Cytotoxic \( \gamma \delta \) T Lymphocytes Associated with an Epstein–Barr Virus-Induced Posttransplantation Lymphoproliferative Disorder

Marc E. Rothenberg,*§ Wim E. J. Weber,** Janina A. Longtine,** and David A. Hafler**

*Division of Immunology and Division of Hematology/Oncology, Department of Pediatrics, Children’s Hospital, Boston, Massachusetts 02115; †Center for Neurologic Diseases, Department of Neurology, and ‡Department of Pathology, Brigham and Women’s Hospital, Boston, Massachusetts 02115; and §Harvard Medical School, Boston, Massachusetts 02115

T cells expressing the \( \gamma \delta \) T cell receptor have been implicated in anti-Epstein–Barr virus (EBV) immunity and in the pathogenesis of autoimmune diseases of the central nervous system. However, they have never been isolated from human brain tissue for direct analysis. We now report a 6-year-old girl with EBV-associated posttransplantation lymphoproliferative disease involving inflammatory brain lesions. A high proportion of \( \gamma \delta \) T cells was found in the blood and in a brain lesion. Cultured T cell lines were found to have a remarkably high frequency of responsiveness to an EBV-transformed line, with a 17-fold enrichment in the brain lesion. These T cells expressed predominantly the \( \gamma \delta \) T cell receptor and mediated non-MHC-restricted cytotoxicity against EBV-infected target cells. These results provide the first demonstration of an association of \( \gamma \delta \) T cells with a posttransplantation lymphoproliferative disease and suggest a role of \( \gamma \delta \) T cells in mediating inflammatory processes in the brain.

**INTRODUCTION**

Epstein–Barr virus (EBV) infections may give rise to different clinical syndromes depending upon the immune status of the host (1). In immunocompetent hosts, EBV infection results in infectious mononucleosis and the EBV-infected B lymphocytes are effectively cleared by specific cytotoxic T lymphocytes. In contrast, in immunocompromised hosts with impaired T cell function, EBV infection may develop into a lymphoproliferative disorder in approximately 1–10% of patients (2–8). For example, EBV can induce a posttransplantation lymphoproliferative disorder (PTLD) following EBV infection secondary to the outgrowth of infected B lymphocytes. In the early stage, PTLD begins with a polyclonal expansion of EBV-infected B cells that can often resolve following reduction in the degree of immunosuppression. However, in the advanced stage, a monoclonal expansion of EBV-infected lymphocytes occurs, resulting in a high rate of mortality.

T cells expressing the \( \gamma \delta \) T cell receptor (TCR) have been implicated in anti-EBV immunity (9–11). \( \gamma \delta \) T cells recognize heat shock proteins and recent data suggest that a subset (V\( \gamma \)2/V\( \delta \)2) may selectively recognize a nonprotein phosphonucleotide antigen found in a number of microorganisms. We have observed a high frequency of \( \gamma \delta \) T cells in brain lesions of patients with multiple sclerosis (12) and in the CSF of patients with subacute sclerosing panencephalitis (13); however, viable \( \gamma \delta \) T cells have not been directly isolated from brain tissue.

We now report the case of a 6-year-old girl suffering from systemic PTLD with the unusual feature of multifocal central nervous system (CNS) gray matter lesions. \( \gamma \delta \) T cells responsive to an EBV-infected B cell line were identified at high frequency in her blood and in a brain lesion and were found to mediate HLA nonrestricted cytotoxicity of Epstein–Barr virus-infected target cells.

**CASE REPORT**

The patient was a female full-term product of healthy unrelated parents who was noted to have progressive irritability, failure to thrive, and organomegaly during the first year of life. After the diagnosis of tyrosinemia, an orthotopic liver transplantation from an unrelated donor was performed at 18 months of life. The initial transplantation course was complicated by two mild episodes of acute rejection treated with OKT3, prednisone, and cyclosporin A. Over the next 3 years, the patient had recurrent mild viral illnesses associated with cervical adenopathy and gingivoglossitis treated with acyclovir, one episode of cytomegalovirus colitis, and uncomplicated chicken pox. She did well developmentally and excelled to the top of her school class. She was eventually weaned to every other day prednisone (5 mg) and maintained on cyclosporin A (150 mg bid).

At the age of 6 years, she began to develop recurrent fevers every 2–3 weeks. After 6 months, these fevers were associated with severe generalized lymphadenop-
lesions. At 7 1/2 years old, she was taken off all immuno-
suppressive therapy in attempt to boost her anti-EBV
T cell immunity. However, her seizure frequency in-
creased and new MRI lesions were found. A cervical
lymph node biopsy showed features characteristic of
PTLD (described below). The patient continued to de-
velop cyclic episodes of episodelia partialis continua
(lasting 1–2 weeks) associated with generalized lymph-
adenopathy, high fevers, and massive splenomegaly;
she subsequently became quadriplegic and barely re-
sponsive to commands. The patient was treated with
high dose-IVIG (250 mg/kg/day) and interferon-α (3
million units/m²/day) (14, 15). After 2 weeks of this
additional therapy, the patient showed no improve-
ment; the IVIG was reduced to 1 g/kg/week and the
interferon-α was increased to 5 million units/m²/day.
The patient continued to have persistent symptoms in-
ducing fever, hepatosplenomegaly, thrombocytopenia,
intermittent leukopenia, refractory status epilepticus,
and progressive lesions on MRI. Further testing at this
time included a bone marrow biopsy which showed no
malignant cells and normal cytogenetics; urine and ce-
rebrospinal fluid analysis for succinylactetone (a
marker for tyrosinemia) revealed only trace amounts
in the urine (kindly performed by Dr. Peiro Rinaldo,
M.D., Yale); and a liver biopsy did not show any signs
of rejection and only minimal fibrosis.

After 6 weeks of failed therapy, at the age of 8 years,
the patient underwent biopsy of a left occipital brain
lesion (Fig. 1). Two days following the brain biopsy,
the patient developed rapidly progressive generalized
adenopathy, particularly in the neck, worsening of
baseline seizures, macroglossia, and required emergent
intubation for airway protection. This occurred during
empiric dexamethasone therapy to reduce periopera-
tive brain edema. She subsequently received whole
brain and neck irradiation (1800 cGy in eight divided
fractions) with no significant improvement in seizure
activity or MRI lesions, and only a modest improve-
ment in the adenopathy. Her clinical course continued
to worsen and aggressive medical therapy was gradu-
ally reduced. She died 3 months later.

METHODS

Preparation of DNA and Southern Analysis

DNA was extracted from peripheral blood cells on
three occasions over the course of 10 months, from bone
marrow, and from the lymph node tissue by standard
techniques. Ten micrograms of DNA was digested with
restriction endonucleases, electrophoresed in 0.8%
agarose gels, transferred to nylon membranes, and hy-
bridized to 32P-labeled probes. Clonality of the EBV-
infected lymphocytes was assessed by analysis of im-
munoglobulin and TCR rearrangement (16, 17), and
analysis of EBV genome clonality was based on the structure of the terminal repeat unit (18).

Immunophenotyping

Immunophenotyping studies were performed by indirect immunoperoxidase staining on paraffin sections (19). Quantification of lymphocyte phenotypes in the brain was performed by analysis of serial sections following staining with specific antisera against the \( \alpha \) or \( \delta \) chain. Two reviewers counted three regions within the perivascular cuff and determined the percentage of immunopositive infiltrative cells. Peripheral blood leukocytes or cell lines were stained with FITC- or PE-labeled antibodies (all from Becton–Dickinson) either alone or in combination for dual immunofluorescence. Analysis was performed by examining fluorescence using a Becton–Dickinson FACS IV and gating for lymphocytes based on light scatter as previously reported (20). TCR\( \gamma \delta \), a monoclonal antibody recognizing the \( \gamma \delta \) TCR, was kindly provided by Dr. M. Brenner (21) and used for staining tissue sections.

T Cell Culture and Assays

EBV-transformed B cell lines were derived in vitro from the patient, her HLA-identical sister, and an unrelated donor as described (22). T cell lines were generated by limiting dilution cultures of mononuclear cells isolated from blood and brain tissue stimulated with HLA-identical EBV-transformed B cells. Briefly, peripheral blood mononuclear cells were separated by Ficoll–Hypaque density gradient. T cells were obtained by mincing brain biopsy tissue through a sieve under sterile conditions. Cell populations were cultured in limiting numbers in microwells containing 10\(^4\) irradiated (7000 rad) EBV-transformed B cells in RPMI 1640 with 10% (v/v) pooled human AB\( ^+ \) serum. Recombinant interleukin 2 (rIL 2, Boehringer Mannheim, 10 U/ml) was added to cultures after 48 hr. Microcultures were fed at weekly intervals with fresh 10\(^4\) irradiated autologous EBV-transformed B cells and rIL 2. After 18–22 days of culture, wells were assessed for proliferation by light microscopy, for EBV-specific cytolysis and for T cells expressing the \( \gamma \delta \) TCR by fluorescent activated cell sorter analysis. Cytotoxicity assays were performed using \( ^{3} \)H-chromium release assay as described, with an effector:target ratio of 20:1 (23). Maximum release was always more than 10\(^{\times}\) spontaneous release. Minimal estimates of precursor frequency were obtained by the maximum likelihood method from the Poisson distribution relationship of the number of cells plated per culture and the logarithm of the percentage of nonresponding cultures (23).

RESULTS

A cervical lymph node biopsy was performed at 8 years of age and demonstrated histologic features characteristic of polymorphic PTLD (2, 3, 6, 7). The nodal architecture was diffusely effaced by an infiltrate comprised of small lymphocytes with irregular nuclear outlines, plasma cells, plasmacytoid cells, histiocytes, immunoblasts, and occasional Reed–Sternberg-like cells. Immunophenotyping studies showed B cells (L26\(^{+}\) positive) present in small residual follicular centers and throughout the lymph node as small and large transformed cells. Frequent small cells and occasional large transformed cells were present that stained with UCHL-1 (CD45R0) consistent with the presence of T lymphocytes. Immunoreactivity for latent membrane protein (LMP) was present in scattered large transformed cells and immunoblasts.

Hybridization of lymph node DNA with probes to the Ig heavy chain gene (\( J_{\mu} \)) and \( \kappa \) light chain (\( C_{\kappa} \)) revealed two moderately intense clonal rearrangements (Fig. 2) indicating B cell clonal expansion. No clonal rearrangements of the \( \lambda \) light chain gene were identified (data not shown). In addition, a dominant strongly hybridizing band was identified in the lymph node DNA using a probe to the terminal repeat unit of EBV (18). This probe documents the presence of EBV DNA and confirmed the clonality of the EBV-infected cells. A weakly hybridizing band of identical size was also present in the blood (Fig. 2) and bone marrow (data not shown).
This documents systemic involvement by this B cell done (24). A clonal rearrangement of the TCR β chain gene was identified within the lymph node using a probe to the joining region (Fig. 2). No clonal rearrangement of the TCR γ chain was seen using a probe to the Jγ region (data not shown). These data suggest that either EBV transformation occurred in an early lymphoid cell prior to complete gene rearrangement or alternatively a clonal population of T cells may also have been present.

On two occasions (age of 7 and 8 years), the patient demonstrated intact delayed-type hypersensitivity to intradermal injection of Candida antigens indicating preserved T cell function. In addition, T cell proliferation in vitro to both mitogens and antigens averaged about 50% of control (data not shown). FACS analysis of peripheral blood (Table 1) was notable for the presence of a large percentage of activated CD3+ cells as assessed by expression of HLA-DR molecules (52%). There was a modest increase in the percentage of cytotoxic T cells and a decrease in natural killer cells compared to normal reference controls. Most surprising was a fivefold increase in the CD3+ cells that expressed the TCR-γδ (19%). Most of these cells were double-negative T cells (CD4−/CD8−); no lymphocytes coexpressed the TCR-γδ and CD4 and only 5% coexpressed CD8.

Cerebrospinal fluid analysis revealed protein of 40 mg/dl, glucose of 40 mg/dl, and a leukocyte counts of 35 cells/mm³ (which remained stable at four separate times), which were predominantly lymphocytes. FACS analysis of these lymphocytes revealed that they were 100% T cells as assessed by CD3 staining. Cerebrospinal fluid lymphocytes were found not to express EBV nuclear antigen-2 (antiserum from Dr. Eliot Kieff) or LMP, two EBV proteins expressed in infected cells, by immunohistochemical staining (data not shown).

Microscopic analysis of the brain biopsy specimen revealed severe and widespread reactive gliosis with numerous gemistocytic astrocytes and destroyed axons and myelin (Fig. 3). There was a lymphoid infiltrate composed of small cells with round or irregular nuclei admixed with occasional large cells. The infiltrate was present as dense perivascular cuffs, within the leptomeninges and scattered throughout the cortex. Immunophenotyping studies performed on cryostat sections revealed that the perivascular cuff was composed of B cells (CD20 [L26], CD22 [Dako pan-B] positive) with no definite immunoglobulin light chain immunoreactivity. T cells (CD2 and CD5 positive) were present scattered within the perivascular cuff and predominantly associated with reactive changes. The T cells included a mixture of CD4- and CD8-positive cells. Immunophenotyping for the TCR revealed the presence of CD3-positive T cells that were a mixture of αβ T cells and γδ T cells. The γδ T cells accounted for 18–36% of these cells depending upon the brain area that

### TABLE 1

<table>
<thead>
<tr>
<th>CD determinant</th>
<th>Target</th>
<th>Percentage of cells</th>
</tr>
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<tbody>
<tr>
<td>CD3</td>
<td>TCR complex</td>
<td>93 ± 2 (n = 8)</td>
</tr>
<tr>
<td>CD4</td>
<td>MHC class II-restricted T cells</td>
<td>29 ± 9 (n = 8)</td>
</tr>
<tr>
<td>CD8</td>
<td>MHC class I-restricted T cells</td>
<td>52 ± 6 (n = 8)</td>
</tr>
<tr>
<td>CD16</td>
<td>Natural killer cell</td>
<td>2 ± 1 (n = 8)</td>
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<tr>
<td>CD19</td>
<td>B cell</td>
<td>5 ± 3 (n = 8)</td>
</tr>
<tr>
<td>CD45</td>
<td>Panlymphocyte marker</td>
<td>95 ± 4 (n = 5)</td>
</tr>
<tr>
<td>CD3/DR</td>
<td>CD3 and HLA-class II coexpression</td>
<td>52 ± 10 (n = 3)</td>
</tr>
<tr>
<td>CD19/DR</td>
<td>CD19 and HLA-class II coexpression</td>
<td>4 ± 1 (n = 3)</td>
</tr>
<tr>
<td>CD3/CD25</td>
<td>CD3 and IL-2r coexpression</td>
<td>1 ± 1 (n = 3)</td>
</tr>
<tr>
<td>CD3/TCR-αβ</td>
<td>CD3 and TCR αβ coexpression</td>
<td>72 ± 2 (n = 3)</td>
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<td>CD4 and TCR γδ coexpression</td>
<td>0 (n = 2)</td>
</tr>
<tr>
<td>CD8/TCR-γδ</td>
<td>CD8 and TCR γδ coexpression</td>
<td>5 ± 1 (n = 2)</td>
</tr>
</tbody>
</table>

*The usual lymphocyte count was 3000 cells/mm³. All values are expressed as the percentage of lymphocytes ± standard deviation unless otherwise indicated; values in parentheses are the number of determinations. Normal values were determined on control donors performed in parallel except when indicated by “a” which represent cumulate normal values for the clinical immunology laboratory at Children’s Hospital.

b These determinations are expressed as means ± range.

p < 0.5 as determined using two-way analysis of variance between the patient and control donors analyzed at the same time.
FIG. 3. (a) Brain biopsy reveals perivascular cuffs of predominantly small lymphocytes which extend into the adjacent parenchyma (hematoxylin and eosin, ×100). (b) A higher power magnification reveals predominantly small lymphocytes and reactive gliosis with numerous gemistocytic astrocytes (hematoxylin and eosin, ×300).

was examined. No immunoreactivity for EBV nuclear antigen-2 was present. Studies for LMP revealed cytoplasmic immunoreactivity in the areas of gliosis, apparently in macrophages based on KP-1 (CD68) immunostaining of these cells in serial sections.

In order to further study the T cells involved in this patient's PTLD, a frequency analysis of growth-positive wells after primary stimulation with autologous EBV B cells was performed on peripheral mono-nuclear cells and on cell populations derived from the brain biopsy shown in Fig. 3. A high frequency of growth-positive microcultures was found in both populations, with a 17-fold increase in the brain compared to peripheral blood, 1/45 vs 1/750, respectively (Fig. 4). Most of the brain-derived T cell lines expressed the γδ TCR (Table 2) and some lines had 100% of the cells expressing the γδ TCR (data not shown). Additionally, all of the EBV-stimulated T cell lines were able to mediate potent cytotoxicity against EBV-infected target cells (data not shown). Cytotoxicity was not HLA-restricted as all (n = 110) the lines from the peripheral blood lysed both HLA-matched and HLA-mismatched B cell targets. Representative data for the cultured T cell lines included 31, 29, and 25% specific cytotoxicity against alloreactive targets for lines grown from the wells initially plated at 10^2, 10^3, and 10^4 cells/well, respectively. In all cases, maximum cytotoxicity was at least more than 10× spontaneous cell death with specific cytotoxicity >20%.

DISCUSSION

We have described an unfortunate 6-year-old girl who developed an EBV-associated PTLD 4 years after a liver transplantation. The most unusual clinical aspect was the unique CNS disease that developed. CNS lymphomas are a reported complication of organ transplantation (2, 25, 26). However, they characteristically involve mass lesions that develop in the perivascular spaces and infiltrate the adjacent parenchyma without associated brain tissue destruction and gliosis. There has been one reported case of transient fleeting CNS lesions associated with acute EBV encephalomyelitis (27). In our patient, the CNS lesions were non-mass gray matter lesions that were migratory, leaving minimal residual signs on serial MRI scans. There was no evidence of a CNS lymphoma and EBV could not be demonstrated in the CSF or in a brain lesion biopsy.
Instead the brain lesion contained an inflammatory cell infiltrate composed predominantly of lymphocytes. In areas of brain tissue destruction and reactive gliosis there was a T lymphocyte infiltrate which included γδ T cells. Additionally, there was a high proportion of γδ T cells in the circulation. In previous reports, γδ T cells have been associated with EBV infections in patients with infectious mononucleosis (9–11). The association with EBV-induced PTLD now suggests a more generalized role of γδ T cells in EBV processes.

Active EBV infection could not be definitively identified in the CNS of this patient and we were therefore interested in analyzing the T cells in order to further understand the cause of this patient’s unique PTLD. Two surprising findings were encountered. First, a remarkably high frequency of cells responsive to the EBV-transformed B cell line was found in the blood and brain lesion with a 17-fold enrichment in the brain (nearly 2% of the brain T cell lines being stimulated). Second, most (~79%) of the these T cell lines were found to express the γδ TCR. T cells expressing the αβ TCR are the predominant type and outgrow γδ T cells in culture (11, 28). In fact, γδ T cells expressing the γδ TCR from biological tissue usually requires extensive purification of γδ T cells prior to culture (11, 28). The growth of a high proportion of γδ T cells under these standard culture conditions offered further evidence that the T cells in this patient were unusual.

Other studies have identified γδ T cells in the brain based on TCR rearrangements and immunohistochemistry; however, this is the first report isolating viable γδ T cell lines from the brain. Functional studies revealed that these T cells were able to mediate potent killing of autologous and allogeneic EBV-infected B cells. Since the T cell lines were not cloned and were unable to be maintained in long-term culture, it was not possible to assess which population of T cells was directly mediating the cytotoxicity. However, the alloreactivity of these lines suggests that this killing was not MHC restricted as would be expected for αβ T cells. The γδ T cells in this patients may have been responding directly to an EBV antigen. Alternatively, they may have been responding to an EBV-induced cellular antigen as has been previously demonstrated for other γδ lines in vitro (11, 29). The lack of EBV detection in the brain of this patient suggests that the latter response was more likely. γδ T cells that respond to both autologous and allogeneic EBV-transformed B-lymphoblastoid cell lines have been found in patients with autoimmune diseases such as reactive arthritis and are thought to represent "autoreactive" cells (28). These results demonstrate an association of γδ T lymphocytes with EBV-induced PTLD and suggest a role of these cells in inflammatory processes in the brain.

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