic HLA-mismatched targets (Table 1). Additional characterization of these clones, including blocking experiments with anti-HLA class I or II antibodies, was not possible because of a shortage of cells. Five T-cell clones had a significant proliferative capacity in response to autologous but not allogeneic EBV+ LCLs (Table 1). In particular, clone 4 showed a remarkably high proliferative response (Auto Ri/Allo RI > 5) to different autologous EBV+ LCLs. Some of the clones proliferating in response to autologous but not allogeneic LCLs also showed detectable levels of allocytotoxicity (clones 4, 12, and 14), probably as a result of a certain degree of natural killer-like cytotoxic activity acquired during in vitro culture.

Cytokine Gene Transcription of Individual EBV-specific T-Cell Clones. The cytokine expression profile of EBV-specific T-cell clones was assessed by reverse transcriptase-PCR on RNA samples from unstimulated cells cultured in complete medium. Table 2 shows the results of the analysis of four EBV-specific T-cell clones. The cytokine mRNA expression pattern of clones 1, 2, and 3 was consistent with a mixed Th0-like profile, whereas that of clone 4 was compatible with a Th2-like pattern (Table 2).

DISCUSSION

Clinical and laboratory findings have indicated that HD patients frequently show a derangement of cellular immune responses, and it has been suggested that these alterations may contribute to the development of the disease (1–13). In particular, the frequent occurrence of abnormally elevated titers of antibodies against EBV antigens before the onset of overt disease (45) and the detection of impaired cellular responses to EBV in HD patients (46) have led to the hypothesis that a defective control of EBV-infected cells may be involved in the pathogenesis of the disease. The recent demonstration that RSCs of EBV-associated HD cases usually express EBV-encoded antigens, such as LMP-1 and LMP-2 (27, 28, 47), despite the fact that these proteins can be the sources of target peptides for EBV-specific CTLs (20, 21), again pointed to the presence of an impaired immune response against EBV-infected cells, at least in EBV+ cases. At present, however, it is not clear whether a defective immunity to EBV is a characteristic restricted to EBV-associated HD cases or, on the contrary, is a more generalized phenomenon, part of the inherent immune deficiency of HD patients. In this study, we have critically addressed this issue by investigating the phenotypic and functional characteristics of polyclonal and clonal TIL cultures established from the pathological tissue of an HD in which EBV infection was confined to normal bystander lymphocytes. We reasoned that, if immune suppression is a characteristic feature of HD, EBV-specific responses should not be detected independent of the EBV status of the tumor. The analysis of cellular responses to EBV was carried out on lymphoid cells derived from a lymph node because PBLs from HD patients are frequently reduced in number (1, 4) and show an abnormal distribution of T-cell subsets with respect to lymphoid tissues (48, 49).
Our results are consistent with the presence within the TIL population of an EBV-specific cytotoxic T-cell component restricted mainly through the HLA-A11 and HLA-B44 class I alleles. This cytotoxic response closely resembled that of EBV-specific memory CTLs, which may be reactivated from the PBLs of healthy donors by in vitro stimulation with autologous LCLs (18–21). Moreover, the analysis aimed at defining the antigenic specificity of the cytotoxic effectors present within the TIL culture revealed that these CTLs recognized LMP-2, and to a lesser extent, EBNA-6 antigens. The prevalence of these specificities may be accounted for by the lack of any in vitro restimulation, which presumably allowed the analysis to reveal the antigens recognized by the cytotoxic effectors preactivated in vivo (43). The EBV-specific cytotoxic class I-restricted component probably represented a minority of the in vivo infiltrate because, at the time of the analysis, only 17% of the TIL culture was composed of CD8+ T lymphocytes.

Additional functional analyses revealed that polyclonal TILs also showed a significant proliferative response when cocultured with autologous, but not allogeneic, EBV+ LCLs or autologous EBV− PHA blasts. These findings, together with the isolation of five CD4+ T-cell clones with the same proliferative capacity, indicate that, in addition to displaying an EBV-specific cytotoxic activity, TILs of this HD also comprised a subset of helper T cells able to proliferate in response to EBV antigens. Two CD4+ T-cell clones were shown to exert an EBV-specific cytotoxicity, suggesting that CD4+ T lymphocytes also may partly contribute to the EBV-specific cytotoxic activity displayed by the polyclonal cultures. Consistent with this finding is the previous observation that more than 50% of CD4+ T-cell clones derived from HD spleens or lymph nodes displayed cytolytic activity (50). Characterization of the cytotoxic expression profile of four EBV-specific CD4+ T-cell clones revealed that one proliferative clone probably belonged to the Th2 functional subset, which provides help for immunoglobulin synthesis (51). An unrestricted cytokine mRNA expression profile, resembling that of Th0 cells (51), was observed in the other three EBV-specific CD4+ T-cell clones. These findings suggest that either a proportion of EBV-specific CD4+ T-cell clones does not fit into clearcut Th1 or Th2 phenotypes or, alternatively, that these Th0-like clones represent memory precursors, which may differeniate into Th1 or Th2 cells upon additional antigen stimulation.

Because the EBV-specific cytotoxic and proliferative responses were demonstrated in polyclonal cultures and individual T-cell clones not previously stimulated with EBV antigens or mitogens and established in vitro with low doses of recombinant IL-2, it is likely that the EBV-specific effector cells present in these cultures represent the expansion of T lymphocytes activated previously in vivo. These findings, therefore, indicate that an ongoing immune response against EBV-infected cells was present in this HD lesion. EBV-specific immune responses have been studied almost exclusively in PBLs, and no information is currently available with regard to lymphoid tissues. The identification of functional EBV-specific T cells in a lymph node provides, for the first time, evidence that this may be the site at which memory EBV-specific T lymphocytes exert the physiological control of EBV-infected cells. Although additional investigations are required to conclusively assess whether the immune response to EBV observed in the HD lesion analyzed is qualitatively and quantitatively similar to that detectable in normal lymph nodes, the detection of functional EBV-specific T lymphocytes suggests a conserved immune competence of this patient in terms of memory responses to EBV. In support of this hypothesis, we have also observed that the number of normal EBV-infected bystander lymphocytes present in the pathological tissue of this HD was not abnormally increased when compared with that in reactive lymphadenopathies (data not shown). In addition, at the time of the diagnosis, the patient showed normal antibody titers against EBV-encoded antigens.

In conclusion, although limited to a single HD case, the findings of this study seem to indicate that the presumed impairment of cell-mediated immunity ascribed to HD patients probably does not include a generalized, constitutional defect of EBV-specific T-cell responses. In addition, the identification of functional responses to EBV in a patient with an EBV− HD provides added support to the hypothesis that EBV-associated and EBV-unrelated HD cases have distinct pathogenetic mechanisms.

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