

Brief Report

**IMMUNOLOGIC ANALYSIS OF A
SPINAL CORD-BIOPSY SPECIMEN
FROM A PATIENT WITH HUMAN
T-CELL LYMPHOTROPIC VIRUS
TYPE I-ASSOCIATED
NEUROLOGIC DISEASE**

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HUMAN T-cell lymphotropic virus type I (HTLV-I) is associated with adult T-cell leukemia and a chronic progressive neurologic disease, HTLV-I-associated myelopathy-tropical spastic paraparesis (hereafter referred to as HTLV-I-associated myelopathy).¹⁻⁵ HTLV-I is endemic in Japan, the Caribbean, Africa, and South America.⁵ Risk factors for infection include sexual contact, exchange of blood products, and vertical transmission from mother to child.⁵ HTLV-I-associated myelopathy causes progressive myelopathy with atrophy of the spinal cord.^{5,6} Subcortical white-matter lesions are sometimes present on magnetic resonance imaging.⁵⁻⁷ Cerebrospinal fluid shows pleocytosis, elevated titers of IgG, and oligoclonal bands.⁸⁻¹⁰ Autopsy results correlate with neurologic findings and show spinal cord atrophy with loss of myelin and axons.¹¹⁻¹⁴ The neuropathological findings provide evidence that immune-mediated mechanisms may have a role in the pathogenesis of the disease.¹⁵ Leptomeninges and blood vessels are infiltrated by lymphocytes that penetrate surrounding parenchyma.^{13,14,16-19} Early in the disease, lymphocytes are abundant, with equal numbers of CD8+ cells and CD4+ cells. B lymphocytes and macrophages are present in areas of paren-

chymal damage.^{11,16} Later in the course, there are fewer inflammatory cells, and these are almost exclusively CD8+ cells.^{12,18}

Patients with HTLV-I-associated myelopathy have an activated immune response^{5,11} with exceptionally high levels of CD8+ cytotoxic T lymphocytes specific for HTLV-I in peripheral blood and cerebrospinal fluid.^{20,21} The role of this immune response in relation to HTLV-I infection and central nervous system damage is unclear. Viral load may be a factor, since affected patients have 50 times more HTLV-I proviral DNA in peripheral-blood lymphocytes than do HTLV-I-seropositive persons who are asymptomatic.²² The localization of HTLV-I-infected cells to the central nervous system may also be crucial. Amplification with the polymerase chain reaction (PCR) of HTLV-I DNA from central nervous system specimens obtained at autopsy showed that the DNA was present where lymphocytes predominated²³ and in areas devoid of immune-cell infiltration,^{24,25} implying infection of non-immune cells. In situ hybridization showed that HTLV-I RNA was present in CD4+ lymphocytes²⁶ and astrocytes.²⁷

We had the opportunity to study a spinal cord-biopsy specimen from a patient with rapidly progressive HTLV-I-associated myelopathy who had gadolinium-enhanced lesions of the spinal cord. Analysis of the biopsy specimen demonstrated infiltration of leptomeninges and adjacent spinal cord parenchyma by numerous mononuclear cells. CD8+ T lymphocytes and macrophages predominated. Functional studies of T-cell lines derived from the biopsy specimen showed HTLV-I-specific cytotoxic-T-lymphocyte activity, providing *in vivo* evidence of the role the immune response may have in the pathogenesis of HTLV-I-associated neurologic disease.

CASE REPORT

A 45-year-old black woman from the southern United States reported a "wobbling gait" four years before admission. Urinary incontinence, constipation, and numbness of the legs developed, and she became wheelchair-bound within 15 months as a result of weakness in her legs. A general physical examination revealed only edema of the legs and a hyperpigmented maculopapular rash. The blood pressure was 148/86 mm Hg. Neurologic examination showed dysphagia, dysarthria, moderate weakness of the arms, and paraplegia. Tone was increased in the legs and normal in the arms. Tendon reflexes were brisk. Babinski signs were present. There was a decreased response to pinprick at the midthoracic level and decreased sensitivity to vibration in the legs.

The coagulation profile, complete blood count, and electrolyte levels were normal. The glucose concentration was 174 mg per deciliter (9.7 mmol per liter; normal range, 70 to 115 mg per deciliter [3.9 to 6.4 mmol per liter]). The IgG concentration was 1550 mg per deciliter (normal range, 523 to 1482), and the IgM concentration was 890 mg per deciliter (normal range, 37 to 200). Laboratory tests for Lyme disease, syphilis, rheumatologic disease, vitamin deficiency, and the human immunodeficiency virus were negative. The test for HTLV-I was positive and was confirmed by Western blotting. A cerebrospinal fluid sample contained 2 white cells per cubic millimeter (normal range, 0 to 5), 27 mg of protein per deciliter (normal range, 15 to 45), 81 mg

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of glucose per deciliter (4.5 mmol per liter; normal range, 40 to 70 mg per deciliter [2.2 to 3.9 mmol per liter]), and 7.3 mg of IgG per deciliter (normal range, 0.8 to 4.1), and oligoclonal bands were present; the IgG index was 1.40 (normal range, 0.26 to 0.62).

Cytologic analysis revealed atypical lymphocytes in cerebrospinal fluid. Cultures of cerebrospinal fluid were negative. A barium-swallow examination showed esophageal dysmotility. Neuroelectrophysiologic tests were normal except for abnormal central nervous system responses in somatosensory evoked potentials in the legs. Magnetic resonance imaging showed nonenhancing periventricular white-matter lesions in the brain, spinal cord atrophy, and gadolinium-enhanced lesions along the posterior thoracic cord (Fig. 1). Skin biopsy revealed lymphoid infiltrates with epidermotropism, suggestive of adult T-cell leukemia or mycosis fungoides. The degree of cytologic atypia was minimal, and an assay for interleukin-2 receptor was negative.

A spinal cord biopsy was performed to rule out a malignant condition of the central nervous system. There were no complications of the biopsy, and a postoperative neurologic examination showed no changes.

METHODS

Immunocytochemical Analysis

Tissue blocks were fixed in 10 percent formalin or frozen at -70°C . Frozen sections were fixed with acetone. Formalin-fixed tissues were embedded in paraffin, and the slides were deparaffinized with xylene and rehydrated in saline. We used an avidin-biotin-peroxidase technique with antibodies against Leu3a (CD4+) and Leu2a (CD8+) (Becton Dickinson, Mountain View, Calif.) and KP-1 (macrophages) and CD45RO (activated T cells) (Dako, Carpinteria, Calif.). Secondary antibodies were applied, and diaminobenzidine was used as the chromagen. Slides of frozen sections were enhanced with nickel chloride.

PCR

Serial dilutions of human cells and preparation of DNA for solution-phase PCR were performed.²⁸ PCR for the *HTLV-I-pol* gene used primers SK110 and SK111,²⁹ and the amplified product was detected with an enzyme oligonucleotide assay according to the manufacturer's instructions (Cellular Products, Buffalo,

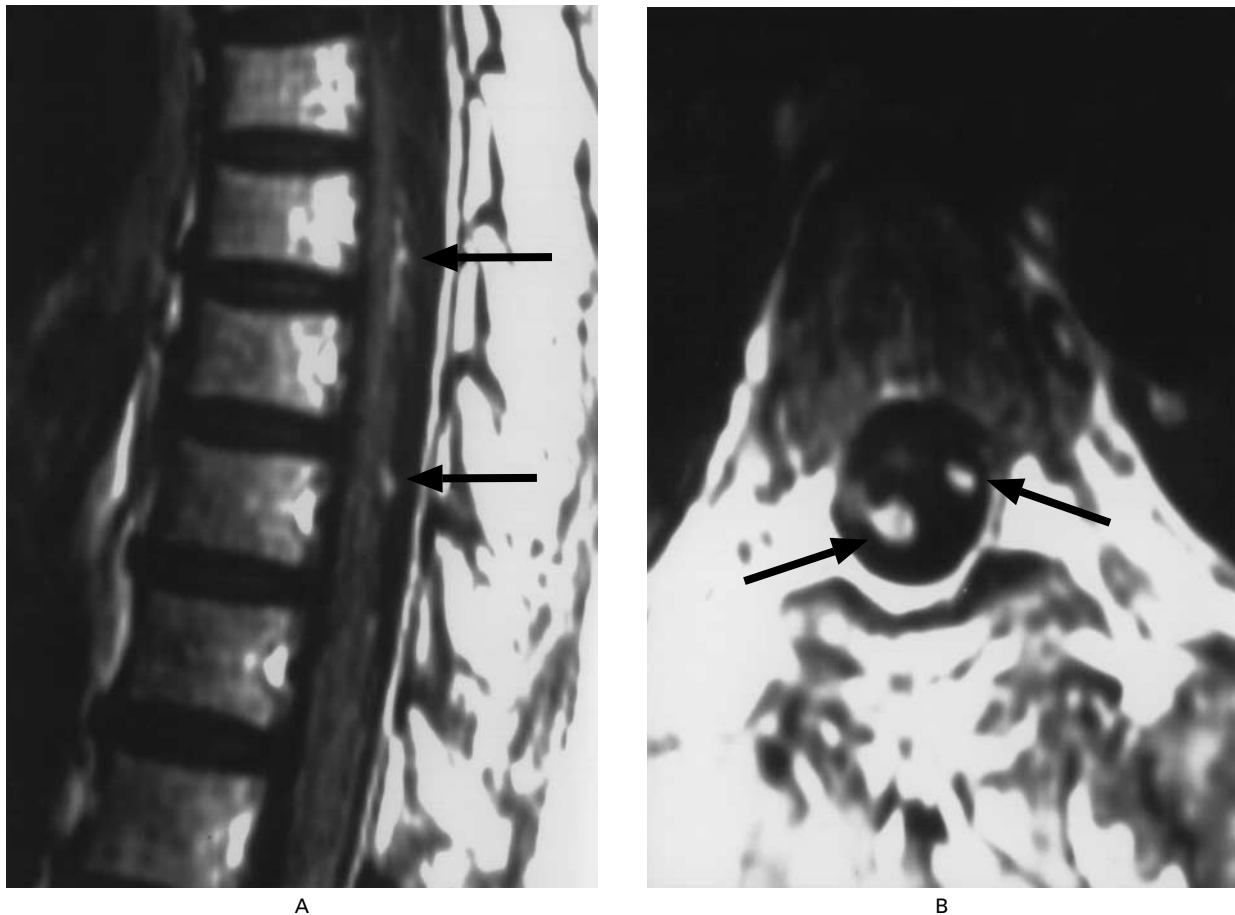
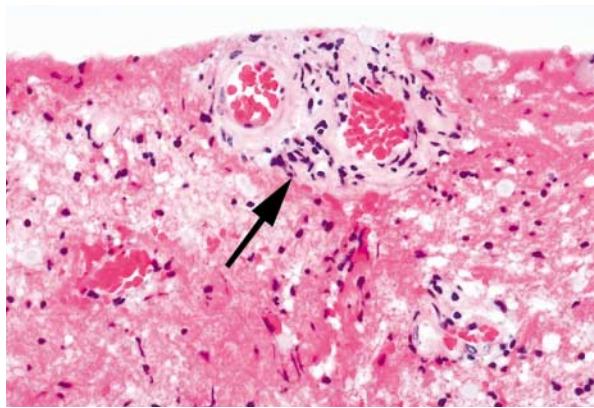
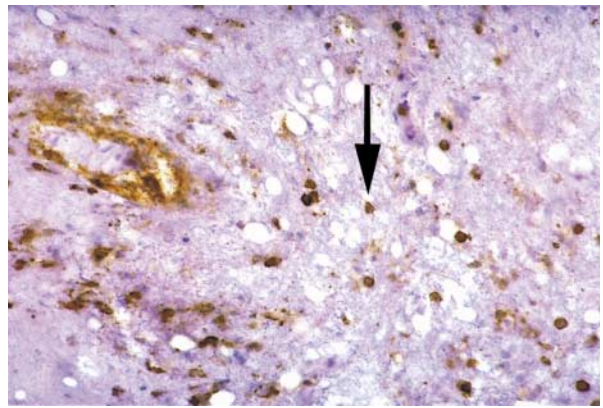


Figure 1. Magnetic Resonance Images of the Spinal Cord of a Patient with HTLV-I-Associated Myelopathy.

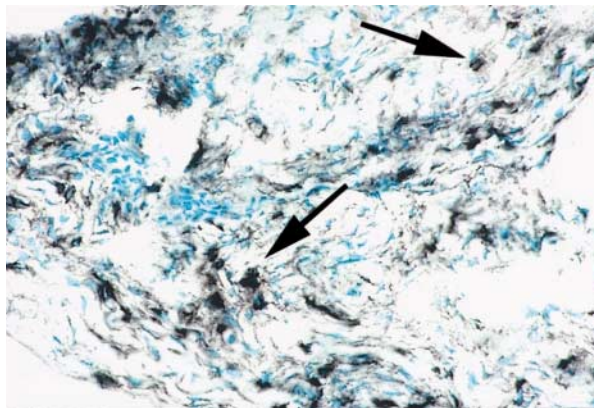
Panel A shows a T_1 -weighted sagittal view after the administration of gadolinium. There are several irregular linear focal areas of enhancement along the posterior aspect of the thoracic spinal cord (arrows). Panel B shows a T_1 -weighted transaxial view of the areas of gadolinium enhancement shown in Panel A. There are areas of focal enhancement associated with the leptomeninges and parenchyma (arrows) in an area of abnormal parenchyma. This is the area from which the spinal cord-biopsy specimen was surgically resected.



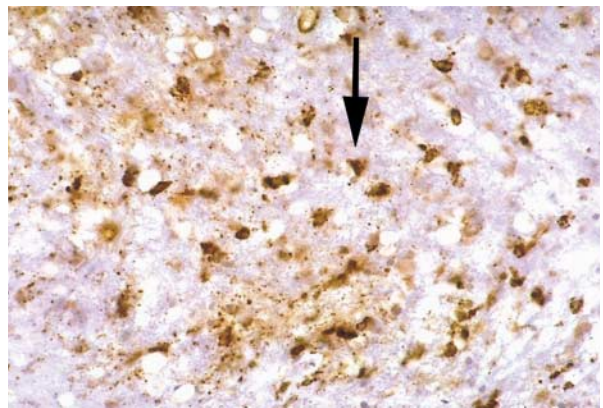
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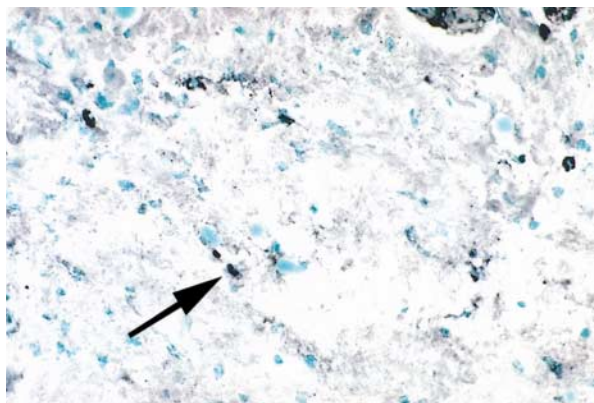
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Figure 2. Immunocytochemical Analysis of the Immune-Cell Infiltrate from the Spinal Cord–Biopsy Specimen ($\times 400$).

There were numerous mononuclear cells (arrow in Panel A) in the biopsy specimen (hematoxylin and eosin). The majority of cells were T cells, as demonstrated by their reactivity to CD3 (data not shown). Most were CD8+ cells (arrows in Panel B), with a few CD4+ cells (arrow in Panel C). In Panels B and C, the areas in which there was an immunocytochemical reaction stained black (methyl-green counterstain). The T cells were also positive for CD45RO, which recognizes activated T cells (arrow in Panel D). There was also a robust macrophage response (arrow in Panel E). In Panels D and E, the areas in which there was an immunocytochemical reaction stained brown (hematoxylin counterstain). Essentially no staining for B cells was observed (data not shown).

N.Y.). A response that was more than 0.075 optical-density unit above the response of the negative control was deemed positive.

Cell Culture

The fresh spinal cord–biopsy specimen was washed repeatedly with RPMI-1640 medium containing 10 percent fetal-calf serum, 1 percent glutamine, and 1 percent penicillin–streptomycin before being placed in 24-well plates (Costar, Cambridge, Mass.) in RPMI medium containing 15 percent fetal-calf serum, 5 percent human AB serum, 10 U of recombinant interleukin-2 per milliliter, 5 percent natural interleukin-2 (Cellular Products), and a 1:10,000 dilution of OKT3 ascites. One week later, nonadherent cells were removed and cultured with 300,000 irradiated (3000 rad) autologous cells per milliliter. Cell cultures were maintained with weekly additions of irradiated autologous cells and interleukin-2. After four weeks there were sufficient cells for fluorescence-activated cell sorting and immunologic assays.

Cytotoxic T-Lymphocyte Assays

Cytotoxicity assays were performed,^{20,21} and the degree of lysis by cytotoxic T lymphocytes specific for HTLV-I was calculated as described previously.²⁰ As targets, 1 million CD4+ cells expressing HTLV-I or autologous B-cell lines⁷ transformed by Epstein–Barr virus and infected with HTLV-I vaccinia recombinants were used at a concentration of 5000 cells per well.

Flow Cytometry

The expression of cell-surface antigen was analyzed by flow cytometry (FACScan, Becton Dickinson)⁸ with primary antibodies against Leu3a, Leu2a, and Leu4 (Becton Dickinson). The control antibody was mouse IgG (Becton Dickinson), with secondary goat antimouse IgG conjugated with fluorescein isothiocyanate (Cappel, West Chester, Pa.).

RESULTS

Magnetic resonance imaging showed atrophy of the cervical and thoracic spinal cord.¹¹ An injection of gadolinium revealed irregular focal areas of enhancement along the posterior thoracic cord (Fig. 1). Magnetic resonance imaging of the brain demonstrated nonenhancing periventricular white-matter lesions on T₂-weighted images and degeneration of the corticospinal tract.^{11-14,16-18}

After thoracic laminectomy, multiple biopsy specimens were obtained from the arachnoid membrane, surrounding nerve root, and dorsal parenchyma. The arachnoid membrane was abnormally thick, but no discrete mass was identified. Histologic examination showed thickened arachnoid membranes, pallor of the white matter, and parenchymal hypercellularity. Meninges, parenchyma, and perivascular areas were infiltrated by numerous mononuclear cells (Fig. 2A). There were no neoplastic or leukemic cells, such as those associated with adult T-cell leukemia. The mononuclear infiltrates were mostly activated T cells (Fig. 2D). Most T cells were CD8+ cells (Fig. 2B), with some CD4+ cells (Fig. 2C). There were numerous macrophages (Fig. 2E) and no B cells.

To assess the viral load, semiquantitative PCR of *HTLV-I-pol* DNA from serial dilutions of the patient's unstimulated peripheral-blood lymphocytes and cerebrospinal fluid cells was performed. *HTLV-I-pol* DNA was detected in as few as 100 cerebrospi-

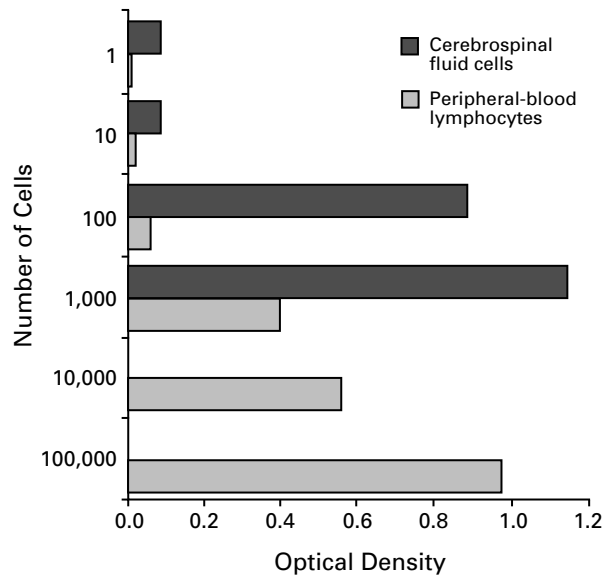


Figure 3. Results of Enzyme Oligonucleotide Assay of *HTLV-I-pol* DNA.

The DNA was obtained from fresh, unstimulated peripheral-blood lymphocytes and cerebrospinal fluid cells from the patient. The optical-density value is proportional to the quantity of amplified *HTLV-I-pol* DNA. The assay was not done on 10,000 or 100,000 cerebrospinal fluid cells because not enough cells could be generated. A total of 100,000 peripheral-blood lymphocytes and 1000 cerebrospinal fluid cells were serially diluted, and PCR was performed on all samples simultaneously. Amplified *HTLV-I-pol* DNA was detected with an enzyme oligonucleotide assay that uses an enzymatically labeled oligonucleotide probe internal to the *HTLV-I-pol* primers, and optical density was measured. There was a higher viral load in cerebrospinal fluid cells than in peripheral-blood lymphocytes. Furthermore, *HTLV-I-pol* DNA could be detected in as few as 100 cerebrospinal fluid cells, whereas at least 1000 peripheral-blood lymphocytes were necessary for detection.

nal fluid cells but not in an equivalent number of peripheral-blood lymphocytes (Fig. 3). This shows that the viral load of the cerebrospinal fluid cells was at least 10 times greater than that of peripheral-blood lymphocytes.

Cytotoxic T lymphocytes specific for HTLV-I (Fig. 4A) recognized the *HTLV-I-pX* gene and to a lesser extent the *HTLV-I-env* gene. In addition, T-cell lines were derived from lymphocytes obtained from the biopsy specimen and tested for cytolytic activity with two types of target cells: the same autologous B-cell lines infected with the HTLV-I vaccinia recombinants that were used to assess the cytotoxic-T-lymphocyte activity of the peripheral-blood lymphocytes and an HTLV-I-expressing CD4+ T-cell line that was derived from the patient's cerebrospinal fluid lymphocytes. A predominantly CD8+ T-cell line (Fig. 4C) derived from the biopsy specimen lysed the HTLV-I-infected cerebrospinal fluid target

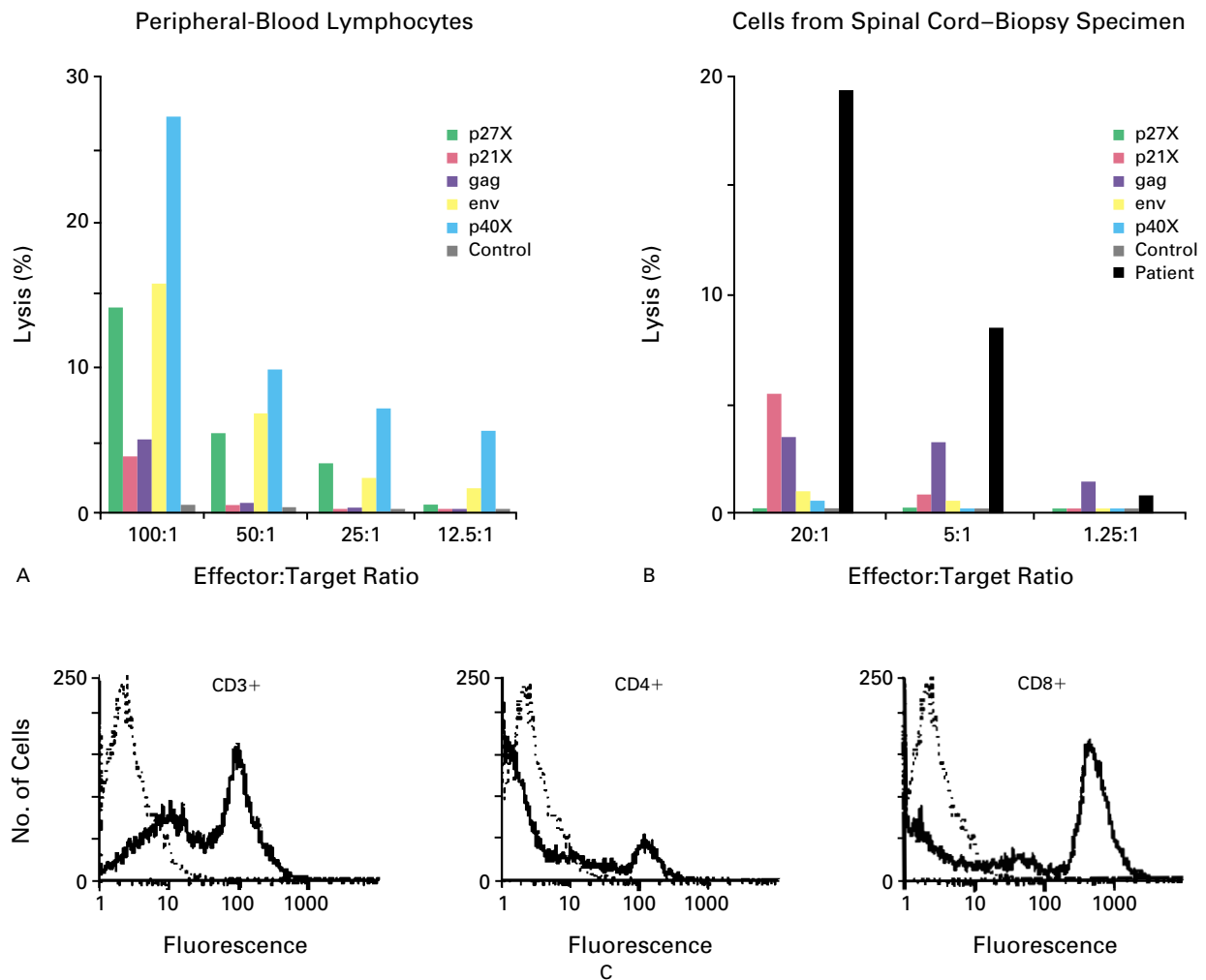


Figure 4. Cytotoxic-T-Lymphocyte Profile of Peripheral-Blood Lymphocytes and Cells Derived from the Spinal Cord–Biopsy Specimen and Results of Flow Cytometry of the Cells Derived from the Biopsy Specimen.

In Panel A, the effector cells are peripheral-blood lymphocytes isolated directly from the patient's blood (without in vitro stimulation)^{20,21} and the target cells are autologous B cells infected with various HTLV-I vaccinia recombinants and a control vaccinia recombinant containing the hemagglutinin of influenza virus. The effector cells were incubated with chromium-labeled target cells, and the degree of lysis of each target was calculated by measuring the release of radioactivity into the supernatant. Peripheral-blood lymphocytes from the patient recognized a number of HTLV-I target cells, particularly in the case of the *HTLV-I-pX* region (p27X and p40X) and the *HTLV-I-env* region. In these experiments there was no lysis by peripheral-blood lymphocytes from HTLV-I-infected patients with no neurologic symptoms or HTLV-I-seronegative normal controls (data not shown).^{20,21}

Panel B shows the cytotoxic-T-lymphocyte profile of the central nervous system cells derived from the spinal cord–biopsy specimen. The effector cells are mononuclear cells derived from the biopsy specimen. The target cells are identical to those in Panel A, with the addition of a CD4+ HTLV-I-infected line derived in vitro from the patient's cerebrospinal fluid (Patient). HTLV-I infection was confirmed by flow cytometry with antibody against HTLV-I-gp46 (data not shown). Cells derived from the spinal cord–biopsy specimen had a strong response to the CD4+ HTLV-I-infected target cells (lysis of almost 20 percent) and a smaller response to an area of the *HTLV-I-pX* region (p21X).

Panel C shows the results of flow cytometry of the cells derived from the patient's spinal cord–biopsy specimen. The intensity of fluorescence of the cells (solid lines) is plotted relative to that of the mouse IgG control (dotted lines). Most of the cells were CD3+ and CD8+ cells, with a few CD4+ cells.

cell line, whose specificity (as defined by the autologous B-cell constructs) may be confined to *HTLV-I-pX* gene products (Fig. 4B), although the degree of lysis was low.

DISCUSSION

We provide evidence of immunologic events in the central nervous system of a patient with HTLV-I-associated myelopathy that is based on studies of a spinal cord–biopsy specimen. There was a direct correlation between the presence of gadolinium-enhanced lesions and the immunopathological findings in the spinal cord specimen. Leptomeningitis was present in which activated CD8+ T lymphocytes predominated. By contrast, few CD4+ cells were detected. B lymphocytes were virtually absent, and there were numerous macrophages. In addition, the inflammatory response extended into the parenchyma. Moreover, the patient had a much larger viral load in cerebrospinal fluid than in peripheral-blood lymphocytes. Since cerebrospinal fluid lymphocytes may reflect events in the central nervous system better than peripheral-blood lymphocytes, the large viral load in cerebrospinal fluid lymphocytes may expose the central nervous system to HTLV-I infection. *HTLV-I-tax* RNA was demonstrated in the biopsy specimen by in situ hybridization, although the cell phenotype was not identified. The rapid deterioration in the patient's condition may have been related to a large viral load in the central nervous system, which triggered an enhanced CD8+ cell immune response.

The demonstration of activated CD8+ cells in the spinal cord–biopsy specimen of this patient is consistent with the results of previous reports¹²⁻¹⁸ and supports the hypothesis that HTLV-I-associated myelopathy is an immunopathologically mediated disorder.^{5,11,15} However, it is still difficult to prove that the CD8+ cells present in these lesions are the same cytotoxic T lymphocytes as those in peripheral-blood lymphocytes²⁰ or cerebrospinal fluid lymphocytes.²¹ For a more accurate determination of the in vivo specificity of T cells in the lesion, a tissue culture of the central nervous system specimen was nonspecifically expanded in vitro without biasing the selection toward HTLV-I reactivity. A T-cell line generated from this material was composed predominantly of CD8+ cells and lysed HTLV-I-infected target cells. Lower levels of lysis were also demonstrated with recombinant HTLV-I-expressing target cells, most notably those expressing the *HTLV-I-pX* gene. These results support the view that CD8+ HTLV-I-specific cytotoxic T lymphocytes may be present in central nervous system lesions of patients with HTLV-I-associated myelopathy and contribute to the damage caused by the disease.

The exact mechanism by which the central nervous system is damaged in HTLV-I-associated myelopathy

has yet to be determined. HTLV-I-specific cytotoxic T lymphocytes may damage resident central nervous system cells, such as HTLV-I-infected astrocytes, directly.²⁷ The HTLV-I-specific cytotoxic T lymphocytes may cause indirect damage by secreting toxic levels of cytokines.²⁶ Finally, molecular mimicry may occur, in which cytotoxic T lymphocytes recognize cross-reactive autoantigen expressed on target cells, leading to central nervous system damage. Although it is uncertain which mechanism predominates, it is clear that the HTLV-I-associated immune response may have an important role in the immunopathogenesis of HTLV-I-associated neurologic disease and may be useful in therapeutic interventions.

Dr. Levin is the recipient of a National Multiple Sclerosis Society fellowship award.

We are indebted to James Corbett, M.D., for the referral and care of this patient.

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