both alleles of the perforin gene. These findings indicate that some cases of CAEBV are due to defects in the perforin gene.

Materials and methods

Cell culture

Blood was drawn after obtaining informed consent from the patient, his parents, and additional patients with CAEBV studied at the National Institutes of Health (Bethesda, MD) under a protocol approved by the institutional review board of the National Institute of Allergy and Infectious Diseases. Blood was also obtained from anonymous healthy blood bank donors and from patients in Japan with CAEBV in whom patient identifiers had been removed from the specimens. Cryopreserved PBMCs were thawed, cultured in RPMI 1640 containing 10% fetal bovine serum supplemented with 10 IU/mL interleukin 2 (IL-2; Roche Molecular Biochemicals, Mannheim, Germany) and 1 × phytohemagglutinin (PHA; Invitrogen, Carlsbad, CA) for 2 days, followed by IL-2 without PHA for 2 weeks. PBMCs and EBV-transformed B cells from patients in Japan with CAEBV[5] were also studied.

Cytokine assays

Cells were stimulated for 36 hours with plate-bound anti-CD3 antibody (1 μg/mL, UCHT1; BD Pharmingen, San Diego, CA) plus anti-CD28 antibody (1:5000, CD28.2; BD Pharmingen) and cell culture supernatants were collected as previously reported.[18] IL-4, -5, -10, interferon-γ (IFN-γ), and tumor necrosis factor α (TNF-α) levels were all assayed with a Luminex 100 multiplex analyzer, with Luminex IS analysis software (Luminex, Austin, TX) using multianalyte microsphere kits (LINCO Research, St Charles, MO). IL-13 levels were determined by enzyme-linked immunosorbent assay (ELISA; Pierce-Endogen, Pierce Biotechnology, Rockford, IL).

Real-time PCR for EBV

Real-time quantitative polymerase chain reaction (PCR) assay with a fluorogenic probe was performed using a TaqMan PCR kit and a Model 7700 Sequence Detector (Applied Biosystems, Foster City, CA). A portion of the *Bam*HI W fragment of EBV was amplified using forward primer 5′-GAGGGGGACCACTGCCCCTGG-3′ and reverse primer 5′-CGCTCTGATGCGACCAGA-3′ and detected with fluorogenic probe 5′-(6FAM)-TCCTGCAGCTATTTCTGGTCGCATCA-(TAMRA)-3′. The bcl-2 gene was amplified using forward primer 5′-CCTGCCCTCCTTCCGC-3′ and reverse primer 5′-TGCATTTCAGGAAGACCCTGA-3′ and detected with fluorogenic probe 5′-(6FAM)-CTTTCTCATGGCTGTCC-(TAMRA)-3′.

Reverse transcriptase (RT)–PCR and Southern blotting for EBV

Total RNA was isolated from stimulated PBMCs and cDNA was synthesized. Primers and probes for EBV genes and PCR conditions were used as previously described.[19] PCR products were separated by electrophoresis, transferred to nylon membranes, and hybridized with [^{32}P]-labeled oligonucleotide probes.

Sequencing of the perforin gene

DNA was extracted from stimulated PBMCs and the perforin gene was amplified by PCR and sequenced as previously described.[20] Nucleotide positions of perforin are numbered according to GenBank accession number X13224. cDNA of perforin exon 3 was amplified by RT-PCR using forward primer (F577) 5′-AACTTTGCAGCCCAGAAG-3′, and reverse primer (ADR)[20] 5′-TTGCATCTCACCTCATGGGAAC-3′ and the sequence of the cDNA at nucleotides 577 and 1229 of perforin was determined.

Mutagenesis of the perforin gene and expression in cells

RNA was extracted from PBMCs of a healthy donor and cDNA was synthesized with reverse transcriptase. Perforin cDNA was amplified by PCR using forward primer 5′-ATTCTCGAGATGGCAGCCCGTCTGCTCCT-3′ and reverse primer 5′-TATGTCGACTCACCACACGGCCCCACTCC-3′. The PCR product was inserted into the *Eco*RI site of plasmid pCIneo (Promega, Madison, WI). Site-directed mutagenesis was performed to change nucleotide 577 from T to C (mother's perforin mutation), nucleotide 1229 from G to C (father's perforin mutation), and nucleotide 1122 from G to A (FHL mutation) using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Mutation of each clone was confirmed by DNA sequencing. 293T cells were transfected with plasmid(s) encoding perforin (2 μg/6-cm dish) using the Fugene 6 transfection system (Roche Biochemicals) and cell lysates were prepared 48 hours after transfection for immunoblotting or immunoprecipitation.

Results

Case report

A 7-year-old white boy presented with heterophile-positive infectious mononucleosis followed by persistent splenomegaly and lymphadenopathy. One year later he underwent splenectomy for hypersplenism; at surgery, mesenteric lymphadenopathy and chylous ascites were noted. Histiocytosis with erythrophagocytosis was demonstrated in the spleen. He was treated with adriamycin, vincristine, prednisone, and cyclophosphamide for lymphadenopathy and presumptive lymphoma. Three years later at age 11, at his first evaluation at NIH, he was found to have T-cell lymphoproliferative disease involving the liver, bone marrow, and a supraclavicular lymph node. His EBV serologies were markedly elevated with an anti-EBV VCA immunoglobulin G (IgG) titer of 20 480, anti-EA IgG titer of 20 480, and anti-EBNA IgG titer of 10, indicating the possibility that the lymphoproliferative process was driven by an EBV infection. Hypertriglyceridemia (2.8 mM) and hypofibrinogenemia (1.4 g/L) were noted. He was treated with acyclovir for 4 weeks without clinical benefit. On initial evaluation and over the ensuing several months, progressive pancytopenia and hypogammaglobulinemia were noted and he received intravenous immunoglobulin. The following year he presented with cholestatic hepatitis secondary to T-cell lymphoproliferative disease of the liver. Although he received interferon alpha, the T-cell lymphoproliferative process persisted and cutaneous leukocytoclastic vasculitis, pneumonia, and chronic sinusitis were noted. Computerized tomography showed reduction in the size of his abdominal and peripheral lymphadenopathy on prednisone and cyclophosphamide; these effects were sustained on prednisone and azathioprine. Lymphocyte phenotype of the peripheral blood showed 98.9% T cells and 0.5% B cells. Markedly elevated EBV titers (VCA IgG 20 480, EA-IgG 20 480) persisted, and a monoclonal IgG kappa gammopathy and Coombs positive hemolytic anemia were present. He died at age 18 due to lymphoproliferative disease and disseminated candida infection. Autopsy was limited to the liver, lymph nodes, lungs, and brain and showed polyclonal T-cell infiltration of the liver and mesenteric lymph nodes with loss of cortical, paracortical, and germinal center architecture. Rare T-cell infiltrates were present in the lungs and disseminated candidiasis involving the lungs, liver, and brain was noted.

The patient fit the diagnostic criteria of CAEBV[3,4,21] based on (1) symptoms of persistent lymphadenopathy, hepatosplenomegaly, and bone marrow hypoplasia that began after infectious mononucleosis; (2) extremely high EBV-specific antibody titers; and (3) EBV-positive cells in the lymph nodes on in situ hybridization. He also fit the diagnostic guidelines for hemophagocytic

lymphohistiocytosis[22] based on his fever, splenomegaly, pancytopenia, hypertriglyceridemia, and histologic evidence of hemophagocytosis. However, he did not fit the criteria for familial hemophagocytic lymphohistiocytosis (FHL), since there was no family history of hemophagocytic lymphohistiocytosis. The patient's brother and both parents are EBV seropositive and have no history of EBV-associated or immunologic diseases. None of the other family members whose history could be ascertained, including the patient's aunts, uncles, grandparents, and maternal great-grandparents, have a history of EBV-associated or immunologic diseases. Furthermore, patients with FHL are usually diagnosed in infancy or early childhood and die within a few years of the diagnosis,[23,24] whereas our patient lived for 18 years. Therefore, our patient was diagnosed with CAEBV, rather than FHL.

Pathology and immunohistochemistry

Multiple lymph node biopsies (cervical, mesenteric, splenic hilar) obtained during the course of the patient's disease showed similar histologic findings (Figure 1). The architecture was effaced by an infiltrate composed predominantly of small normal-appearing lymphocytes with admixed histiocytes. A similar infiltrate was seen in the bone marrow and liver. The spleen showed marked histiocytosis with erythrophagocytosis of red pulp.

Immunohistochemical studies in all sites showed predominately $CD3^+$ T cells, with a CD4/CD8 ratio of approximately 1:4. Numerous cells were positive for granzyme B; however, cells were consistently negative for perforin (Figure 1G-H). Germinal centers were absent; however, scattered individual $CD20^+$ cells and small aggregates were seen. In situ hybridization with the EBV encoded RNA (EBER) probe showed only rare positive small lymphocytes (< 1 per high-power field). Southern blot analysis for clonal T-cell receptor beta gene rearrangements showed no evidence of a clonal population (data not shown).

Lymphocyte phenotype, EBV infection, and cytokine profile in PHA and IL-2–stimulated PBMCs from the patient with fatal CAEBV

PBMCs from the patient described above, his parents, and healthy donors were stimulated with PHA and IL-2 for 2 days followed by IL-2 alone for 2 weeks in vitro. More than 50% of the stimulated cells were $CD8^+$ (Figure 2A). While blood from the patient's mother and a healthy donor contained more than 30% $CD56^+$ and $CD8^-$ cells (normal range of healthy donors, 7.8%-35%; average, 14.3%; standard error, 2.5%), blood from the patient and his father had less than 5% $CD56^+$ and $CD8^-$ cells (Figure 2B). The patient's father also had a small population of $CD56^+$ and $CD8^+$ cells (NK T cells).

Patients with CAEBV frequently have elevated levels of EBV DNA in their PBMCs, T cells, NK cells, or tissues.[5,25,26] Real-time PCR showed that stimulated PBMCs from the patient contained high levels of EBV DNA (Figure 2C). EBV DNA was not detected in PHA and IL-2–stimulated PBMCs from the patient's parents and healthy donors; however, stimulated PBMCs from an unrelated patient with CAEBV had elevated levels of EBV DNA. EBV-infected cells from patients with CAEBV consistently express the latency-associated genes EBV nuclear antigen 1 (EBNA-1) and EBERs; however, other latency-associated genes such as EBNA-2 and latent membrane protein 1 (LMP-1) are expressed in cells from some, but not all, patients.[26] Stimulated PBMCs from our patient expressed EBNA-1, but not EBNA-2, LMP-1, or LMP-2A (Figure 2D, data not shown). These results indicate that the stimulated PBMCs from the patient had a type I EBV latency program.

Some patients with CAEBV have been reported to have dysregulation of cytokines with increased expression of both T-helper (Th) 1 and Th2 cytokine transcripts, termed an "unbalanced cytokine profile."[25] Stimulation of cells from the patient with antibodies to CD3 and CD28 resulted in high levels of both Th1 (IFN-γ) and Th2 (IL-4, IL-10, and IL-13) cytokines (Figure 2E). These cytokines are involved in regulation of cytokine production, activation, and proliferation of lymphocytes and monocytes. Cells from the patient's father showed similar elevations in some (IL-4, IL-13), but not all (IFN-γ, IL-10) of the cytokines found to be elevated in his son. In contrast, cells from an unrelated patient with CAEBV did not show cytokine elevations. These data suggest that stimulated PBMCs from the patient had an unbalanced cytokine profile.

Mutations in the perforin gene of the patient and his parents

Since many patients with CAEBV have hemophagocytic syndrome, and since perforin mutations have been found in patients with FHL,[20] we determined the sequence of the entire coding region of the perforin gene (exons 2 and 3) in our patient as well as

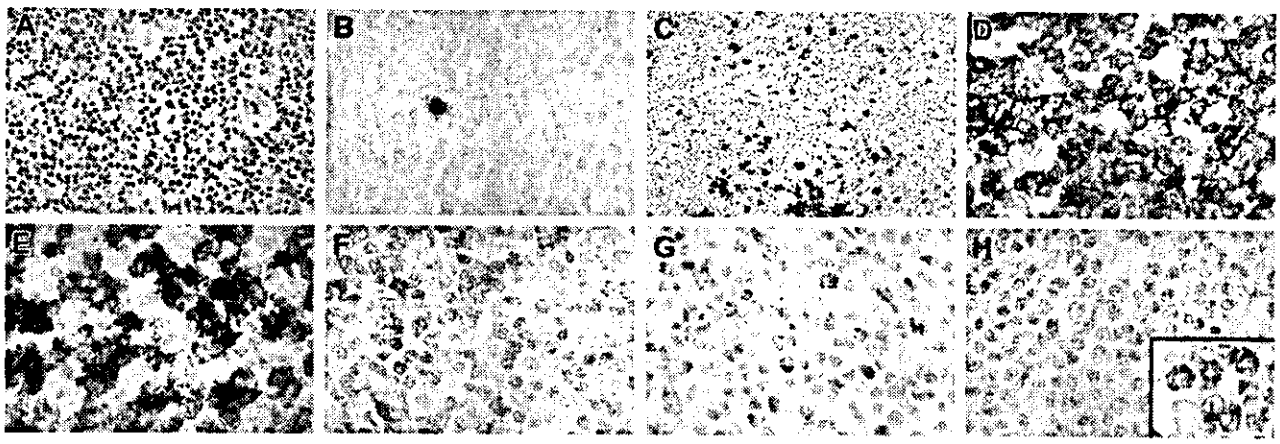

Figure 1. Characterization of T-cell lymphoproliferative process in lymph node. (A) Architecture is diffusely infiltrated by small lymphocytes with admixed histiocytes. (B) In situ hybridization with an EBV-encoded RNA (EBER) probe shows rare positive small lymphocytes (< 1 per high power field) that correspond to the distribution of B cells in the same area. (C) CD20 stain shows scattered small positive lymphocytes and small aggregates, but absence of follicular structures. The majority of the lymphocytes are $CD3^+$ (D), with a predominance of $CD8^+$ cells (E), over $CD4^+$ cells (F). Numerous lymphocytes are granzyme B positive (G), but negative for perforin (antibody KM585-P1-8; Kamiya Biochemical, Seattle, WA) (H). Inset in panel H shows positive perforin control in a patient with large granular lymphocyte leukemia. Original magnification, × 400, except panel C (× 250) and panel H inset (× 1000).

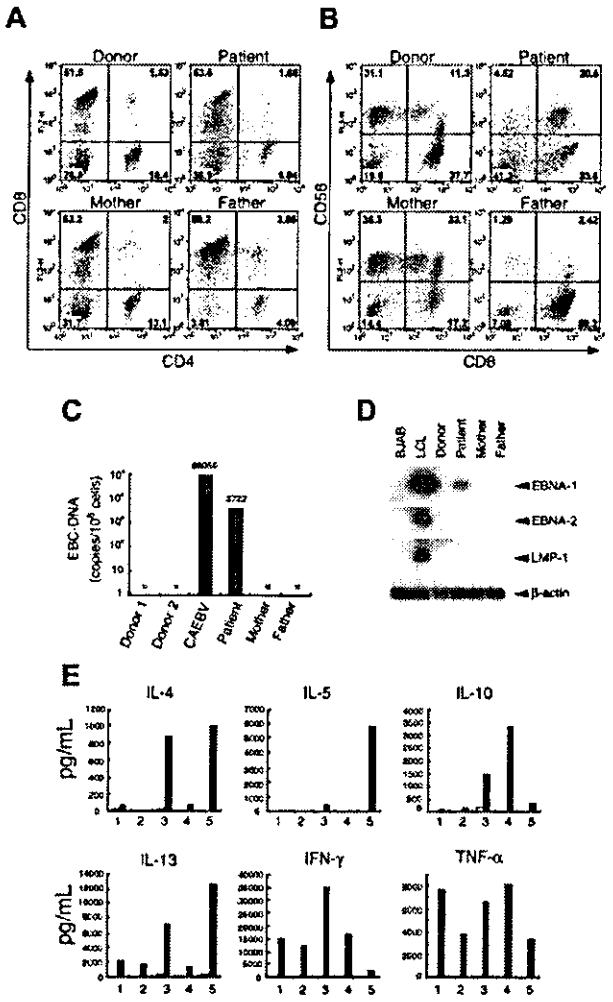

Figure 2. Lymphocyte phenotype, EBV DNA levels with latent gene expression, and cytokine levels in stimulated PBMCs from the patient, his parents, and a healthy blood bank donor. (A) CD4/CD8 and (B) CD8/CD56 expression of PBMCs stimulated with PHA and IL-2 for 2 days followed by IL-2 alone for 2 weeks. (C) EBV DNA levels in stimulated PBMCs. DNA from the EBV *Bam*HI W region DNA was quantified using real-time PCR. The EBV *Bam*HI W fragment copy number per cell was calculated by the formula N = 2 × (W/B), where N is the EBV *Bam*HI W copy number/cell, W is the EBV *Bam*HI W copy number, and B is the bcl-2 copy number. Copy numbers of EBV-*Bam*HI W gene per 1×10^6 cells are indicated. U indicates undetectable. (D) RT-PCR analysis of EBV latency genes in stimulated PBMCs. Southern blots of PCR products were hybridized with [^{32}P]-labeled probes. (E) Cytokine levels were measured in culture supernatants of stimulated PBMCs from a healthy donor (1), an unrelated patient with CAEBV (2), the patient with the perforin mutations (3), the patient's mother (4), and the patient's father (5). Cells were stimulated with anti-CD3 antibody (black bars) or isotype control antibody (white bars). The experiment was performed 3 times with similar results.

14 other patients with CAEBV (12 patients from the United States, 2 patients from Japan). Whereas none of the 14 other patients with CAEBV showed any mutations in their perforin genes, sequence analysis showed that our patient had mutations in both alleles of his perforin gene. Of 6 cloned PCR products from the patient's genomic DNA, 2 clones had a T to C mutation at nucleotide 577 of the perforin gene, and 4 had a G to C mutation at nucleotide 1229 (Figure 3A). The change in nucleotides 577 and 1229 are predicted to change amino acid 193 from phenylalanine to leucine, and amino acid 410 from arginine to proline, respectively. To verify that these changes were due to mutations and not to polymorphisms, DNAs were obtained from 54 blood bank donors (predominantly white, like our patient) and PCR analyses (corresponding to 108 chromosomes) showed that none had the nucleotide changes noted in the patient. Thus, these nucleotide changes were likely due to mutations and not to polymorphisms in the perforin gene. These 2 nucleotide changes in perforin have not been reported previously.[27]

The nucleotide sequence of the perforin genes from the patient's mother and father were also determined. Sequence analysis of DNA from the patient's mother showed a T to C change in nucleotide 577 in one allele of the perforin gene, whereas analysis of DNA from the patient's father showed a G to C change in nucleotide 1229 in one allele of the gene. These data indicate that mutations in nucleotides 577 and 1229 in the patient's perforin gene were inherited from his mother and father, respectively.

To determine if transcripts of mutant and wild-type perforin were expressed at similar levels in the patient and his parents, RT-PCR products of exon 3 in the perforin gene were sequenced. Direct sequencing and analysis of the chromatogram showed that T and C at nucleotide 577, and the G and C at nucleotide 1229 produced peaks of similar heights, indicating that the perforin mRNAs were expressed at similar levels in the patient (Figure 3B). In addition, the similar heights of peaks for these nucleotides in the chromatograms of the cDNAs from the patient's mother and father indicates that both the wild-type and mutant transcripts were expressed at similar levels in the patient's parents.

Expression of the native form of perforin is impaired in the patient with CAEBV

To determine whether the mutations in the perforin gene alter the level of expression of perforin protein in the patient's cells, we performed flow cytometry on permeabilized cells from the patient, his parents, and from blood bank donors. PHA- and IL-2–stimulated PBMCs were tested, since perforin is expressed in $CD8^+$ T cells and NK cells. Incubation of the cells with fluorescein isothiocyanate (FITC)–conjugated antiperforin antibody dG9,[28] which recognizes only the native form of perforin,[28,29] showed markedly reduced levels of perforin in cells from the patient, whereas perforin was readily detected in stimulated cells from his mother and father, 2 blood bank donors, and another (unrelated) patient with CAEBV (Figure 4A, data not shown). In contrast, granzyme A expression was observed in more than 80% of cells from all of the individuals tested. Immunofluorescence assay of stimulated PBMCs using FITC-conjugated antiperforin antibody

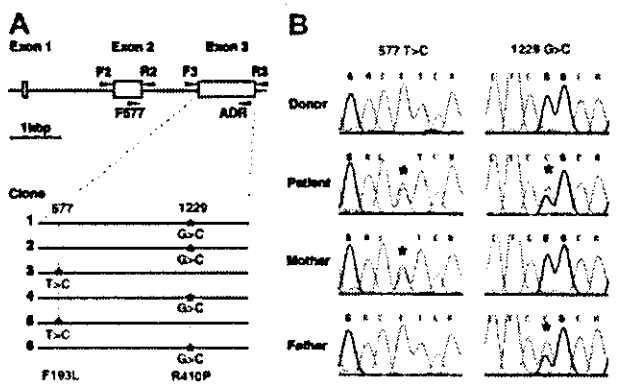

Figure 3. Perforin mutations in the patient with CAEBV and in his parents. (A) The perforin gene contains 3 exons and the open reading frame is encoded by the second and third exons. Exons 2 and 3 were amplified by PCR from genomic DNA using the indicated primers (arrowheads),[20] cloned in plasmid pCR2.1, and sequenced. Mutations were found in both alleles of exon 3 in the patient. Four of 6 clones had a G to C mutation at nucleotide 1229 and 2 clones had a T to C mutation at nucleotide 577, indicating that each allele had a separate mutation. (B) Chromatograms of RT-PCR products. RT-PCR was performed using mRNA isolated from stimulated PBMCs of the patient, his parents, and a healthy donor. RT-PCR products were directly sequenced (not having been cloned) and asterisks indicate mutations.

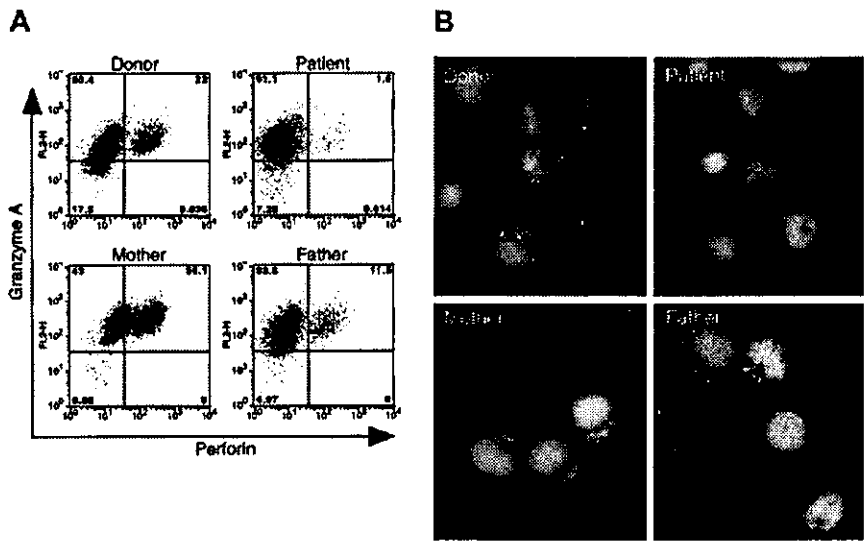

Figure 4. Expression of the native form of perforin in stimulated PBMCs from the patient, his parents, and a healthy blood bank donor. (A) PHA- and IL-2–stimulated PBMCs were fixed and permeabilized, stained with FITC-conjugated antiperforin antibody (dG9; Ancell, Bayport, MN) or phycoerythrin (PE)–conjugated anti–granzyme A antibody (CB9; BD Pharmingen), and analyzed by flow cytometry. The experiment was performed 3 times with similar results. The normal range of perforin-positive cells in 3 healthy donors was 3.4% to 56% (average 14%) in 4 separate experiments. (B) Immunofluorescence assay shows expression of perforin in PBMCs stimulated with PHA and IL-2, fixed, permeabilized, incubated with FITC-conjugated antiperforin antibody dG9 (green), and counterstained with propidium iodide (red). Original magnification, × 1000.

dG9 showed punctate staining in the cytoplasm in 40% to 60% of stimulated cells from the patient's mother and father and from healthy blood bank donors; however, very rare cells from the patient stained with the perforin antibody (Figure 4B). Perforin usually shows a bright, punctate staining pattern in the cytoplasm, since the protein is present in granules in the cytoplasm.[20] In contrast to the punctate distribution of perforin seen in his parent's cells, the patient's cells showed a weaker and more diffuse pattern of staining with the antibody. Expression of perforin in the mother's cells demonstrated not only the punctate staining pattern, but also weak diffuse staining in the cytoplasm. Since antibody dG9 recognizes the native form of perforin, these data suggest that expression of the native form of perforin is markedly reduced in the patient with the perforin mutations.

Proteolytic cleavage of perforin is inhibited in the patient with fatal CAEBV

Perforin is synthesized as a 70-kDa inactive glycosylated precursor which is subsequently cleaved at the C-terminus to yield a 60-kDa active, mature form.[29] To determine the level of expression of the precursor and mature forms of perforin, we performed immunoblot analyses using the 2d4-perf monoclonal antibody to perforin, which recognizes both mature and precursor forms of perforin.[29,30] Stimulated PBMCs from the patient expressed predominantly the 70-kDa precursor protein that reacted with the antibody that was larger in size than the 60-kDa mature protein from the healthy donor that bound to the antibody (Figure 5). To verify that the perforin molecule from the patient corresponds to the 70-kDa inactive precursor form, concanamycin A, which inhibits the cleavage of the precursor form of perforin, was added to the cells to block cleavage of perforin. Incubation of cells from the healthy blood bank donor or from the patient with CAEBV with concanamycin A followed by immunoblotting with antibody 2d4-perf resulted in detection of only the 70-kDa precursor form of perforin. Thus, cells from the patient with fatal CAEBV expressed predominantly the precursor form of perforin, indicating that proteolytic cleavage of perforin is inhibited. Since the cleavage site in perforin is located at amino acids 520-521,[29] and the perforin mutations are located at amino acids 193 and 410, these findings suggest that his mutations in perforin result in a conformational change in the protein that prevents cleavage to the mature protein.

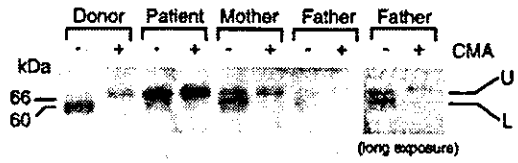

Figure 5. Inhibition of proteolytic cleavage of perforin in the patient with CAEBV. Stimulated PBMCs were cultured in the presence (+) or absence (–) of 200 nM concanamycin A (CMA) for 5 hours. Lysates were prepared from the cells and perforin was detected by immunoblotting with 2d4-perf antibody under nonreducing conditions.[29,30] Two bands corresponding to perforin are seen. The upper (U) and lower (L) bands indicate precursor and mature form of perforin, respectively. Protein concentrations were equal in each sample. The right panel indicates a darker exposure from a repeat experiment using the father's cells.

Immunoblotting of perforin in stimulated PBMCs from the patient's mother showed 2 forms of perforin, the 70-kDa inactive precursor form and the 60-kDa mature form. This is consistent with heterozygosity in the mother. Immunoblotting of perforin in PBMCs from the patient's father showed 2 faint bands of 70 kDa and 60 kDa, even though equal amounts of cell lysates were used.

Mutation in the patient's perforin changes the reactivity with an antiperforin antibody

The inhibition of proteolytic cleavage of mutant perforin in cells from the patient suggested that a dynamic conformational change in perforin occurred in his stimulated PBMCs due to the mutations in perforin. To provide further evidence for this hypothesis, we cloned cDNA for perforin derived from a healthy donor and inserted the gene into expression vector pCIneo. Site-directed mutagenesis of the plasmid was performed to construct perforin genes with each of the patient's mutants as well as a perforin mutant with stop codons previously identified in a patient with FHL.[20] These plasmids were transfected either individually or in combinations into 293T cells. Immunoblotting using antiperforin antibody 2d4-perf, which recognizes both mature and precursor forms of perforin, showed that cells transfected with plasmids encoding perforin with the mother's or father's mutation, or cotransfected with 2 plasmids together (wild-type and mother's mutant perforin, wild-type and father's mutant perforin, or mother's and father's mutant perforin), expressed perforin in 293T human embryonic kidney cells (Figure 6, top panel). Since perforin is not processed to its mature form in non-T cells,[29] the 70-kDa bands represent the precursor form of perforin. Transfection of cells with the plasmid encoding mutant perforin from a patient with FHL

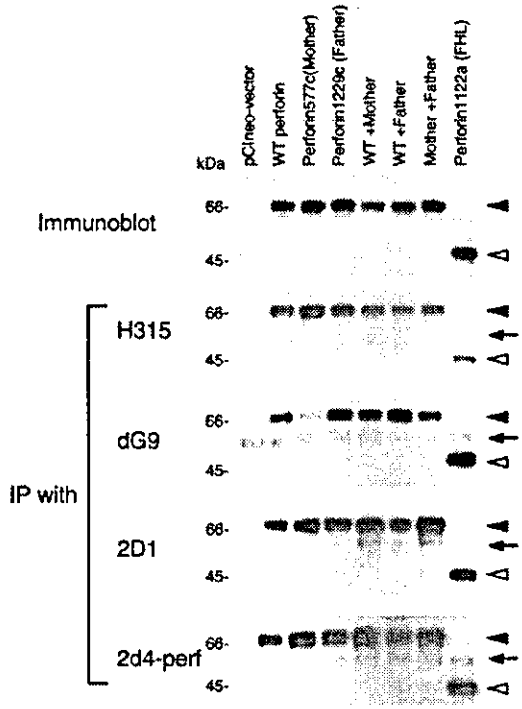

Figure 6. Binding activity of perforin mutants in 293T cells with antibodies. 293T cells were transfected with plasmid(s) expressing wild-type (WT) and/or mutant perforin genes, cell lysates were prepared, and immunoblots were performed using 2d4-perf antibody (top panel). Lysates were immunoprecipitated with perforin antibodies H315 (Santa Cruz Biotechnology, Santa Cruz, CA; second panel), dG9 (Endogen, Woburn, MA; third panel), 2D1 (US Biological, Swampscott, MA; fourth panel) and 2d4-perf (the bottom panel). Immunoprecipitates were immunoblotted under reducing conditions and stained with 2d4-perf antibody. Solid and open arrowheads indicate full-length and truncated forms of perforin, respectively. Arrows correspond to the heavy chain of immunoglobulin (IgH).

produced a truncated form of perforin. To see if mutations in perforin might induce conformational changes, transfected cell lysates were immunoprecipitated with various antibodies recognizing different portions of perforin. Rabbit polyclonal antibody H315 recognizes the carboxy terminal region (amino acids 241-555) of perforin; murine monoclonal antibodies 2D1 and 2d4-perf recognize a domain within amino acids 189-320 of mouse perforin and amino acids 264-279 of human perforin, respectively.[30,31] Murine monoclonal antibody dG9 recognizes only the native form of perforin, but the epitope detected by the antibody is unknown.[28,29] Mutant perforin from the patient's mother was weakly immunoprecipitated from 293T cells with antibody dG9, whereas the wild-type and other mutant perforins were immunoprecipitated to a similar extent (Figure 6). Immunoprecipitation of wild-type perforin and each of the mutant forms of perforin were similar with antibodies H315, 2D1, and 2d4-perf. These data suggest the mutant perforin from the patient's mother may have a conformational change, resulting in its reduced ability to bind antibody dG9. Alternatively, the perforin epitope recognized by antibody dG9 might overlap the mutation in the mother's perforin, resulting in reduced immunoprecipitation of the protein.

Cytotoxicity is impaired in cells from the patient with fatal CAEBV

Perforin is important for the cytotoxic activity of $CD8^+$ T and NK cells. Since the patient with CAEBV had mutations in both perforin genes that resulted in reduced expression of the native form of the protein and that inhibited maturation of the protein, we postulated that cytotoxicity by his $CD8^+$ T and NK cells might be reduced. Incubation of PHA- and IL-2–stimulated PBMCs from the patient with Fas-deficient target cells showed a marked diminution in cytotoxicity compared with cells from blood bank donors (Figure 7A). Cells from the patient's father demonstrated an intermediate level of cytotoxicity, whereas cells from the patient's mother or from an unrelated patient with CAEBV did not show a reduction in cytotoxicity. The reduced level of cytotoxicity with the father's cells may be related to the lower number of NK cells in his stimulated PBMCs (Figure 2B). Although stimulated PBMCs from the patient contained a larger number of NK cells than PBMCs from his father, the cytotoxicity of the patient's PBMCs was markedly lower than PBMCs from his father. Similar cytotoxicity results for cells from the patient, his parents, and the blood bank

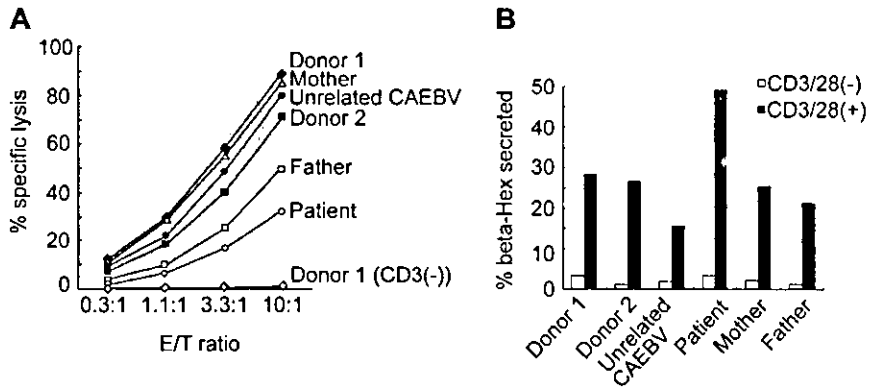

Figure 7. Cytotoxicity and granule exocytosis assays using stimulated PBMCs from the patient, his parents, an unrelated patient with CAEBV, and healthy donors. (A) PBMCs were stimulated with PHA and IL-2, cultured in IL-2 (effector cells), and flow cytometry showed lymphocyte phenotypes described in Figure 2B. The cells were incubated with [^{51}Cr]-labeled L1210 target cells (Fas-deficient target cells) in the presence of anti-CD3 (UCHT-1; BD Pharmingen) and anti-Fas blocking antibody (ZB4; Beckman Coulter, Fullerton, CA) in a Fas-independent CTL killing assay. Cytotoxicity in this assay system is CD3-dependent, since T-cell receptor activation is required to trigger release of granules containing perforin and granzymes.[20] The effector-to-target (E/T) ratio is indicated on the x axis and the percent specific lysis is indicated on the y axis. The percent lysis was calculated as follows: % lysis = (E − S)/(M − S) × 100, where E is the release from experimental samples, S is the spontaneous release, and M is the maximum release upon lysis with 2% NP-40. The gray band indicates the normal range of cytotoxicity seen in 4 healthy donors (data not shown). Cells from a healthy blood bank donor incubated in the absence of anti-CD3 antibody were assayed as a negative control. Flow cytometry showed the following lymphocyte phenotypes: donor 1: CD8 = 49%, CD56 = 42%; donor 2: CD8 = 55%, CD56 = 42%; patient: CD8 = 54%, CD56 = 25%; mother: CD8 = 50%, CD56 = 68%; father: CD8 = 91%, CD56 = 4%; unrelated patient with CAEBV: CD8 = 55%, CD56 = 40%. (B) PBMCs were stimulated with PHA and IL-2 followed by incubation in the presence (black bars) and absence (white bars) of plate-bound anti-CD3/anti-CD28 antibody. Secretion of β-hexosaminidase in the cell culture supernatant was measured to quantify the level of granule exocytosis.[33] β-hexosaminidase release was expressed as a percentage of the enzyme in the supernatant divided by the total enzyme from cells lysed in 0.1% Triton X-100. Experiments in panels A and B were performed 3 times and a representative result is shown.

donors were observed in 3 separate experiments performed on different days. Taken together, these results suggest that the mutations in the patient's perforin genes result in defective Fas-independent cytotoxic killing of target cells.

Granule exocytosis is another important process required for CTL activity. Reduced CTL activity has been demonstrated in humans and mice that have mutations in the Rab27A gene that controls granule exocytosis.[32,33] In addition, hemophagocytic syndrome has been noted in patients with Rab27A mutations.[34] To determine if granule exocytosis is impaired in cells from the patient with fatal CAEBV, PHA- and IL-2–stimulated PBMCs were stimulated with antibodies to CD3 and CD28 and the level of β-hexosaminidase secreted into the culture media, a marker of degranulation, was measured (Figure 7B). Elevated levels of β-hexosaminidase were detected in culture supernatants of cells from the patient with perforin mutations, compared with cells from the patient's parents, 2 blood bank donors, and an unrelated patient with CAEBV. These results suggest that granule exocytosis was up-regulated in stimulated cells from the patient.

Discussion

We have identified mutations in both alleles of the perforin gene in a patient with fatal CAEBV. The patient's PBMCs showed reduced expression of the native form of perforin and the unprocessed precursor was the predominant form detected. Expression of perforin mutants in 293T cells suggested that the mother's mutant perforin might have a conformational change. Stimulated PBMCs from the patient were markedly impaired for cytotoxic activity in vitro. Taken together, these data imply that the reduced cytotoxicity of CTL and NK cells in the patient is due to the mutations in his perforin genes. This is the first case of CAEBV in which genetic mutations have been linked to the disease.

Perforin is present in cytolytic granules of CTL and NK cells and has a critical role in their cytotoxic activity. Perforin comprises one of 2 major pathways that CTLs use to kill virus-infected cells.[35] In the Fas pathway, Fas ligand on CTLs binds to Fas on virus-infected cells, which initiates caspase-mediated killing of the cells. In the perforin pathway, engagement of the T-cell receptor on the surface of CTLs with viral peptides associated with major histocompatibility complex (MHC) class I molecules triggers release of granules containing perforin and granzymes. Perforin is known to be important for control of certain virus infections by the immune system.[36] Perforin knock-out mice have a normal phenotype, but when they are infected with lymphocytic choriomeningitis virus they show immune defects.[35,37] Similarly, perforin-deficient mice show increased mortality after infection with herpes simplex virus and ectromelia virus compared with control mice.[38,39]

Perforin has been implicated in the killing of EBV-infected cells. EBV-specific T-cell cytotoxicity is mediated through the perforin pathway in patients with lymphoproliferative disorders after allogeneic bone marrow transplantation.[40] The perforin/granzyme pathway, rather than the Fas or TRAIL pathways, is the major pathway for MHC class II–restricted lysis of EBV-transformed lymphoblastoid cell lines by virus-specific $CD4^+$ T cells.[41,42] Recent studies indicate that expression of perforin is impaired in $CD8^+$ cells from patients with EBV-positive nasopharyngeal carcinoma and HIV infection, suggesting that low expression of perforin in $CD8^+$ T cells may constitute an important mechanism for immune escape by tumors or virus-infected cells.[43-45] Perforin-deficient mice also have a higher incidence of T- or B-cell lymphomas, confirming that perforin is critical in immune surveillance against cancer.[46]

Perforin mutations have been identified in patients with FHL.[20] Patients with FHL may share certain clinical findings seen in some patients with CAEBV such as fever, splenomegaly, lymphadenopathy, hepatomegaly, neurologic abnormalities, and hemophagocytosis.[47,48] Patients with FHL usually present in infancy or very early in childhood and in the absence of bone marrow transplantation most patients die within 1 year after diagnosis.[23,27,49] Patients with FHL and nonsense mutations tend to present within the first few months of life, whereas those with missense mutations usually present by 2 years of age.[23] Although our patient showed many of the features of FHL early in the course of his disease,[22] he presented at an older age (7 years) than most patients with FHL, survived for 10 years after diagnosis, and did not have a family history of hemophagocytic lymphohistiocytosis. While a variety of virus infections have been reported in patients with FHL at the time of diagnosis, including cytomegalovirus, parvovirus, EBV, hepatitis B virus, and adenovirus, no single agent has been identified that triggers the disorder in most cases. FHL and EBV-associated hemophagocytic lymphohistiocytosis (EBV-HLH) have overlapping clinical manifestations, and CAEBV is often associated with EBV-HLH at some point during the course of disease. HLH is considered the prototype of hemophagocytic syndrome.[50] In a study of patients with EBV-associated hemophagocytic syndrome from Japan, no mutations were detected in the perforin gene of 14 patients.[51] Thus, whereas perforin defects have been associated with FHL, mutations in the gene do not appear to be responsible for most cases of EBV-associated hemophagocytic syndrome.

Perforin is synthesized as an inactive precursor that must be cleaved at its carboxy terminus to yield the active, mature form of the protein.[29] The precursor form of perforin consists of 555 amino acids and contains 2 N-linked glycosylation sites and an approximately 300 amino acid C2 domain that is homologous to the C2 domain of protein kinase C. The C2 domain of perforin is important for binding of the protein to the phospholipid bilayer of the cytoplasmic membrane.[29] The C2 domain in the uncleaved form of perforin cannot bind to the membrane, due to the presence of a bulky N-linked glycan on the carboxy terminal domain of the protein. Proteolytic cleavage of the carboxy terminal domain allows the C2 domain to bind to the membrane. We demonstrated that the inactive precursor form of perforin accumulated in the patient's PBMCs, implying that the proteolytic cleavage was inhibited. Reduced expression of the active, mature form of perforin was likely the cause of the defect in Fas-independent cytotoxicity of T cells and NK cells from the patient. Mutations in the patient's perforin may have altered its conformation and inhibited proteolytic processing of the protein to its mature form. Although we wanted to examine processing of perforin encoded by each allele separately, processing of perforin has never been demonstrable in cells transfected with the perforin gene. We were unable to show processing of perforin in 293T or T cells transfected with our perforin expression vector (data not shown). Similarly, Uellner et al[29] could not detect processing of the protein in rat leukemia cells transfected with the perforin gene. Since precursor (uncleaved) forms of perforin were present in cells from both of the patient's parents (Figure 5), we postulate that both mutations inhibit cleavage of perforin. In addition, since both parents were seropositive for EBV and asymptomatic, and both had a mutant and normal perforin allele, one wild-type copy of the gene may be sufficient for control of EBV infection. Some perforin mutations reported in patients with FHL induce only single amino acid

missense mutations as was the case in our patient, and low-level expression of perforin in T cells and NK cells from patients with FHL has been reported using the dG9 antibody.[20,52] Our data suggest that at least some missense mutations in FHL may also induce conformational changes and inhibit proteolytic cleavage of the precursor form of perforin.

Our patient with mutations in both alleles of the perforin gene died from T-cell lymphoproliferative disease. Perforin mutations may contribute to uncontrolled lymphoproliferative disease.[53-55] In the absence of functional perforin, T and NK cells might be unable to contain the expansion of EBV-infected cells, resulting in a persistent active infection. This process might be similar to the massive T-cell proliferation that occurs in response to acute EBV infection during infectious mononucleosis; however, in infectious mononucleosis cell proliferation is down-regulated when virus replication is reduced. The persistent activation of T cells in our patient may have increased the likelihood of development of uncontrolled T-cell lymphoproliferative disease. Since dysregulation of cytokine expression has been reported in both CAEBV and FHL,[24,25] it may be associated with the persistent activation of T cells, and might not be specific for mutations in perforin.

We have identified the genetic basis for CAEBV in one patient, and postulate how mutations in the perforin protein may have led to his disease. The observation that CTLs and NK cells in other cases of CAEBV have defective cytotoxic activity[13,14,56] suggests that other proteins in the perforin or Fas pathways may be responsible for CAEBV in other patients. Thus, the diverse clinical expressions of CAEBV may be due to unique, heritable disorders with the shared inability to contain proliferation of EBV-infected cells.

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BCL-6-positive Human Herpesvirus 8-associated Solid Lymphoma Arising from Liver and Spleen as Multiple Nodular Lesions

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We report a case of BCL-6-positive B cell lymphoma with human herpesvirus 8 (HHV-8) infection. A human immunodeficiency virus-infected patient developed a diffuse large B cell lymphoma, which was found exclusively in the liver and spleen with the absence of lymphadenopathy and effusion in any body cavities. The lymphoma cells were composed of medium to large-sized cells positive for CD20, CD45, and BCL-6, and negative for epithelial cell membrane antigen, CD30, CD45RO, and CD138 syndecan-1, suggesting a germinal center B cell origin. The patient was serologically positive for HHV-8, and HHV-8 was detected in the liver biopsy tissue both by polymerase chain reaction and by immunohistochemistry for HHV-8-encoded latency-associated nuclear antigen. Other HHV-8-associated diseases, such as Kaposi's sarcoma, primary effusion lymphoma, or multicentric Castleman's disease were not detected in the patient. Chemotherapy was effective and reduced the size of the lymphoma dramatically. This is the first case report of a germinal center B cell-originating lymphoma with HHV-8 infection.

Keywords: BCL-6; HHV-8; Malignant lymphoma; AIDS

INTRODUCTION

Acquired immunodeficiency syndrome (AIDS)-associated malignant lymphoma (AIDS lymphoma) usually develops at extranodal sites, and 95% of lymphomas are B-cell type of non-Hodgkin lymphoma (NHL). Epstein-Barr virus (EBV) has been thought to be the causative agent for a large portion of AIDS lymphoma. Recently, human herpesvirus 8 (HHV-8; or Kaposi's sarcoma-associated herpesvirus; KSHV) has been also thought to play a role in the pathogenesis of some AIDS lymphoma. HHV-8 is associated with Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and some cases of AIDS-related multicentric Castleman's disease (MCD) [1–4]. Malignant lymphoma associated with HHV-8 was first described as PEL or body cavity-based lymphoma (BCBL) in AIDS patients [2,3]. In the cases of PEL, the lymphomas were found exclusively in serous effusions without detectable mass lesions in the body cavities and in the absence of lymphadenopathy or organomegaly. These lymphomas exhibited virtually identical morphologies, which were consistent with immunoblastic and/or anaplastic large cell lymphoma (ALCL)-like morphology; also, HHV-8 genomes, and sometimes EBV genomes, were detected in the lymphoma cells [5]. Gene array analysis revealed that HHV-8-infected PEL has a plasma cell gene expression profile, suggesting post-germinal center B cells as an origin of PEL [6,7].

In addition to PEL, 18 cases of solid lymphoma associated with HHV-8 have been reported so far [8–12]. These cases of HHV-8-associated solid lymphoma occurred as lymphoma exhibiting mass lesions without effusion in the lung, skin, bone marrow, gastrointestinal tract, large bowel, and central nervous system of the patients with HIV infection and/or MCD. Immunohistochemistry revealed that the HHV-8-associated solid lymphoma demonstrated a profile of cellular protein expression, e.g., positive for CD45, CD30, CD43, CD138, and immunoglobulin light chain lambda, but negative for CD3, CD4, CD8, CD19, CD20, and immunoglobulin

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light chain kappa, suggesting a similar origin to that of PEL (post-germinal center B cells) [10,11,13,14]. In addition, since both HHV-8-associated PEL and solid lymphoma express the HHV-8 encoded latency-associated nuclear antigen (LANA) constantly, similar oncogenic mechanisms involving HHV-8 is suggested for both types of lymphomas.

Here we report a case of BCL-6-positive solid lymphoma arising from the liver and spleen occurring in a Japanese man with HIV infection. BCL-6 is a marker of germinal center B cells, follicular lymphoma, and diffuse large B cell lymphoma originating from germinal center B cells [15]. HHV-8 infection was demonstrated in the lymphoma cells. This is the first case report of HHV-8-infected B cell lymphoma originating from the germinal center.

CASE REPORT

A 50-year-old Japanese bisexual man presented with weakness, gastric discomfort and abdominal side pain. Imaging examination by magnetic resonance imaging (MRI) and computed tomography (CT) scan revealed growing multiple solid nodular lesions both in the liver and spleen with the absence of lymphadenopathy (Fig. 1A). Liver biopsy yielded a diagnosis of diffuse large B-cell lymphoma. The first course of treatment with a 50% dose of normal CHOP treatment (cyclophosphamide 700 mg, doxorubicin 70 mg, vincristine 2 mg, methylprednisolone 125 mg) was started and found to be effective. The CT scan of the abdomen including liver and spleen after the treatment for 10 days revealed a marked reduction in the size of the tumor lesions (Fig. 1B). HIV antibody testing was found to be positive both by ELISA and Western blotting methods. A CD4 count was 44/μl, and HIV-RNA viral load was 7.3×10^4 copy/ml. The patient was transferred to another hospital for further treatment of HIV infection. Subsequently, the HIV infection was well controlled by highly active antiretroviral therapy (HAART), and the lymphoma lesions also disappeared after the treatment.

The specimen obtained from the liver biopsy showed diffuse proliferation of atypical lymphocytes in the parenchyma of the liver. The sinusoidal structure of the liver was dramatically damaged. The lymphoma

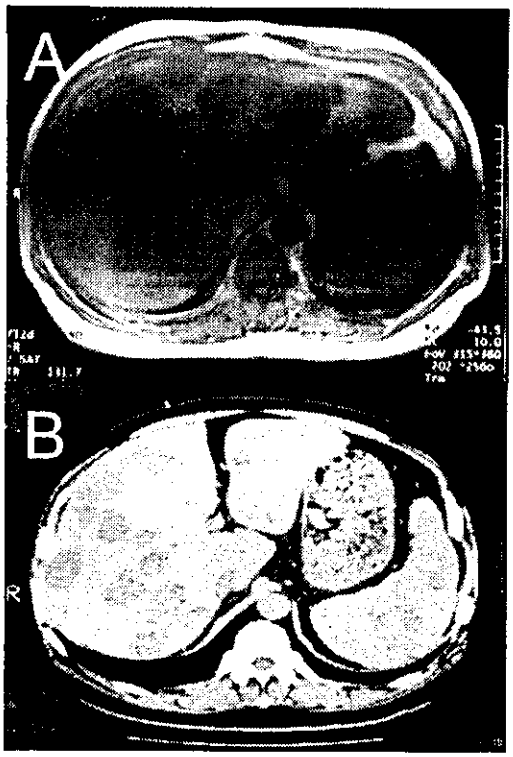

FIGURE 1 Images of MRI and CT scans. (A) MRI demonstrated multiple nodular lesions in liver and spleen on initial examination. (B) CT scan, 10 days after the CHOP therapy showed the reduction of tumor mass size both in the liver and spleen.

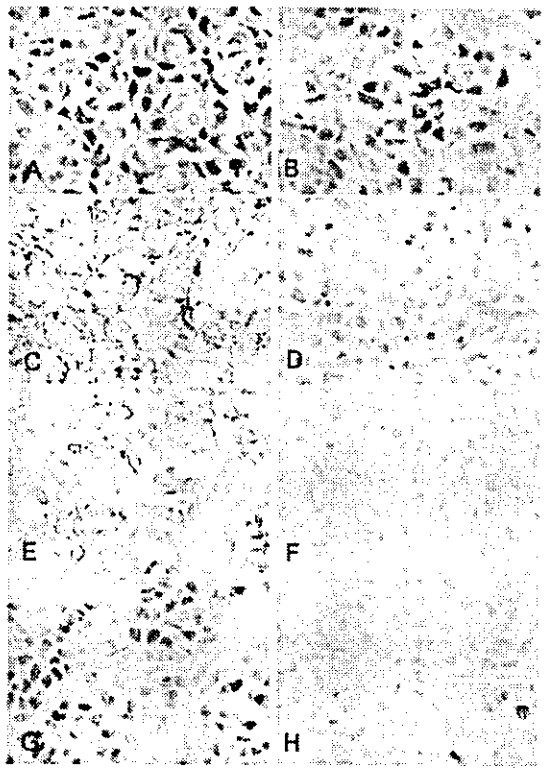

FIGURE 2 Histology. Liver biopsy specimens showed diffuse proliferation of medium- and large-sized lymphocytes with angulated, elongated or cleaved nuclei. H.E. stain (A). Immunohistochemistry of the liver shows that lymphoma cells were positive for BCL-6 (B), CD20 (C), and immunoglobulin light chain lambda (E) and negative for CD45RO (D) and immunoglobulin light chain kappa (F). LANA was detected in most of the nuclei of the lymphoma cells with a dot-like staining pattern (G). EBER-1 was negative (H). Inset in (H) is a positive control of EBER-1 *in situ* hybridization.

contained various types of medium- and large-sized atypical lymphocytes with irregular nuclei (Fig. 2A). Immunohistochemistry revealed that these cells were positive for CD45 (leukocyte common antigen; LCA, Dako, Kyoto, Japan) and CD20 (L26, Dako), but negative for epithelial cell membrane antigen (EMA, Dako), CD43 (Dako), CD45RO (UCHL-1, Dako), and CD30 (Ki-1, Dako) (Fig. 2C, D). The tumor cells expressed immunoglobulin light chain lambda (Dako), but not kappa (Dako) (Fig. 2E, F). These results corresponded to the diagnosis of B cell lymphoma, diffuse large cell type. From the point of view of histogenesis of this lymphoma, the expression pattern of BCL-6 and CD138/syndecan-1, a marker of pre-terminal and terminal B cell differentiation, respectively, were examined. The present case showed positive expression for BCL-6 (Dako) (Fig. 2B) and negative expression for CD138/syndecan-1 (Dako). This pattern reflects the physiological stage of germinal center (GC) B cells. Almost all atypical cells were positive as a diffuse or dot-like pattern in the nuclei by anti-HHV-8 LANA antibody [12,16,17], whereas non-lymphoma cells were not stained (Fig. 2G). Immunohistochemistry also demonstrated that the tumor cells lacked the expression of lytic proteins encoded by HHV-8, e.g., K8, ORF50, and ORF59 [12,17]. *In situ* hybridization using EBER-1 probe (Dako) and immunohistochemistry for latent membrane protein (LMP-1, Dako) for EBV infection were negative in the lymphoma cells (Fig. 2H).

PCR products for HHV-8 DNA using K1VR1 primer sets (K1VR1F1: TTG CCA ATA TCC TGG TAT TGC, K1VR1R1: CAA GGT TTG TAA GAC AGG TTG) were identified as specific sized bands, 162 bp [14] (Fig. 3). The sequence analysis confirmed the sequence to be similar to the HHV-8 sequence in database, and it was phylogenetically identified as being subtype C of HHV-8.

The patient's serum was positive for anti-HHV-8 antibody, which was examined by indirect immunofluorescence assay on acetone-fixed TY-1 cells (primary effusion lymphoma cell line, HHV-8-positive and EBV-negative cells) [18] (Fig. 4).

DISCUSSION

In this report we described an AIDS case with the development of a BCL-6-positive lymphoma associated with HHV-8 infection exhibiting diffuse large cell morphology. The lymphoma was found exclusively in the liver and spleen with the absence of lymphadenopathy and any effusions in any body cavities. This is the first report in which HHV-8 infection has been associated with germinal center B cell-originating lymphoma cells.

The present case is different from those of PEL and HHV-8-associated solid lymphomas reported previously in many aspects [3,8,12]. At first, the morphological features of the present case are similar to those of centrocytes in follicular lymphoma (Grade 1) rather than PEL [13,19,20]. Second, the site of lesion is unique. Oksenhendler *et al.* reported 14 cases of NHL in HIV-infected patients associated with MCD [21]. These included 3 cases of PEL, 5 large/anaplastic cell lymphoma cases in skin, bone marrow, gastrointestinal tract, and central nervous system, and 6 plasmablastic lymphoma cases in blood or spleen [21]. Prior to this paper, only 7 solid lymphoma cases had been reported. These included 1 case in the lung, 3 cases on the skin, 2 cases of bowel origin and one case of unknown origin [8,9,11]. There are no reports describing an HHV-8-associated lymphoma occurring only in the liver and spleen as in the present case. Third, the present case was not complicated with other HHV-8-related diseases such as KS, PEL, and MCD [8,12]. Fourth, the present case did not express ORF59, ORF50, and K8, which are expressed in a small population of cells in PEL and HHV-8-associated solid

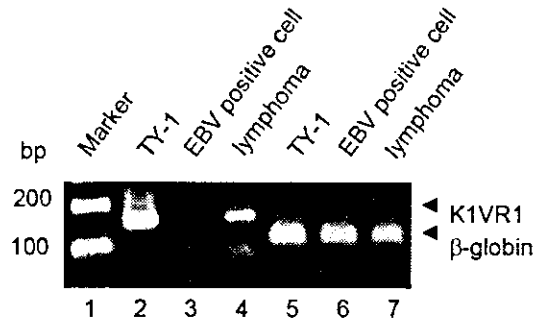

FIGURE 3 Polymerase chain reaction analysis. A 162-bp of the amplified DNA was detected in paraffin-embedded tissue sections of the lymphoma (lane 1; 100 bp DNA ladder marker, lane 2–4; K1VR1 primer sets). TY-1 is HHV-8-positive, and Ramos is HHV-8-negative and EBV-positive lymphoma cell line. These cells were used as positive and negative controls. Lanes 5–7 show amplification of β-globin gene (110 bp) as a control for DNA quality.

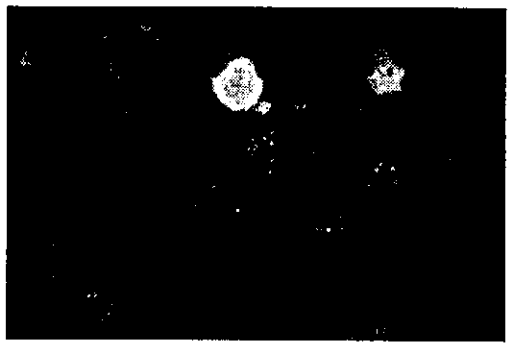

FIGURE 4 Serum anti-HHV-8 antibodies were detected by indirect immunofluorescence assay using acetone-fixed TY-1 cells, showing mainly latency-associated nuclear antigen (LANA) in their nuclei. Some cells showed diffuse cytoplasmic staining, indicating the presence of lytic antigens to HHV-8.

lymphoma reported previously [12,17]. These features indicate that the present case is an unusual and unique one as an HHV-8-associated lymphoma.

The most important difference between the present case and PEL and/or HHV-8-associated lymphoma is the origin of the lymphoma. While PEL cells have a profile of post-germinal center B cell [6,7], the present case of lymphoma expressed BCL-6 but did not express CD138, suggesting it to be of germinal center B cell origin [22]. In the literature, HHV-8 DNA has been detected in a few cases of follicular lymphoma [23]; however, expression of HHV-8-encoded proteins is constantly negative in germinal center B cell-originating lymphomas containing follicular lymphoma and diffuse large B cell lymphoma with BCL-6 expression [22]. The present case suggests the possibility that HHV-8 might infect and transform germinal center B cells, resulting in diffuse large B cell lymphoma. However, since morphological features and expression of virus proteins were different from those of PEL or HHV-8-associated solid lymphoma reported previously, we presume that HHV-8 occasionally infected the lymphoma cells and might not be directly associated with lymphomagenesis. Alternatively, HHV-8 might infect normal germinal center B cells and the HHV-8-infected B cell might be transformed by other factors besides HHV-8 infection. Nevertheless, this case suggests that HHV-8 might infect germinal center B cells as well as post-germinal center B cells.

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研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yamada, T., Watanabe, N., Nakamura, T and Iwamoto, A.	Antibody-dependent cellular cytotoxicity via a humoral immune epitope of Nef protein expressed on the cell surface.	J. Immunology.	172	2401-6	2004
Takeshi Fujii, Tetsuya Nakamura, Aikichi Iwamoto.	Current Concept of SARS Treatment.	J Infect Chemother.	10	1-7	2004
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D Zhu, H Taguchi-Nakamura, M Goto, T Odawara, T Nakamura, H Yamada, H Kotaki, W Sugiura, A Iwamoto & Y Kitamura.	Influence of single-nucleotide polymorphisms in the multidrug resistance-1 gene on the cellular export of nelfinavir and its clinical implication for highly active antiretroviral therapy.	Antiviral Therapy	9	929-35	2004

Antibody-Dependent Cellular Cytotoxicity via Humoral Immune Epitope of Nef Protein Expressed on Cell Surface[1]

Takeshi Yamada,[2]* Nobukazu Watanabe,† Tetsuya Nakamura,‡ and Aikichi Iwamoto[3]*‡

Antibodies against various proteins of HIV type 1 (HIV-1) can be detected in HIV-1-infected individuals. We previously reported that the level of Ab response against one Nef epitope is correlated with HIV-1 disease progression. To elucidate the mechanism for this correlation, we examined Ab-dependent cellular cytotoxicity (ADCC) against target cells expressing Nef. We observed efficient cytotoxicity against Nef-expressing target cells in the presence of patient plasma and PBMCs. This ADCC activity was correlated with the dilution of plasma from HIV-1-infected patients. Addition of a specific synthetic peptide (peptide 31: FLKEKGGLE) corresponding to the Nef epitope reduced cell lysis to ~50%. These results suggest that PBMCs of HIV-1-infected patients may exert ADCC via anti-Nef Abs in the patients' own plasma and serve as a mechanism used by the immune system to regulate HIV-1 replication. ***The Journal of Immunology,*** **2004, 172: 2401–2406.**

Highly active antiretroviral therapy dramatically suppresses HIV-1 replication and has thereby contributed to decrease the incidence of AIDS-related opportunistic infections and subsequent mortality (1, 2). However, elimination of HIV-1 from infected individuals has not yet been achieved by highly active antiretroviral therapy alone (3–5). Therefore, the development of different therapeutic approaches is mandatory.

Ab-dependent cellular cytotoxicity (ADCC)[4] as well as CTL play an important role in protective immunity against viral infections (6, 7). ADCC can inhibit viral replication and cell-to-cell infection by killing HIV-1-infected cells before maturation of virus particles (8, 9). Therefore, ADCC activity could benefit the prevention of disease progression. In early studies, Rook et al. (10) and Ljunggren et al. (11) demonstrated that sera from HIV-1-infected individuals were able to mediate ADCC against HIV-1-infected T cells, and there was a positive correlation between ADCC activity and disease progression. When HIV-1-infected cells produce virus particles, viral envelope glycoproteins are abundantly exposed to the cell surface through the plasma membrane. In fact, ADCC via Abs against gp120 or gp41, HIV-1 envelope protein, has been well documented (12–20). It has been described that gp120 or gp120/41-specific ADCC correlates with rate of disease progression (19, 21). But, in contrast, ADCC via envelope proteins could potentially kill the uninfected $CD4^+$ T cells with free viral envelopes on their surface, and therefore ADCC could contribute to depletion of $CD4^+$ T cells and AIDS pathogenesis (22, 23). In addition, gp120 is prone to high frequency of mutations; thereby, viral escape mutants may evolve easily (24–26). In view of these disadvantages, envelope proteins appear to be unsuitable as targets for ADCC against the progression of disease in HIV-1-infected patients. Conserved proteins may be better targets if one considers ADCC as a durable therapeutic weapon against HIV-1. With regard to this, Gag and Pol are very conserved proteins, and if their epitopes were expressed on the cell surface, these proteins could be good candidates for specific ADCC. Rook et al. (10) described that Ab reactivity with the p24 (Gag) protein of patient's serum correlates inversely with disease progression. It has been reported that Gag proteins are expressed on the cell surface (27, 28); nevertheless, the inductions of ADCC via Gag have never been succeeded (29). And, furthermore, there has been no evidence that Pol proteins are expressed on the HIV-1-infected cells; therefore, Pol Ags could not be exposed to the extracellular enviroment as ADCC target. Thus, the contribution of other HIV-1 proteins except envelope proteins to ADCC has remained unclear.

Nef protein is an HIV-1 accessory protein with important roles for pathogenesis of HIV-1 infection (30–35). Nef protein is partially expressed on the surface of HIV-1-infected cells (36–38). We previously reported that highly conserved amino acid residues (FLKEKGGLE) are expressed on the surface of HIV-1-infected cells. The peptide residues served as an epitope for Ab response, and the plasma level of the Abs against the epitope was correlated with HIV-1 disease progression (39, 40). To elucidate the mechanism of this correlation, we studied ADCC activities using patients' peripheral mononuclear cells (PBMCs) and a patient's plasma, which contained high amount of anti-Nef Abs. We also analyzed characteristics of patients' NK cells that should be the key player in ADCC against virus-induced target cells.

Materials and Methods

Cells

Five HIV-1-infected subjects whose PBMCs were used as effector cells for the ADCC assay are listed in Table I. PBMCs were freshly isolated by centrifuging heparinized blood over Ficoll-Hypaque (Meneki-seibutsuken, Gunma, Japan). PBMCs were counted and adjusted to the concentration of 2×10^6 cells/ml in RPMI 1640 medium supplemented with 10% heat-inactivated FCS (RPMI 10). A portion of the cells was used for phenotypic analysis using flow cytometry. For the flow cytometric analysis of NK

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[4] Abbreviations used in this paper: ADCC, Ab-dependent cellular cytotoxicity; LTNP, long-term nonprogressor.

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Table I. *Patient profiles*

Patient	Age	Sex	$CD4^+$ Count (cells/μl)	$CD8^+$ Count (cells/μl)	NK Cell Count (cells/μl)	% NK Cell in FBMC	HIV RNA (copies/ml)[a]	Antiretroviral Drugs[b]
P1	37	M	754	996	155	8.0	<400	d4T + 3TC + NFV
P2	32	M	63	214	20	3.7	770	d4T + 3TC + NFV
P3	45	M	204	620	220	12.6	<400	AZT + ddC + IDV
P4	37	M	638	1034	102	5.7	<400	d4T + 3TC + NFV
P5	35	M	372	877	73	5.0	2200	AZT + ddC + IDV

[a] Amplicor HIV monitor test (Roche Diagnostics Systems, Somerville, NJ).
[b] AZT, zidovudine; d4T, stavudine; 3TC, lamivudine; ddC, zalcitabine; NFV, nelfinavir; IDV, indinavir.

cells, PBMC samples from another 40 HIV-1-positive subjects and 16 uninfected donors were included in this study.

For the ADCC assay, we used CEM-NK^R cells that were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health from J. Corbeil (41). Nef proteins were expressed in these cells by using a recombinant Sendai virus system, which has been shown to express large amounts of heterologous recombinant proteins in 24 h after infection in suspension cells (42). CEM-NK^R cells were infected with SeV-Nef to express HIV-1 (NL43 strain) Nef proteins or wild SeV at a multiplicity of infection of 10 for 1 h at 37°C, as previously described (43), and cultured for 24 h in RPMI 10. These cells were designated CEM-NK^R-Nef or CEM-NK^R-mock cells, respectively.

Subjects and reagents

For ADCC assay, we used the plasmas from long-term nonprogressor 2, 5, and 6 (LTNP 2, 5, and 6), whose characterization was published previously (39). $Na_2[^{51}Cr]O_4$ was obtained from NEN Life Science Products (Boston, MA). mAbs N901 (NKH-1) (anti-CD56; FITC conjugated) and 3G8 (anti-CD16; PE) were obtained from Coulter (Miami, FL). mAbs SJ25C1 (anti-CD19; PerCP) and SK7 (anti-CD3; allophycocyanin) were obtained from BD Immunocytometry Systems (San Jose, CA). mAb δG9 (anti-perforin) was a generous gift of E. Podack (University of Miami, Miami, FL). δG9 was conjugated with FITC in our laboratory. Nine-mer peptide 31 (=FLKEKGGLE) and control peptide (=GGGGGGGGG) were synthesized using a Multipin peptide synthesis kit (Chiron Mitotopes, Clayton, Victoria, Australia). The yields were analyzed by gas-liquid chromatography to confirm the correct synthesis.

Immunofluorescent staining

For analysis of Sendai virus-infected CEM-NK^R cells, cells (10^5) were centrifuged over silan-coating glass coverslips (DAKO, Carpenteria, CA), fixed with 2% paraformaldehyde in PBS for 5 min, blocked with BlockAce (Snow-Brand, Tokyo, Japan) for 30 min, and incubated for 1 h with plasma of LTNP 5 1/2.5 diluted in PBS. Then cells were incubated for 30 min with FITC-conjugated goat anti-human Igs (IgG, IgA, and IgM) $F(ab')_2$ (BioSource International, Camarillo, CA) after wash with PBS, and were mounted in 85% glycerol, 10 mM of Tris-HCl (pH 8), and 5% *n*-propylgallate. These stained cells were inspected with a confocal microscope (MRC 1024; Bio-Rad, Hercules, CA).

ADCC assays

ADCC assays were performed in 200 μl, total volume. Patient plasma used in the ADCC assay was incubated for 30 min at 56°C to inactivate the complement system. Plasmas from randomly selected healthy donors were used as control. A total of 1×10^6 target cells was labeled by incubation with medium containing $Na_2[^{51}Cr]O_4$ (0.5 mCi/ml) at 37°C for 1 h. Cells were washed three times with plain RPMI 1640 medium and resuspended in RPMI 10 at 2×10^5 cells/ml. A total of 50 μl of resuspended cells was added to each well of a 96-well microtiter plate (U bottom). Then, 50 μl of heat-inactivated healthy or patient's plasma diluted to 1/2.5 (thus, final concentration equals to 10^{-1} of original in 200 μl, total volume) in RPMI 10 was added to the plate before incubating for 30 min at 37°C. For the dilution assay of plasma, final concentration of plasma was adjusted to 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} of original with RPMI 10, respectively. After incubation, either 100 μl of patients' PBMCs (2×10^6 cells/ml) (for sample count), 100 μl of RPMI 10 containing 2% Triton solution (for maximum count), or 100 μl of RPMI 10 (for spontaneous release count) was added to each well. The mixtures of reaction were incubated at 37°C in a humidified 5% CO_2 atmosphere for 4 h as in previous reports (8, 41). A total of 100 μl of supernatant was collected from each well, and γ emission was counted using a gamma counter. The percentage of dead cells was calculated using the following formula: cell death (%) = 100 × (sample count − spontaneous release)/(maximum count − spontaneous release).

Blocking of ADCC by peptide 31

After diluted plasma was added with 0, 10, or 100 μg/ml peptide 31 (=FLKEKGGLE) or 100 μg/ml of control peptide (=GGGGGGGGG), 50 μl of the solution was added to resuspended target cells. ADCC assay was performed as above.

Flow cytometric analysis

For analysis of NK cell subsets, we used the following Ab combinations: 1) FITC-conjugated anti-CD56, PE anti-CD16, PerCP anti-CD19, allophycocyanin anti-CD3; 2) FITC anti-perforin, PE anti-CD56, PE anti-CD16, PerCP anti-CD19, allophycocyanin anti-CD3. For phenotypic analysis of NK cells, PBMCs were suspended in 50 μl of culture medium, and stained with Ab combination 1, for 20 min on ice. After incubation, cells were washed twice with cold PBS. Cells were resuspended in 200 μl of PBS containing 0.5% formaldehyde. For intracellular staining of perforin, cells were stained with Ab combination 2 (without anti-perforin Ab) for 20 min. After incubation, cells were washed twice with cold PBS, and resuspended in 100 μl of PBS. After addition of 100 μl of 4% formaldehyde and incubation for 20 min at room temperature, cells were pelleted and supernatants were removed. Cells were washed once with PBS/0.5% BSA/1 mM of sodium azide (PBS/BSA/azide buffer), and resuspended in 150 μl of permeabilization buffer (PBS/BSA/azide buffer containing 0.5% saponin). After pipetting gently to mix and incubating for 10 min at room temperature, cells were pelleted and supernatant was removed. A total of 25 μl of permeabilization buffer containing the appropriate amount of Abs against intracellular perforin was added to the cell pellets and incubated at room temperature for 30 min in the dark. Cells were washed once with 0.5 ml of permeabilization buffer and once with 1 ml of PBS/BSA/azide buffer. Finally, cells were suspended in 200 μl of PBS/BSA/azide buffer. All samples were kept at 4°C and protected from light until analysis on the flow cytometer.

Six-parameter flow cytometric analysis was done on a FACSCalibur flow cytometer (BD Immunocytometry Systems) using CellQuest software (BD Immunocytometry Systems) with FITC, PE, PerCP, and allophycocyanin as the four fluorescent parameters. FlowJow software (Tree Star, San Carlos, CA) was used to make configurations. Light scatter gates were designed to include only lymphocytes, and up to 100,000 events in this gate were collected. The absolute lymphocyte count was determined from the complete blood count. The number of NK cells per microliter of whole blood was calculated by multiplying the fraction of lymphocytes that were $CD16^+$ or $CD56^+$ by the absolute lymphocyte per microliter of blood. For analysis and display of statistical comparisons, we used JMP software for the Apple Macintosh (SAS Institute, Cary, NC). Comparisons of distributions were performed by the nonparametric two-sample Wilcoxon rank test.

Results

Nef protein expression on the cell surface infected with SeV-Nef

LTNP 5 in the previous study had a high titer of the Abs against peptide 31 (39). When CEM-NK^R-Nef cells fixed with paraformaldehyde were stained with diluted plasma from healthy donor or LTNP 5, and FITC-conjugated anti-human Ig secondary Abs, positive fluorescent signals were given on the surface of CEM-NK^R-Nef cells by plasma from LTNP 5, but not from a healthy donor

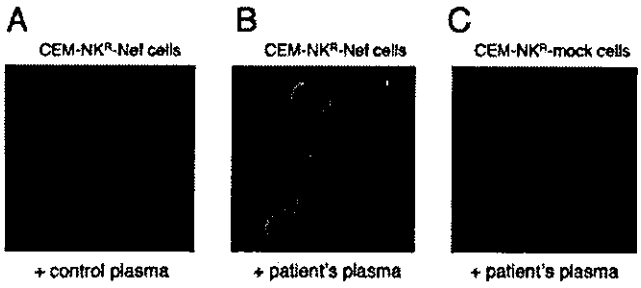

FIGURE 1. Immunological staining of CEM-NK^R cells infected with SeV-Nef (CEM-NK^R-Nef cells). Cells were stained with 1/2.5 diluted plasma and FITC-conjugated anti-human Ig secondary Abs. The stained cells were observed by confocal microscopy. *A*, CEM-NK^R-Nef cells stained with plasma of a healthy donor. *B*, CEM-NK^R-Nef cells stained with plasma from LTNP 5. *C*, CEM-NK^R-mock cells stained with plasma from LTNP 5.

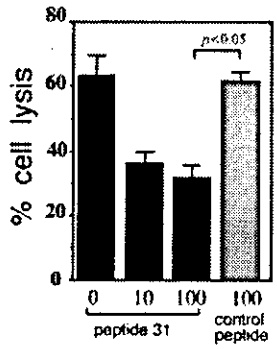

FIGURE 3. Inhibition of ADCC by peptide 31. Percentage of cell lysis by PBMCs of P3 was examined by ADCC assay in the presence of peptide 31 (■) or control peptide (hatched column) at an E:T ratio of 20:1. Data are shown as the mean of triplicate determinations (bars represent SDs). There is a significant difference between peptide 31 and control peptide at the concentration of 100 μg/ml (Student's *t* test, $p < 0.05$).

(Fig. 1, *A* and *B*). Plasma from LTNP 5 did not recognize proteins on the cell surface of CEM-NK^R-mock cell (Fig. 1*C*).

ADCC assay

An ADCC assay was conducted using plasma from LTNPs (LTNP 2, 5, and 6) (39) and PBMCs of either a healthy volunteer or from a patient 1–5 whose profiles are provided in Table I. As shown in Fig. 2*A*, CEM-NK^R-Nef incubated with plasma of LTNP 5 (final concentration, 10^{-1} of original) was efficiently lysed with PBMCs of a healthy volunteer at an E:T ratio of 20:1 (mean percentage of cell lysis, 58%) and 50:1 (66%). When the E:T ratio was lowered to 5:1, percentage of cell lysis decreased to 30% (Fig. 2*A*). The plasmas from LTNP 2, 5, and 6 (final concentration, 10^{-1} of original) induced ADCC activity via Nef, and the plasma of LTNP 6 indicated lower activity compared with that of LTNP 2 or LTNP 5 (Fig. 2*B*). Cytotoxic activity against CEM-NK^R-Nef was observed when PBMCs of five HIV-1-infected patients (p1–5) were used as effector cells at an E:T ratio of 20:1 (Fig. 2*C*). This cytotoxicity was specific to plasma of HIV-1-infected patients, because cell lysis was less than 10% when plasma from a healthy donor was used instead of patient plasma (Fig. 2*C*). In addition, the observation that dilution of patient plasma reduced the percentage of CEM-NK^R-Nef cell lysis (Fig. 2*D*) also suggested that lysis was mediated by the Ab in the plasma. To examine whether the cell lysis is specific to Nef, we added synthetic peptide 31 to the mixture of ^{51}Cr-labeled CEM-NK^R-Nef, PBMCs of patient 3, and LTNP 5 plasma at an E:T ratio of 20:1. Addition of 10 or 100 μg/ml peptide 31 decreased the percentage of cell lysis by 42 or 48% when compared with cell lysis without peptide 31, respectively, whereas addition of 100 μg/ml of control peptide did not show any effect on cytotoxicity (Fig. 3).

NK cells of HIV-1-infected patients

We analyzed NK cells in the peripheral blood using flow cytometry. NK cells were defined as CD3$^-$, CD19$^-$, CD16$^+$, or CD56$^+$ lymphocyte (44). PBMCs from 41 HIV-1-infected patients and 16 healthy donors were examined. There was a significant difference between HIV-1-infected patients and normal controls in total counts of NK cells (mean ± SD = 131 ± 85 and 198 ± 87 cells/μl, respectively, $p = 0.014$) (Fig. 4*A*). When HIV-1-infected individuals were divided into two groups by CD4$^+$ T cell counts (CD4 ≥ 200 or CD4 < 200 cells/μl), there was no significant difference between these two groups in absolute counts of NK cells (CD4 ≥ 200 and CD4 < 200 cells/μl; mean ± SD = 125 ± 94 and 142 ± 82 cells/μl, respectively, $p = 0.643$). For the functional analysis of NK cells, we next examined the expression of intracellular perforin in NK cells of HIV-1-infected patients. As shown in Fig. 4*B*, there was no significant difference between HIV-1-infected patients and healthy controls in frequency of perforin high-positive cell (%) of total NK cells (CD4 ≥ 200, CD4 < 200 cells/μl, and healthy controls; mean ± SD = 83 ± 12, 90 ± 6, and

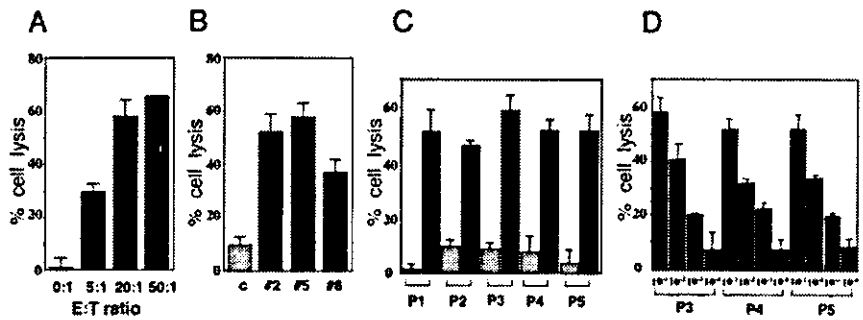

FIGURE 2. ADCC assay using diluted plasma, PBMCs, and radiorabeled CEM-NK^R-Nef. The values are given as percentage of specific cell lysis = 100 × (sample count − spontaneous release)/(maximum count − spontaneous release). *A*, Various E:T ratio with healthy donor PBMCs in the presence of plasma from LTNP 5. *B*, Plasma from a healthy donor (hatched column) or LTNPs (LTNP 2, 5, and 6) (■) at an E:T ratio of 20:1 with healthy donor PBMCs. *C*, PBMCs from five patients (P1-P5, Table I) at an E:T ratio of 20:1 in the presence of either plasma from a healthy donor (hatched column) or LTNP 5 (■) in *C*. *D*, Plasma Ab titration. Percentage of cell lysis by PBMCs from patient P3, P4, or P5 was examined with serially diluted plasma from LTNP 5 at an E:T ratio of 20:1. The values along the *x*-axis represent final concentration, 10^{-1}~10^{-4} of original plasma. Data are shown as the mean of triplicate determinations (bars represent SDs).

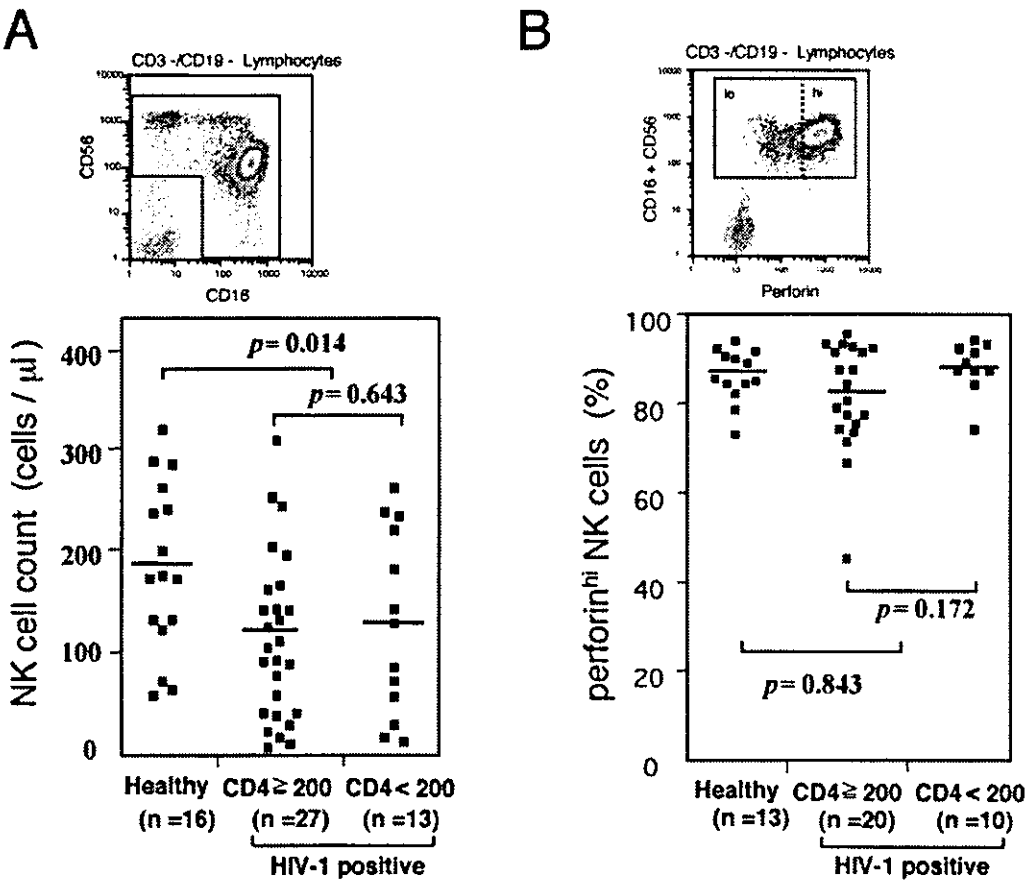

FIGURE 4. Flow cytometric analysis of NK cells. NK cells were defined by $CD3^-$, $CD19^-$, $CD16^+$, or $CD56^+$ expression. *Upper panels*, Show flow cytometry profiles gated on $CD3^-$ and $CD19^-$ lymphocytes. NK cells were gated by red filled line. *A*, *Lower panel*, Comparison of NK cell counts was conducted between 16 healthy donors and 40 HIV-1-positive individuals. *B*, *Upper panel*, NK cells are distingushed between perforin high/positive (hi) and low (lo) populations by red dotted line. *Lower panel*, Frequency of perforin high-positive cells (%) of total NK cells for each donor was calculated. Comparison was conducted between 13 healthy donors and 30 HIV-1-positive individuals. Median values are shown as bars.

88 ± 6%, respectively), suggesting that NK cells in HIV-1-infected patients were as functionally active as those in non-HIV-1-infected individuals.

Discussion

In a previous report, we showed that the progression of disease in HIV-1-infected patients was correlated with Ab titers against peptide 31 (39). In an effort to elucidate the mechanism for this correlation, we studied the role of ADCC against peptide 31 in this study. The interaction between plasma Abs of LTNP 5 and Nef proteins was specific (Fig. 1). We showed that PBMCs from HIV-1-infected donors as well as healthy donors could exert specific ADCC against the cells expressing Nef protein (CEM-NK^R-Nef cells) with patient's plasma even in the face of less than normal NK cell count (Table 1; Fig. 2, *A*, *B*, and *C*). Thus, the ADCC activity may contribute to the elimination of HIV-1-infected cells in vivo. Because ADCC activity is dependent on the titer of plasma Ab (Fig. 2*D*), the lower activity of LTNP 6 (Fig. 2*B*) could be attributed to the lower titer of Ab against Nef epitope compared with LTNP 2 or 5, based on our previous data (39). The ADCC activity was inhibited up to ~50% by peptide 31 compared with control peptide (Fig. 3), suggesting that specific Abs against peptide 31 may contribute substantially to eliminate the HIV-1-infected cells. However, other Nef-derived peptides may also contribute to the residual 50% activity as epitopes we have not yet isolated. It was previously shown that selective down-regulation of MHC class I molecules protects HIV-1-infected cells from CTL and NK cells (45–49). In contrast, ADCC via Abs against the conserved cell surface HIV-1 epitopes such as peptide 31 may be an alternative armor against HIV-1 infection.

Although percentages of NK cells varied in the five patients examined (3.7~12.6%) (Table I), they showed almost the same levels of ADCC activity (Fig. 2*C*). This result may be due to the high E:T ratio that we used in the cytotoxicity assay (Fig. 2*A*); however, it is possible that ADCC activity may be retained until late in the clinical stage, as previously reported (50, 51). Flow cytometric analysis revealed a reduction of total NK cell counts in HIV-1-infected individuals, similar to the previous reports (52, 53) (Fig. 4*A*). There was no significant difference between the two groups of HIV-1-positive patients (CD4 ≥ 200 cells/μl and CD4 < 200 cells/μl); therefore, NK cells appear to be retained even late in the disease progression. With regard to Nef epitope expressing on the cell surface, we previously documented that HIV-1-infected cells were lysed by the combination of rabbit polyclonal Abs against peptide 31 and rabbit complements (39). Thus, we speculate that the level of Nef expression could be sufficient for the induction of ADCC via Nef epitope on the cell surface. However, it could be too difficult to estimate ADCC via Nef epitope with HIV-1-infected cells and patient's plasma because of the existence of abundant anti-envelope Abs as well as anti-Nef Abs in the plasma from HIV-1-infected patient.

We and others showed that HIV-1-specific CD8 T cells contain less perforin (54–56). NK cells may function as better effector cells in the HIV-1-infected individuals. Although the number of

NK cells was lower in HIV-1-infected patients than healthy controls, NK cells retained the high expression of perforin until late in the clinical course (Fig. 4*B*). Rukavina et al. (57) demonstrated that perforin expression significantly correlates with NK cytotoxicity against K562 cells. The fact that LTNPs had higher anti-peptide 31 Abs than progressors may indicate that ADCC against conserved cell surface HIV-1 epitopes such as peptide 31 may have favorable influence on the clinical course. Finally, therapeutic intervention that contributes to raise specific Ab levels against the conserved cell surface HIV-1 epitopes may prove to have a clinical benefit.

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REVIEW ARTICLE

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Current concepts in SARS treatment

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Abstract The outbreak of severe acute respiratory syndrome (SARS) has drawn enormous attention and caused fear worldwide since early 2003. The disease appears to be under control now; however, the possible return of SARS must be emphasized. Although many clinical experiments have been reported, the treatment of SARS is largely anecdotal, and so far no treatment consensus has been reached. We summarize 14 clinical reports and attempt to assess the effectiveness of various treatment regimens. A combination treatment of steroids and ribavirin was widely used empirically from the outset of the epidemic. In general, the use of steroids for SARS seemed beneficial, but the optimal timing, dosage, and duration of treatment have not yet been determined. On the other hand, ribavirin administration apparently reduced neither the rate of intratracheal intubation nor that of mortality. Moreover, significant toxicity, such as hemolytic anemia, has been attributed to ribavirin. A few preliminary trials and in vitro data suggest the possibility of treating SARS with interferon. Other agents, including the HIV protease inhibitor glycyrrhizin and convalescent plasma, remain to be evaluated.

Key words SARS · Treatment · Steroids · Ribavirin · Interferons

Introduction

Severe acute respiratory syndrome (SARS) is a newly emerging, readily transmissible, and predominantly pneumonic disease caused by a novel coronavirus referred to as the SARS coronavirus (SARS-CoV).[1–3] It first appeared in Guangdong Province, China, in November 2002[4] and rapidly spread to a total of 29 countries all over the world since late February 2003. This outbreak affected 8098 people and resulted in 774 deaths (mortality rate: 9.6%) by 31 July 2003,[5] drawing enormous attention and causing fear worldwide. The World Health Organization (WHO) declared the end of the worldwide SARS outbreak in July 2003. Although the disease appears to be under control at the time of writing (December 2003), the possible return of SARS should be considered.

Numerous articles on SARS, describing its epidemiology, etiology, diagnosis, clinical features, and management, have been published internationally. Much has been learned about SARS during the several months since the end of the outbreak, but many questions remain unanswered. In particular, the treatment of SARS remains largely anecdotal, and no treatment consensus has yet been reached, since randomized controlled treatment trials were understandably not possible during the outbreak of this novel acute disease. Until we have efficacious vaccines and specific anti-SARS-CoV agents, SARS is likely to remain a major health threat to the world. Here, we review the diverse treatment experiences and controversies to date in order to consolidate our current knowledge and prepare for a possible resurgence of the disease.

Antibiotics

At the first signs of the disease, the administration of broad-spectrum antibiotics such as a fluoroquinolone or β-lactams plus macrolide is warranted because presenting features are nonspecific. Efficient and rapid diagnostic tests are not yet available, especially ones effective in the first few days after onset.[6–8] Upon identification of SARS-CoV, the antibiotic therapy may be withdrawn. In addition to their antibacterial action, macrolides[9] and fluoroquinolones[10] are known to have immunomodulatory properties, but their effect on the course of SARS has not been determined.

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