Nuclear Extraction and Electrophoretic Mobility-Shift Assays. Nuclear extracts were prepared from 1×10^7 cells as described (28). Activation of NF-κB was determined by using 5 μg of nuclear extract in the gel-shift assay system (Promega) according to the manufacturer's instructions.

Apoptosis Assays. DNA fragmentation by apoptosis was detected by PI staining as described (29). Terminal deoxytransferase-mediated dUTP nick end labeling (TUNEL) assays were performed by using the *in situ* cell death detection kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. DNA ladder formation was performed by extracting DNA from cells with the genomic DNA purification kit (Gentra Systems), and electrophoresis was performed.

Animal Experiments. Simvastatin tablets (ZOCOR, Merck) were mixed with mouse food at a ratio of 160 mg of simvastatin per 65 g of powdered food. Untreated animals received powdered food without simvastatin. LCLs (0.25×10^6, 1×10^6, or 4×10^6) were inoculated i.p. into 8-week-old SCID mice. Simvastatin was given either 3 days before (pretreatment group) or 7 days after (treatment group) inoculation of cells and continued until 4–6 weeks after inoculation. Thereafter, food without simvastatin was given to all mice, because of the side effects of prolonged high-dose simvastatin. All dead mice were autopsied and examined for the presence of lymphomas.

Results

Simvastatin Inhibits Clump Formation and Induces Apoptosis of LCLs. LCLs and Burkitt lymphoma cells that express each of the EBNAs, LMPs, and LFA-1 grow in tight clumps. Binding of anti-LFA-1 antibody TS1/22 to the I domain of LFA-1 inhibits clumping of phorbol myristate acetate-stimulated LCLs after 18 h (6). To determine whether simvastatin affects clumping of unstimulated LCLs, we treated an LCL (12A1) with various concentrations of the drug. Five days after the addition of simvastatin, LCL clumps broke apart in wells treated with ≥ 2 μM simvastatin, whereas clumps remained in wells treated with ≤ 1 μM simvastatin (Fig. 1*A*). Dissociation of clumps was also observed in other LCLs (6B10 and 295H) and in Mutu-3 cells (data not shown). Cell viability assays were performed on various cell lines expressing different levels of LFA-1, ICAM-1, and LMP-1 (Table 1 and Fig. 5, which is published as supporting information on the PNAS web site). XTT cell proliferation assays showed that simvastatin decreased the number of viable cells in a dose-dependent manner (Fig. 1*B*). A loss in viability of LCLs was induced by 0.5 μM simvastatin, whereas ≥ 2 μM simvastatin was required to reduce viability of other cells. Trypan blue staining showed that LCLs 12A1 and 295H had >50% reduction in viability with 2 μM simvastatin, whereas Akata and P3HR-1 cells required higher concentrations of the drug (4 μM) to achieve >50% reduction in viability (Fig. 1*C*). The number of dead cells began to increase 5 days after addition of simvastatin at the time when clumps started to dissociate. PI staining followed by flow cytometry was also used to determine cell viability with 2 μM simvastatin. Cells expressing LMP-1 such as LCLs (12A1, 6B10, and 295H) and Mutu-3 showed low (<50%) viability after culture in 2 μM simvastatin for 7 days (Fig. 1*D*). Thus, incubation of cells with 2 μM simvastatin for 5 days inhibits clump formation and induces death in cells expressing LMP-1 (Table 1).

Some statins induce apoptosis of certain tumor cells *in vitro* (30). PI staining showed that treatment of LCLs with simvastatin for 5 days induced a dose-dependent increase in fragmented DNA that was smaller than G_0–G_1 DNA (2n), indicative of apoptosis (29) (Fig. 1*E*). TUNEL assay confirmed that simvastatin induced DNA fragmentation in LCLs (Fig. 1*F*). Gel electrophoresis showed that simvastatin induced DNA fragment in LCLs resulting in formation of a DNA ladder (Fig. 1*G*). Thus, simvastatin induces apoptosis in LCLs.

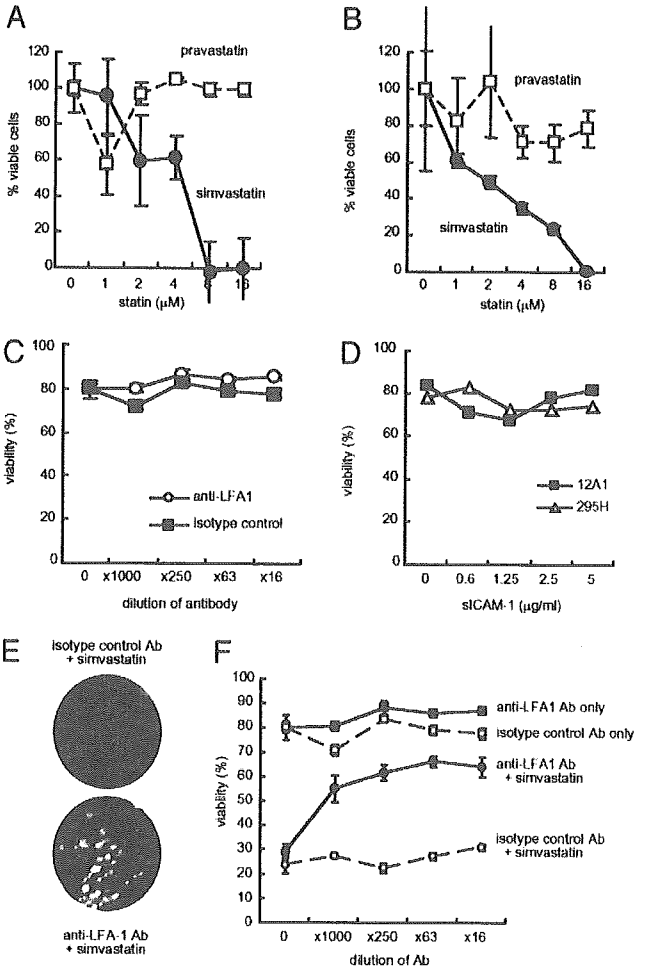

Fig. 2. Apoptosis-inducing effect of simvastatin depends on binding to the I domain of LFA-1. (*A* and *B*) XTT cell proliferation assay was performed for LCLs 12A1 (*A*) and 6B10 (*B*) in the presence of simvastatin or pravastatin after 5 days. (*C*) Cell viability was assayed by trypan blue staining for LCL 12A1 in the presence of anti-LFA-1 antibody TS1/22, which recognizes the I domain of LFA-1, or an isotype control antibody for 7 days. (*D*) Cell viability was measured by trypan blue staining of LCLs 12A1 and 295H cultured with soluble ICAM-1 (sICAM-1) for 7 days. (*E*) LCL 12A1 was cultured with anti-LFA-1 (TS1/22) or isotype control antibody for 1 h, simvastatin (2 μM) or no compound was added, and the cells were cultured for 7 days. Cell clumping is reduced with isotype control antibody and simvastatin (*Upper*) but not with anti-LFA-1 antibody and simvastatin (*Lower*). (*F*) Cell viability was assayed by trypan blue staining of LCLs cultured with an anti-LFA-1 or isotype control antibody. Error bars indicate standard deviations of three separate experiments.

The Apoptosis-Inducing Effect Is Specific for Simvastatin and Depends on Binding to the I Domain of LFA-1. To determine whether loss of cell viability of LCLs occurs with a statin with different binding properties than simvastatin, LCLs were incubated with pravastatin, which does not bind to LFA-1 (13). Pravastatin had little effect on viability of LCL 12A1 or 6B10 (Fig. 2 *A* and *B*). Anti-LFA-1 monoclonal antibody TS1/22 and soluble ICAM-1 (sICAM-1) bind to the I domain of LFA-1 (26, 31), which is the site on LFA-1 targeted by simvastatin (13). Anti-LFA-1 TS1/22 antibody bound to LFA-1 on the cell surface at a 1:100 dilution by flow cytometry (Fig. 5); however, the antibody did not affect viability of 12A1 cells at concentrations up to 1:16 (Fig. 2*C*). sICAM-1 used at concentrations (5 μg/ml) that block rhinovirus infection (31) did not affect viability of LCLs 12A1 and 295H (Fig. 2*D*). Thus, antibody or another ligand that binds to the I domain of LFA-1 does not induce death of LCLs.

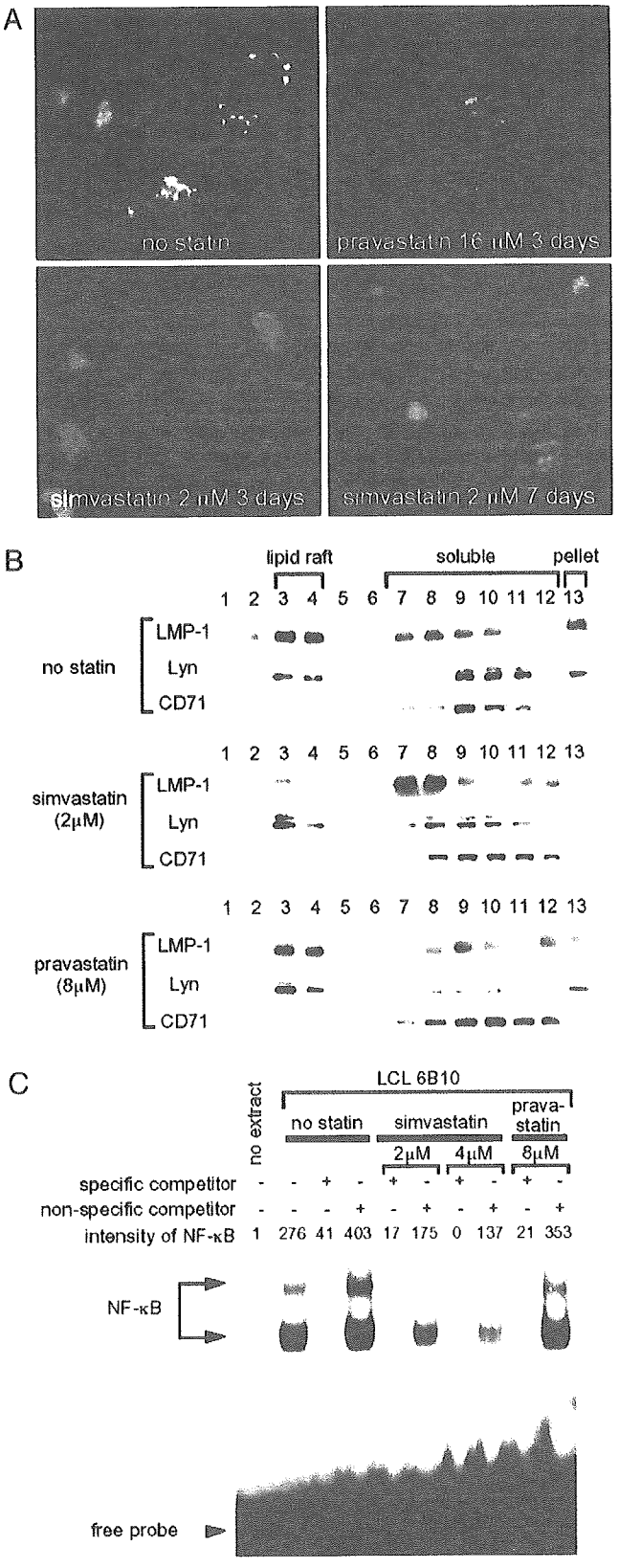

Fig. 3. Simvastatin alters the localization of LMP-1, displaces LMP-1 from lipid rafts, and inhibits NF-κB activation in LCLs. (*A*) Immunofluorescence of LMP-1 in LCL 6B10 in the absence or presence of simvastatin or pravastatin. Cells were fixed, permeabilized, and incubated with anti-LMP-1 antibody (CS1–4, DakoCytomation, Carpinteria, CA), followed by FITC-conjugated secondary antibody and PI. LMP-1 is green (FITC) and nuclei are red (PI). (*B*) Immunoblotting of LCL fractions. LCLs were cultured in the absence or presence of simvastatin or pravastatin for 3 days, and cell lysates were fractionated by sucrose gradient ultracentrifugation. Equal aliquots of each fraction beginning at the top of the centrifuge tube were probed with antibodies to LMP-1, Lyn (a tyrosine kinase that localizes in lipid rafts), and CD71 (a transferrin receptor that does not localize in rafts). (*C*) Electrophoretic mobility-shift assays for NF-κB. LCL 6B10 was cultured in the absence or presence of simvastatin or pravastatin for 3 days, and nuclear extracts were used in electrophoretic mobility-shift assays with a radiolabeled NF-κB probe. Specific or nonspecific nonradioactive competitor oligonucleotides were added to some assays. The intensity of NF-κB-specific bands was measured by using a phosphorimager.

Antibody to LFA-1 (TS1/22) has been shown to induce signal transduction in lymphocytes (32). Therefore, pretreatment of LCLs with the antibody might inhibit the effects of simvastatin. Pretreatment of cells with anti-LFA-1 antibody TS1/22 blocked the ability of simvastatin to dissociate clumps of LCLs (Fig. 2*E*) and to reduce viability of LCLs (Fig. 2*F*).

Simvastatin, but Not Pravastatin, Dissociates LMP-1 from Lipid Rafts and Reduces Activation of NF-κB. *In vitro* assays indicate that cholesterol depletion by high doses of statins disrupts lipid rafts and alters the localization and function of proteins in lipid rafts on the cell membrane (33–35). EBV LMP-1 localizes in lipid rafts and constitutively activates NF-κB via tumor necrosis factor receptor-associated factors (3, 27, 36). Therefore, we postulated that simvastatin may affect localization of LMP-1 in lipid rafts and impair signal transduction by LMP-1. To determine whether simvastatin alters localization of LMP-1, we treated LCLs with simvastatin and performed immunofluorescence assays for the viral protein. LMP-1 localized to large punctate structures in LCLs in the absence of simvastatin (Fig. 3*A*). After treatment with simvastatin for 3 or 7 days, LMP-1 showed a fine granular pattern in most of the cells that was much fainter and more diffuse than in untreated cells. In contrast, LMP-1 maintained its large punctate structures in cells treated with pravastatin. To further examine the effect of statins on LMP-1 localization, cell extracts were fractionated by using centrifugation and floatation in sucrose gradients (27). Lyn localizes to lipid rafts, whereas CD71 is in the soluble fraction (27). Immunoblotting showed that lipid raft fractions (fractions 3 and 4) contained the highest concentrations of LMP-1 in untreated or pravastatin-treated LCLs. At 2 μM simvastatin, the highest concentrations of LMP-1 shifted to soluble fractions (fractions 7–12), but Lyn remained in lipid rafts (fractions 3 and 4) (Fig. 3*B*). Quantitative analysis showed that 47% of LMP-1 was localized in lipid rafts of untreated cells; after treatment with simvastatin for 3 days, only 7% of LMP-1 was located in rafts. These data indicate that simvastatin, but not pravastatin, alters the localization of LMP-1 in the cell and dissociates LMP-1 from lipid rafts.

Each of the LCLs used in the present study showed constitutive activation of NF-κB (Fig. 5). Electrophoretic mobility-shift assays showed that treatment of LCLs with simvastatin (2 μM or 4 μM) for 3 days reduced the level of activated NF-κB, whereas pravastatin (8 μM) did not reduce NF-κB (Fig. 3*C*). Cell viability at 3 days ranged from 80–90% in cells treated with either statin or in untreated cells. Because inhibition of NF-κB induces apoptosis of LCLs (28), our results suggest that reduction of NF-κB by simvastatin results in induction of apoptosis in LCLs.

Simvastatin Delays the Onset of EBV Lymphomas and Prolongs Survival in SCID Mice Inoculated with EBV-Transformed LCLs. Because simvastatin induced apoptosis of LCLs, we tested the effect of the drug on SCID mice with EBV lymphomas. Simvastatin (250 mg/kg/day) was given orally to SCID mice beginning 3 days before i.p. inoculation with EBV-transformed LCLs. Control animals did

Table 2. SCID mouse experiments with simvastatin

LCL	Group	No. of cells	No. of mice*	Time of therapy†	Simvastatin days (mg/kg/day)	50% survival, days	Day of death, range in days	P (logrank)‡
12A1	1	0.25×10^6	6	Pretreatment	−3 to +28 (250)	81	70 to >105	0.0496
	2	0.25×10^6	10	No treatment	−(0)	56	45–105	
	3	1×10^6	6	Pretreatment	−3 to +28 (250)	56	53 to >105	0.0350
	4	1×10^6	12	No treatment	−(0)	53	48–56	
	5	4×10^6	10	Pretreatment	−3 to +28 (250)	56	49–57	<0.0001
	6	4×10^6	12	No treatment	−(0)	46	43–50	
6B10	7	1×10^6	10	Pretreatment	−3 to +44 (250)	>100	64 to >100	0.0375
	8	1×10^6	11	Treatment	+7 to +44 (250)	85	76 to >100	0.2135
	9	1×10^6	11	No treatment	−(0)	71	54 to >100	

*Number of mice analyzed. At 4 weeks, six animals receiving 0.25×10^6 of LCL 12AI and simvastatin, six animals receiving 1×10^6 of 12A1 and simvastatin, and two animals receiving 4×10^6 12A1 and simvastatin died or were sacrificed because of simvastatin side effects. These included severe weight loss, hunched body, or red swollen eyes. Autopsies showed no evidence of ascites or lymphomas in these mice and they were not included in the table and were excluded from the analysis.

†Time of therapy. Animals received simvastatin 3 days before (pretreatment) or 7 days after (treatment) inoculation with LCLs.

‡Significance. *P* values indicated are for mice treated with simvastatin versus mice not treated with the drug for each cell number.

not receive simvastatin. Four or 6 weeks later, simvastatin was discontinued because of side effects from the drug (Table 2) and mice were followed for development of tumors. Seven weeks after inoculation with LCLs, 80% of mice in the control group that received 0.25×10^6 cells developed ascites, whereas none of the animals treated with simvastatin that received the same number of cells had ascites (Fig. 4*A*). Nine weeks after inoculation, >70% of the control mice were dead of lymphoma, whereas all mice treated with simvastatin that received the same number of cells were alive, although a few had ascites. Mice pretreated with simvastatin that received different doses of LCLs all survived significantly longer compared to mice that did not receive the drug (Table 2). At autopsy all of these control mice had immunoblastic lymphomas (Fig. 4*A*). Flow cytometry showed that expression of LFA-1 in tumor cell ascites was reduced in mice treated with simvastatin compared with mice that did not receive the drug (data not shown). In a separate series of experiments using LCL 6B10, we compared mice that received simvastatin 3 days before inoculation with LCLs (pretreatment group) with mice that received the drug 7 days after inoculation (treatment group). Mice in the pretreatment group survived significantly longer ($P < 0.04$) compared with animals in the control group; however, the difference in survival was not significant for animals in the treatment group versus the control group ($P = 0.2135$) (Table 2 and Fig. 4*B*).

Discussion

We have shown that simvastatin induces cell death of EBV-transformed LCLs *in vitro* and prolongs survival of SCID mice with EBV lymphomas. This effect was seen with simvastatin, which binds to LFA-1, but not with pravastatin, which does not bind to LFA-1. Simvastatin dissociated LMP-1 from lipid rafts, inhibited NF-κB activation, and induced apoptosis in LCLs. Expression of LMP-1 and LFA-1 and constitutive NF-κB activation were critical for simvastatin-induced death of LCLs (Table 1). To our knowledge, this is the first report in which a drug that targets LFA-1 has been used to treat B cell lymphoma.

Apoptosis of LCLs occurred after 5–7 days of treatment with simvastatin. During the first 2 days of treatment there was no change in the morphology or viability of LCLs. However, 3 days after addition of simvastatin, LCL clumps became smaller, but the cells were still viable. At 5 days most clumps had dissociated and apoptosis was detected; at day 7 >80% of the cells were dead. We postulate that the relatively long time required for simvastatin to induce cell death is likely due to initiation of several processes in the cell. First, simvastatin blocks the binding of ICAM-1 to the I domain of LFA-1. LFA-1 binding to ICAM-1 on T cells induces cell proliferation (37), and because simvastatin inhibits LFA-1 binding

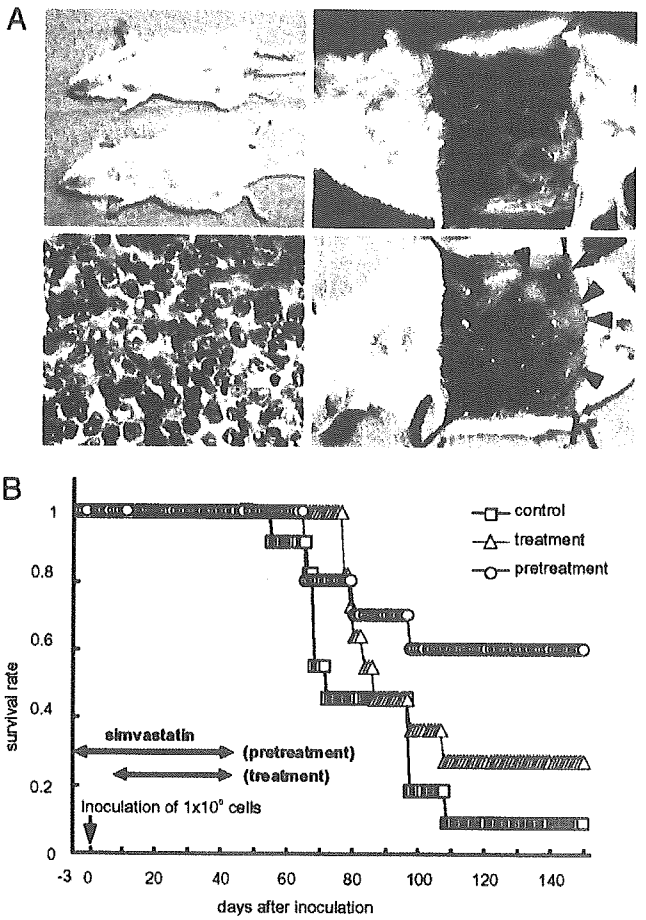

Fig. 4. Simvastatin prolongs survival of SCID mice inoculated with LCLs. (*A*) Appearance of mice at 45 days after inoculation. The mouse that did not receive simvastatin (*Upper Left*, lower mouse) has ascites, but the simvastatin-treated mouse (upper mouse) has no ascites. The simvastatin-treated mouse (*Upper Right*) has no ascites or solid tumors, whereas the untreated mouse (*Lower Right*) has ascites (arrows) and solid tumors (arrowheads). Immunoblastic lymphoma is present in the mouse not treated with simvastatin (*Lower Left*). (*B*) Kaplan–Meier survival curves of SCID mice that were inoculated with 1.0×10^6 6B10 LCLs. Mice were pretreated with simvastatin beginning 3 days before inoculation with cells (blue circles), treated with drug beginning 7 days after inoculation with cells (red triangles), or not treated with drug (squares).

to its receptor, the proliferation signal from LFA-1 is impaired by simvastatin. Second, simvastatin displaces EBV LMP-1 from lipid rafts and prevents signaling by the viral protein. LMP-1 recruits tumor necrosis factor receptor-associated factor 3 to lipid rafts and localization of LMP-1 to rafts activates NF-κB (36). Three days after treatment of LCLs with simvastatin, LMP-1 was dissociated from rafts and activation of NK-κB was inhibited in nuclear extracts from the cells. Because constitutive activation of NF-κB is required for growth of LCLs (28), simvastatin may induce apoptosis by turning off activation of NF-κB in these cells. This hypothesis is consistent with our observations that 2 μM simvastatin induced >50% death only in cells that expressed LMP-1 in which NF-κB was constitutively activated (Table 1). Although LFA-1 also localizes in lipid rafts of stimulated T cells (38) and simvastatin might have an effect on LFA-1, we did not observe a reduction in LFA-1 levels after treatment of LCLs with simvastatin *in vitro* (unpublished data). We did, however, note down-regulation of LFA-1 in EBV-transformed B cells in mice after treatment for 4–6 weeks. It is uncertain whether long-term simvastatin treatment *in vivo* resulted in a direct down-regulation of LFA-1 expression, or whether lower (trough) levels of simvastatin in serum may have allowed for selection and expansion of rare EBV-transformed B cells with low LFA-1 expression on their surface.

Simvastatin prolonged survival in SCID mice inoculated with EBV-transformed LCLs. Statins have previously been reported to have antitumor effects; however, these effects occurred at higher levels of drug (5–400 μM) (39–41) than were used in our study. In addition, other studies of statins showed that the effects were not specific for individual cell types and were seen with a wide variety of tumors both *in vitro* and in animal models (39–44). Clinical trials of lovastatin have shown partial responses in some patients with cancer (30). Simvastatin had an adjuvant effect in some, but not all, studies when given with other antitumor drugs (40, 41). The plasma levels of statins achieved in humans in clinical trials (2.32 ± 1.27 μM at peak concentration) (45) were comparable to the concentration of simvastatin (2 μM) that induced apoptosis of EBV-transformed LCLs *in vitro* and did not result in severe drug toxicity in the patients (30). Although a high dose of simvastatin was used in mice in this study (250 mg/kg/day), administration of simvastatin to mice at 100–400 mg/kg/day resulted in mean plasma drug levels about four to eight times higher than the mean human plasma drug level after an 80-mg oral dose, which is the maximum dose for cholesterol-lowering effects (46). Mice metabolize simvastatin much more rapidly than humans and therefore lower doses might be effective in humans.

Simvastatin, or other statins that block LFA-1 binding to ICAM-1, may have a role in the treatment of EBV-associated immunoblastic lymphomas that occur in immunocompromised persons. These tumor express high levels of LFA-1 (4) and LMP-1 (47) and have constitutive activation of NF-κB *in vivo* (9). Simvastatin might be used in combination with other agents for these patients. Simvastatin has not been reported to cause suppression of bone marrow function, and therefore it might not add to the hematopoietic toxicity that often occurs with other chemotherapeutic agents used to treat EBV-associated lymphomas. Recently we have found that lovastatin and atorvastatin also kill LCLs at similar concentrations as simvastatin (H.K. and J.I.C., unpublished results). Further studies are needed to assess the use of statins as adjunctive therapy for EBV-associated lymphomas in immunocompromised persons.

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Chronic active Epstein-Barr virus infection associated with mutations in perforin that impair its maturation

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Chronic active Epstein-Barr virus infection (CAEBV) is a rare disease in which previously healthy persons develop severe, life-threatening illness. Mutations in the perforin gene have been found in familial hemophagocytic lymphohistiocytosis, which shares some features with CAEBV. We studied a patient who died at age 18, 10 years after the onset of CAEBV. The patient had high titers of antibodies to EBV, EBV RNA in lymph nodes, T-cell lymphoproliferative disease, and hemophagocytic lymphohistiocytosis. DNA sequencing showed novel mutations in both alleles of the perforin gene that resulted in amino acid changes in the protein. The quantity of the native form of perforin from the patient's stimulated peripheral blood mononuclear cells (PBMCs) was extremely low and immunoblotting showed accumulation of an uncleaved precursor form of perforin. Stimulated PBMCs from the patient were defective for Fas-independent cytotoxicity. These data imply that mutations in this patient resulted in reduced perforin-mediated cytotoxicity by his lymphocytes. This is the first case in which perforin mutations have been shown to result in accumulation of the uncleaved, immature form of perforin. Mutations in the perforin gene are associated with some cases of CAEBV with hemophagocytic lymphohistiocytosis. (Blood. 2004;103: 1244-1252)

Introduction

Epstein-Barr virus (EBV) is a member of the human herpesvirus family that infects over 95% of the United States population.[1] Most infections occur in childhood and are asymptomatic; infection of adolescents and young adults with EBV often results in infectious mononucleosis. EBV is associated with a spectrum of lymphoproliferative diseases in patients with congenital or acquired immunodeficiency.

Chronic active EBV infection (CAEBV) is a rare and often fatal disorder that occurs in previously healthy persons and seemingly immunocompetent persons.[2] The disease has been defined by the presence of 3 features.[3,4] First, patients have a severe progressive illness that began as a primary EBV infection, or is associated with abnormal EBV-specific antibody titers that include markedly elevated antibodies to viral capsid antigen (VCA) and early antigens (EAs). Second, histology shows evidence of major organ involvement such as lymphadenitis, hemophagocytosis, meningoencephalitis, or persistent hepatitis. Third, elevated EBV DNA, RNA, or proteins are demonstrable by in situ hybridization or immunohistochemical staining of affected tissues. Recent studies showed that patients with CAEBV can also have markedly elevated levels of EBV DNA in the peripheral blood and this criterion has been used diagnostically in some cases.[5] Patients with CAEBV often develop a progressive cellular and humoral immunodeficiency with pancytopenia and hypogammaglobulinemia that renders them susceptible to opportunistic infections or B- or T-cell lymphoproliferative disease.[3] Therapy for CAEBV is unsatisfactory and, at best, progression of disease is temporarily delayed.

The etiology of CAEBV is unknown. Two studies suggested that persons with CAEBV were infected with unusual lytic strains of virus.[6,7] However, the finding of the same lytic strain of EBV in the unaffected father of one of the patients, and in healthy controls,[8] suggests that other factors, including inherited abnormalities in the response to EBV, contribute to the pathogenesis of the disease. Four observations favor a genetic cause for CAEBV. First, CAEBV is rare in the United States, but relatively common in Japan, Korea, and China. Most patients reported to have fulminant EBV-positive T-cell lymphoproliferative disease following acute and/or chronic EBV infection have been Asian in origin.[9] Second, specific mutations in the signaling lymphocyte activation molecule (SLAM) associated protein (SAP) gene have been identified in boys with a disease that shares many of the features of CAEBV, the X-linked lymphoproliferative disease (XLPD).[10-12] Third, 2 studies showed that cytotoxic T lymphocyte (CTL) or natural killer (NK) cell activity was reduced in patients with CAEBV and in their parents.[13,14] Fourth, gene mutations and polymorphisms have been associated with severe infections with herpesviruses including EBV.[15-17] Taken together, these findings suggest that a genetic abnormality could underlie some cases of CAEBV.

Here we describe a patient with CAEBV in whom we documented a defect in cytotoxic activity in association with markedly reduced levels of the native form of perforin in his stimulated peripheral blood mononuclear cells (PBMCs). Sequence analysis showed him to have compound heterozygous mutations affecting

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Submitted July 1, 2003; accepted October 16, 2003. Prepublished online as *Blood* First Edition Paper, October 23, 2003; DOI 10.1182/blood-2003-06-2171.

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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′-CGCTCT-GATGCGACCAGA-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′-ATTCTCGAGATGGCAGCCCGTCT-GCTCCT-3′ and reverse primer 5′-TATGTCGACTCACCACACGGC-CCCACTCC-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

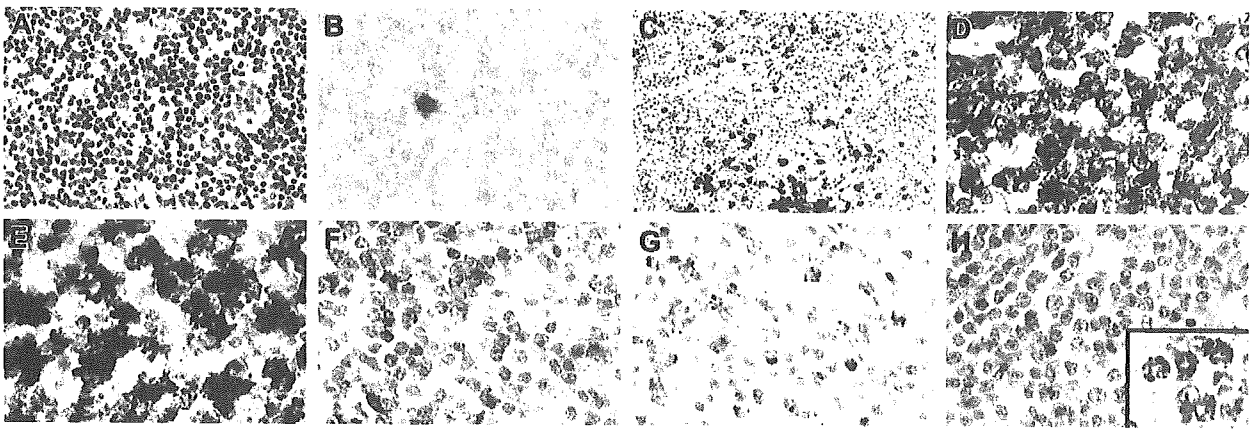

Figure 1. Characterization of T-cell lymphoproliferative process in lymph node. (A) Architecture is diffusely infiltrated by small lymphocytes with admixed histiocytes. (B) In situ hybridization with an EBV-encoded RNA (EBER) probe shows rare positive small lymphocytes (< 1 per high power field) that 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).

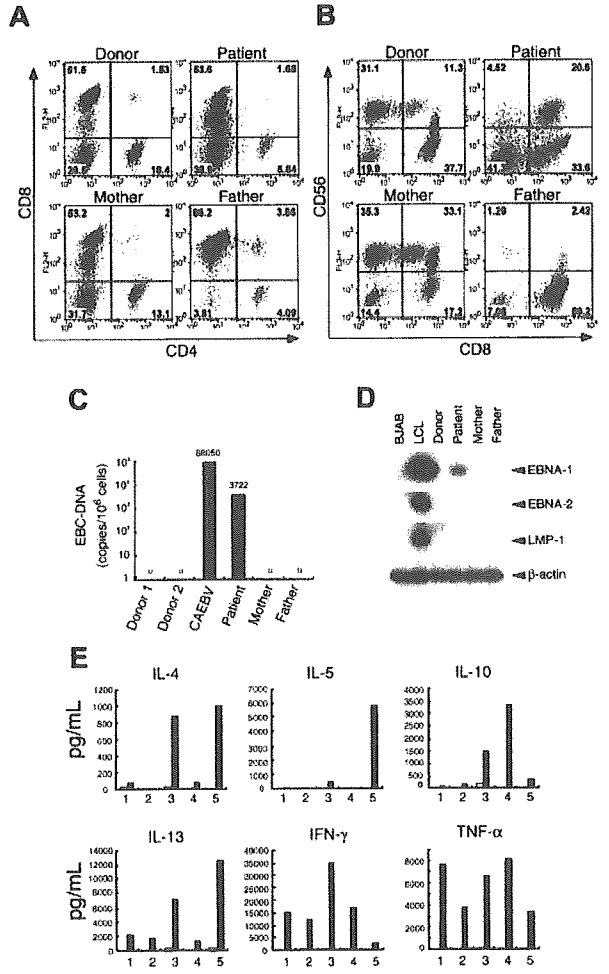

Figure 2. Lymphocyte phenotype, EBV DNA levels with latent gene expression, and cytokine levels in stimulated PBMCs from the patient, his parents, and a healthy blood bank donor. (A) CD4/CD8 and (B) CD8/CD56 expression of PBMCs stimulated with PHA and IL-2 for 2 days followed by IL-2 alone for 2 weeks. (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

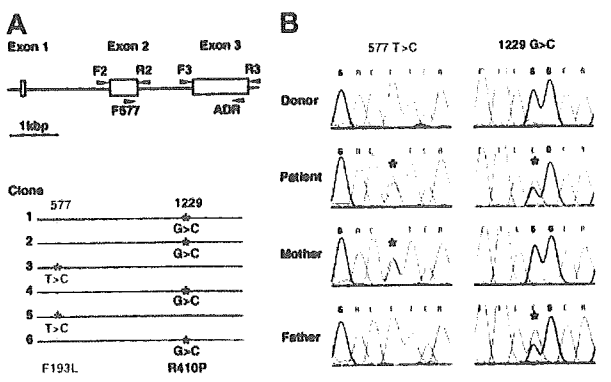

Figure 3. Perforin mutations in the patient with CAEBV and in his parents. (A) The perforin gene contains 3 exons and the open reading frame is encoded by the second and third exons. Exons 2 and 3 were amplified by PCR from genomic DNA using the indicated primers (arrowheads),[20] cloned in plasmid pCR2.1, 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.

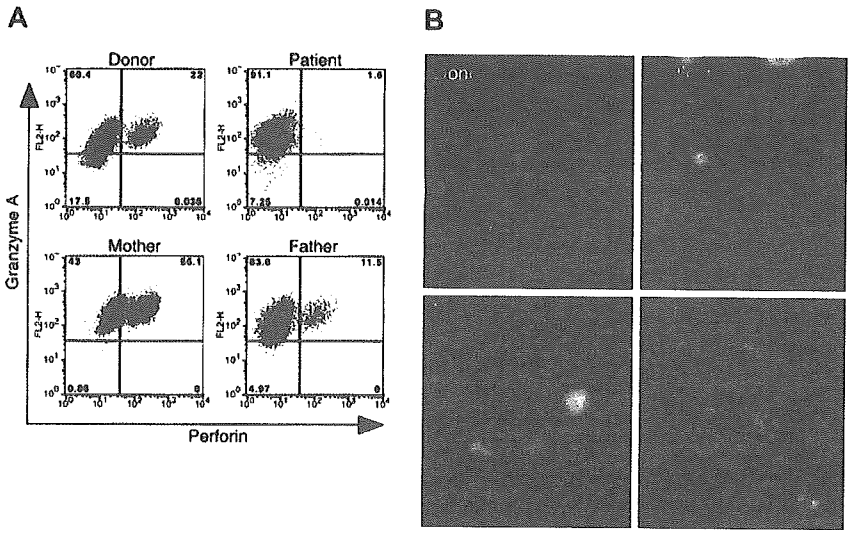

Figure 4. Expression of the native form of perforin in stimulated PBMCs from the patient, his parents, and a healthy blood bank donor. (A) PHA- and IL-2–stimulated PBMCs were fixed and permeabilized, stained with FITC-conjugated antiperforin antibody (dG9; Ancell, Bayport, MN) or phycoerythrin (PE)–conjugated 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.

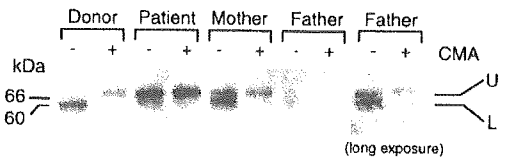

Figure 5. Inhibition of proteolytic cleavage of perforin in the patient with CAEBV. Stimulated PBMCs were cultured in the presence (+) or absence (–) of 200 nM concanamycin A (CMA) for 5 hours. Lysates were prepared from the cells and perforin was detected by immunoblotting with 2d4-perf antibody under 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

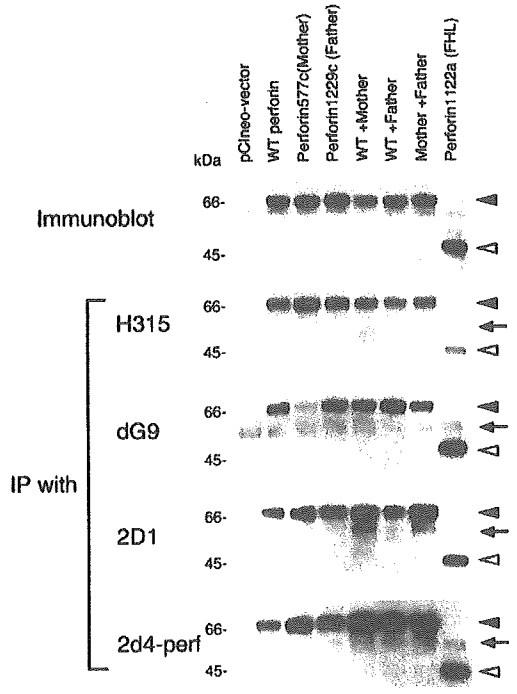

Figure 6. Binding activity of perforin mutants in 293T cells with antibodies. 293T cells were transfected with plasmid(s) expressing wild-type (WT) and/or mutant perforin genes, cell lysates were prepared, and immunoblots were performed using 2d4-perf antibody (top panel). Lysates were immunoprecipitated 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

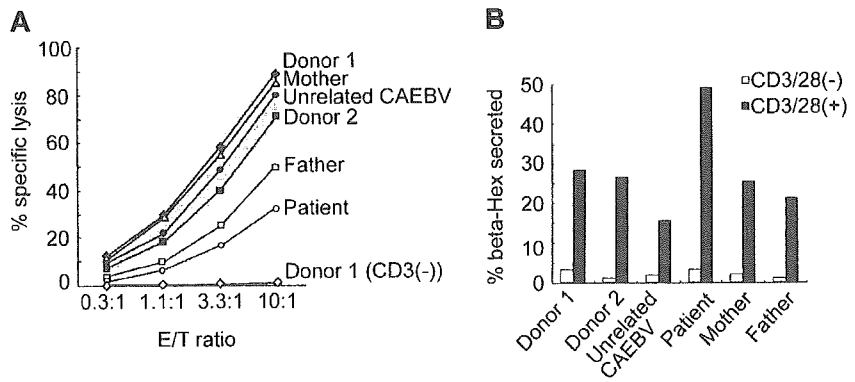

Figure 7. Cytotoxicity and granule exocytosis assays using stimulated PBMCs from the patient, his parents, an unrelated patient with CAEBV, and healthy donors. (A) PBMCs were stimulated with PHA and IL-2, cultured in IL-2 (effector cells), and flow cytometry showed lymphocyte phenotypes described in 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.

Acknowledgments

We thank Susan E. Stepp, Department of Pathology, University of Texas Southwestern Medical School, Dallas, TX, for advice on PCR of the perforin gene; Dong Zhang, Center for Blood Research, Department of Pediatrics, Harvard Medical School, Boston, MA, and Gillian M. Griffiths, Sir William Dunn School of Medicine, Oxford, United Kingdom, for perforin antibody 2d4-perf; Susan F. Leitman, Department of Transfusion Medicine, NIH Blood Bank, NIH, Bethesda, MD, for assistance in obtaining cells from blood bank donors; Pierre Henkart for advice on cytotoxicity assays; Ronald Hornung, SAIC, Frederick, MD, for ELISA assay results; Warren Strober and Yo Hoshino, LCI, NIAID, NIH, for helpful discussions.

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Leukemia & Lymphoma, October 2004 Vol. 45 (10), pp. 2169–2172

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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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(Received 13 May 2004)

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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ISSN 1042-8194 print/ISSN 1029-2403 online
DOI: 10.1080/10428190410001723241

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

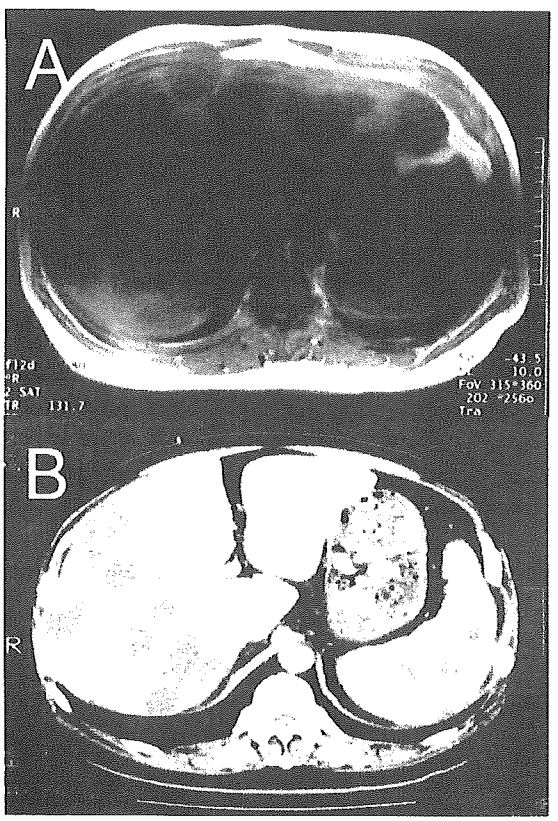

FIGURE 1 Images of MRI and CT scans. (A) MRI demonstrated multiple nodular lesions in liver and spleen on initial examination. (B) CT scan, 10 days after the CHOP therapy showed the reduction of tumor mass size both in the liver and spleen.

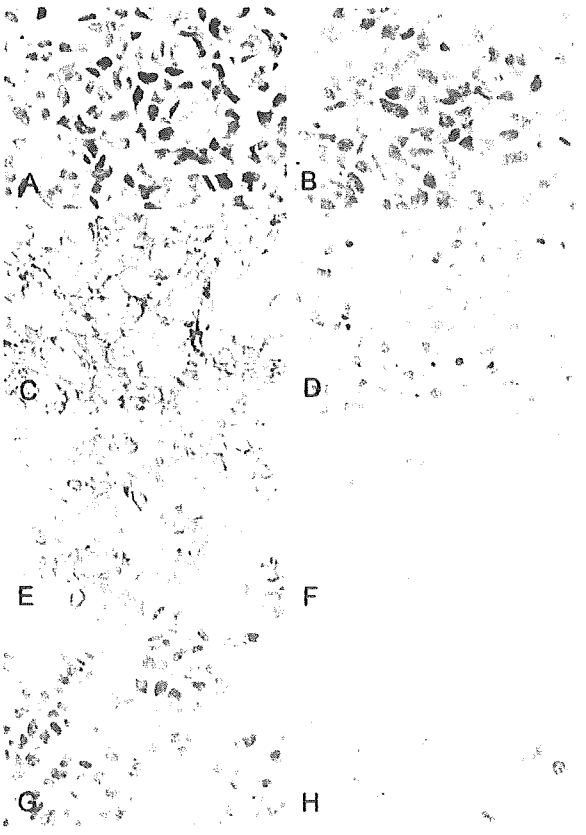

FIGURE 2 Histology. Liver biopsy specimens showed diffuse proliferation of medium- and large-sized lymphocytes with angulated, elongated or cleaved nuclei. H.E. stain (A). Immunohistochemistry of the liver shows that lymphoma cells were positive for BCL-6 (B), CD20 (C), and immunoglobulin light chain 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

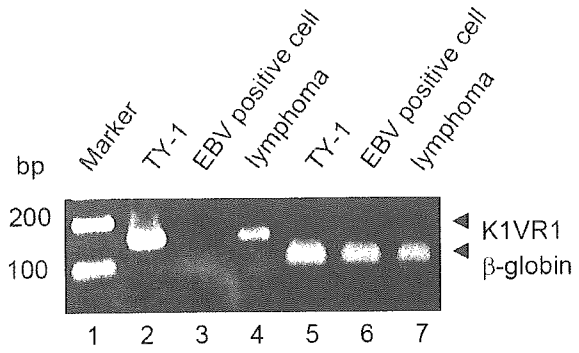

FIGURE 3 Polymerase chain reaction analysis. A 162-bp of the amplified DNA was detected in paraffin-embedded tissue sections of the lymphoma (lane 1; 100 bp DNA ladder marker, lane 2–4; K1VR1 primer sets). TY-1 is HHV-8-positive, and Ramos is HHV-8-negative and EBV-positive lymphoma cell line. These 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.

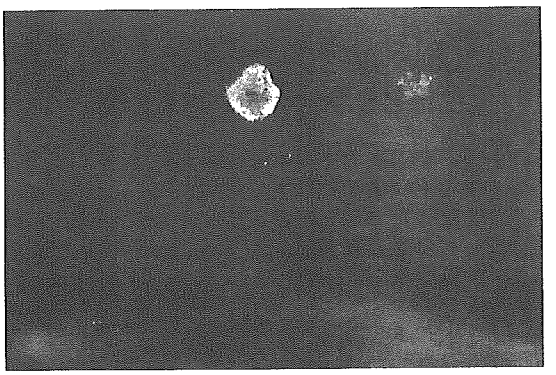

FIGURE 4 Serum anti-HHV-8 antibodies were detected by indirect immunofluorescence assay using acetone-fixed TY-1 cells, showing mainly latency-associated nuclear antigen (LANA) in their nuclei. Some cells showed diffuse cytoplasmic staining, indicating the presence of lytic antigens to HHV-8.

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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bjh review

Perforin and lymphohistiocytic proliferative disorders

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Summary

Perforin is critical for cytotoxicity mediated by granules present in natural killer (NK) cells and cytotoxic T lymphocytes (CTLs). Perforin-deficient mice have impaired cytotoxicity by NK cells and CTLs, resulting in failure to control infections with certain viruses or bacteria. Infection of perforin-deficient mice with lymphocytic choriomeningitis virus results in haemophagocytic lymphohistiocytosis and elevated levels of pro-inflammatory cytokines. Mutations throughout the perforin gene have been identified in patients with familial haemophagocytic lymphohistiocytosis (FHL) type 2. These patients present with fever, hepatosplenomegaly, pancytopenia, have marked elevations of T-helper type 1 and type 2 cytokines, and have impaired NK cell and CTL cytotoxicity. A number of infectious pathogens have been implicated as triggering the onset of disease. Identification of mutations in perforin as the cause of FHL should allow prenatal diagnosis of the disorder. While stem cell transplantation is curative, gene therapy might be effective in the future.

Keywords: perforin, familial haemophagocytic lymphohistiocytosis, haemophagocytosis, histiocytosis, Epstein–Barr virus.

Molecular basis of killing by CTLs and NK cells: the role of perforin

Cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells are critical for host defence against pathogenic microorganisms and malignant cells (Russell & Ley, 2002). When CTLs recognize target cells, the T-cell receptor (TCR) on the cell surface of CTLs engages with major histocompatibility complex (MHC) class I molecules. Signals from the TCR activate both the perforin and Fas pathways in CTLs (Henkart, 2000). Perforin is present in granules of CTLs and NK cells. Cytotoxic granules gather at the site of contact between the CTL and the target cell, known as the immunological synapse. The granule membrane fuses with the cell membrane and releases the contents of the granules in a process referred to as exocytosis. This results in rapid death of the target cell.

Perforin released from CTLs binds to the phospholipid bilayer and forms small (15 nm diameter) pores in the membrane of target cells (Lowin *et al*, 1995). Some investigators have postulated that granzymes, released from cytotoxic granules with perforin, go though the pores, enter the cytoplasm of target cells and induce apoptosis in the target cells (Lieberman, 2003). Granzyme B induces cleavage of caspases, including caspase-3 (Darmon *et al*, 1995). In addition, granzyme B can induce cell death in the presence of caspase inhibitors, suggesting that granzyme B also activates caspase-independent apoptosis (Henkart *et al*, 1997). Granzyme B cleaves BH3-interacting domain death agonist (BID), and the cleaved form of BID induces apoptosis through the mitochondrial pathway (Barry *et al*, 2000). Granzyme A damages DNA by inducing single-stranded nicks with resultant apoptosis of the cells (Shi *et al*, 1992). The activity of granzymes is dependent on the activity of perforin. Transfection of cells that do not have endogenous perforin or granzymes with granzyme A or B cDNA does not induce death of target cells, while transfection of the same cells with perforin cDNA induces death (Shiver *et al*, 1992; Nakajima *et al*, 1995). This suggests that granzymes enter target cells through perforin membrane pores. Other studies, however, suggest that perforin cannot form pores large enough for entry of granzymes (Froelich *et al*, 1996). As a pore size of >50 nm in diameter may be required for entry of granzymes, the usual perforin pore (15 nm in diameter) is too small. A new hypothesis states that perforin is required for the release of granzymes from the endocytic compartment into the cytosol and for tracking to the nucleus (Browne *et al*, 1999).

The perforin gene consists of three exons (Fig 1). The coding sequence is located in exons 2 and 3. Perforin is a 555 amino acid protein that has a 21 amino terminal signal sequence, an approximately 300 amino region that shares homology to the C9 complement protein, a 36 amino acid epidermal growth factor-like domain, and a 132 amino acid domain homologous to the C2 domain of protein kinase C (Lichtenheld *et al*, 1988; Uellner *et al*, 1997). The protein contains two N-linked glycosylation sites. Perforin is synthesized as an inactive precursor, which must be cleaved at its

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  doi:10.1111/j.1365-2141.2004.05305.x

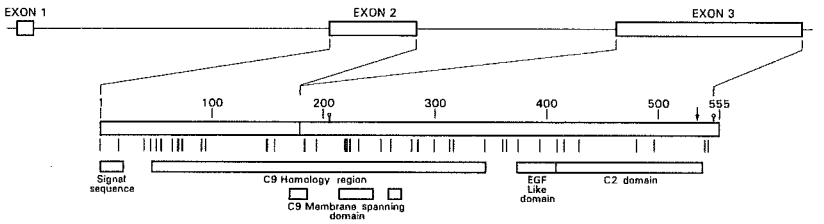

Fig 1. Structure of perforin gene, perforin protein, sites of mutations, and structural domains of the protein. The vertical lines below the perforin protein are the sites of amino acid mutations reported in patients with familial haemophagocytic lymphohistiocytosis. The arrow indicates the carboxy terminal cleavage site and the two lollipops indicate N-linked glycosylation sites.

carboxy terminus, releasing the approximately 20 carboxy terminal amino acids, to yield the active form of the protein (Uellner *et al*, 1997). The C2 domain of perforin is important for its binding to the phospholipid bilayer of cytoplasmic membranes (Fig 2). The C2 domain in the uncleaved form of perforin cannot bind to the membrane, because of 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.

Perforin is harmful to cell membranes; however, the granule membrane encompassing the protein is not destroyed by perforin. The pH inside granules is very acidic (pH <6·0) and this probably keeps perforin in an inactive state. When exocytosis occurs and perforin is released from the granule, the pH becomes neutral and perforin becomes active. In addition, proteoglycans (serglycin and calreticulin) usually cover and inactivate perforin in the granule (Masson *et al*, 1990; Metkar *et al*, 2002). When the granule is released from the cell, perforin is separated from proteoglycans and binds to cell membranes of target cells. Cathepsin B, a lysosomal protease, usually localizes in granules; however, cathepsin B moves to the surface of CTLs during granule exocytosis (Balaji *et al*, 2002). As cathepsin B can cleave perforin, a membrane-bound form of cathepsin B is thought to destroy perforin on the surface of CTLs, preventing the formation of perforin membrane pores and protecting the cell membrane from attack by perforin. Protection of CTLs by cathepsin B is impaired by addition of an inhibitor to the enzyme.

The other mechanism used by CTLs to kill target cells is the Fas pathway. Signalling through the TCR promotes transcription of Fas ligand in CTLs (Henkart, 2000). Fas ligand binds to Fas on the surface of target cells and initiates caspase-mediated killing of the cells. Some proteins function in both the perforin and Fas pathways to mediate cytotoxicity. For example, granzyme B cleaves caspase-3 in both pathways (Darmon *et al*, 1995).

The role of perforin in animal models

Perforin is important for control of pathogens, malignancies and graft-versus-host disease

Perforin-deficient mice were generated by inserting a neomycin cassette into the second or third exons of the perforin gene (Kagi *et al*, 1994a; Kojima *et al*, 1994; Walsh *et al*, 1994). Homozygous mice failed to express perforin protein. Cells from these mice were impaired for lysis of certain virus-infected cells or NK cell targets *in vitro*.

Intravenous or intraperitoneal inoculation of homozygous perforin-deficient mice with lymphocytic choriomeningitis virus (LCMV) resulted in persistent infection of tissues, ultimately resulting in death, while wild-type and heterozygous animals cleared the virus (Kagi *et al*, 1994a; Walsh *et al*, 1994). Perforin-deficient mice are also impaired for control of herpes simplex virus infection (Ghiasi *et al*, 1999) and ectromelia (mousepox) virus (Mullbacher *et al*, 1999). Rossi *et al* (1998)

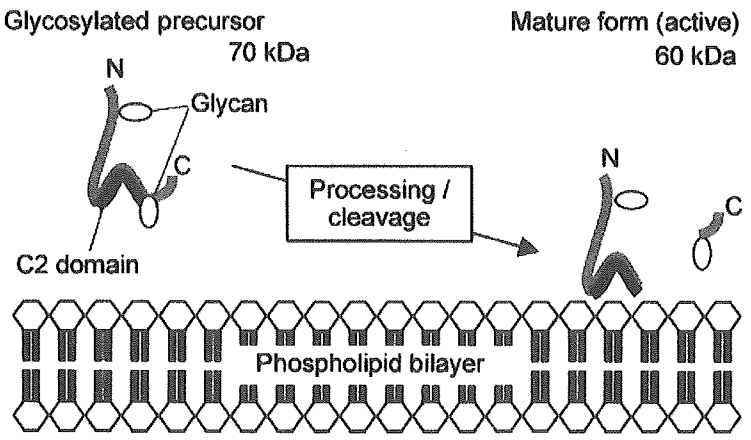

Fig 2. Processing of the perforin protein to its mature form. Perforin is synthesized as a 70 kDa inactive, glycosylated precursor, which is cleaved at its C-terminus to yield a 60 kDa active, mature protein. Only the mature form of the protein can bind to the phospholipid bilayer of cells at the C2 domain of perforin.

showed that perforin-deficient mice infected with Theiler's virus developed encephalomyelitis and inflammatory infiltrates in the spinal cord with high levels of viral RNA in the central nervous system, while control mice remained healthy. Perforin-deficient mice are not impaired for clearance of other viruses, including vaccinia virus, vesicular stomatitis virus, murine cytomegalovirus, Semliki Forest virus (Kagi *et al*, 1995), cowpox virus (Mullbacher *et al*, 1999), rotavirus (Franco *et al*, 1997), coxsackie B virus (Gebhard *et al*, 1998), or murine herpesvirus-68 (Usherwood *et al*, 1997), a mouse homologue of Epstein–Barr virus (EBV).

While perforin-deficient mice are able to ultimately clear infection with the intracellular bacteria *Listeria monocytogenes*, there is delayed clearance from the spleen (Kagi *et al*, 1994b). Perforin-deficient mice showed increased mortality during chronic infection with *Toxoplasma gondii* (Denkers *et al*, 1997) or *Histoplasma capsulatum* (Zhou *et al*, 2001). In contrast, perforin-deficient mice show no delay in clearance of *Chlamydia trachomatis* (Perry *et al*, 1999) or *Chlamydia pneumonia* (Rottenberg *et al*, 1999).

Inoculation of syngeneic fibrosarcoma cells into perforin-deficient mice results in failure to reject the tumour cells, while wild-type animals reject the cells (Kagi *et al*, 1994a). Perforin is important for control of tumour initiation, growth and metastases (Street *et al*, 2001). Perforin-deficient mice are more prone to spontaneous T, B and NK cell tumours (Smyth *et al*, 2000). Growth of implanted tumour cells in mice is controlled by NK cell cytotoxicity, which is dependent on perforin (van den Broek *et al*, 1995).

Perforin also plays an important role for class I-restricted graft-*versus*-host disease (GvHD) in mice. $CD8^+$ T cells from perforin-deficient mice result in less susceptibility to fatal GvHD after infusion into lethally irradiated animals compared with cells from wild-type or granzyme B-deficient animals (Graubert *et al*, 1997; Jiang *et al*, 2001.) In contrast, Fas ligand, but not perforin is important for class II-restricted ($CD4^+$ T cell) GvHD in mice. $CD8^+$ T cells can also prevent rejection of allogeneic bone marrow grafts; perforin-deficient $CD8^+$ T cells are impaired in their ability to prevent rejection in mice (Martin *et al*, 1998).

Absence of perforin results in uncontrolled lymphocyte proliferation and haemophagocytosis

Perforin deficiency increases immunopathology in some, but not all, animal models. Perforin-deficient mice have normal levels of NK and $CD8^+$ T cells and remain healthy in the absence of infection (Kagi *et al*, 1994a). However, infection of these animals with different viruses or bacteria can initiate an uncontrolled immune response resulting in death of the animal, usually because of the inflammatory response rather than directly because of destruction of cells by the pathogen. Infection of perforin-deficient mice with LCMV results in fatal pancytopenia with elevated levels of tumour necrosis factor α (TNF-α) and interferon γ (IFN-γ) and increases in activated virus-specific CD8 T cells (Binder *et al*, 1998; Matloubian *et al*, 1999). These animals develop haemophagocytic lymphohistiocytosis with fever, splenomegaly, haemophagocytosis, hypertriglyceridaemia and hypofibrinogenaemia (Jordan *et al*, 2004). Lymph nodes and spleen from these animals show the depletion of follicles with activated lymphocytes. Periportal infiltrates are present in the liver, the bone marrow shows hypoplasia, and a mononuclear pleocytosis is present in the cerebrospinal fluid. Multiple cytokines, including interleukin (IL)-6, IL-10, IL-18, macrophage colony-stimulating factor (M-CSF), IFN-α, IFN-γ, and TNF-α, are elevated. Antibody to IFN-γ, but not other cytokines, prolongs survival and prevents development of histiocytic infiltrates and cytopenia. Depletion of $CD8^+$ T cells, but not NK or $CD4^+$ T cells, prolongs survival. The $CD8^+$ T cells secrete IFN-γ and depletion of these cells lowers the level of the cytokine. The elevated IFN-γ is thought to be the result of the increased antigen stimulation of $CD8^+$ T cells; neutralizing antibody to LCMV lowers IFN-γ levels and prolongs survival.

Perforin-deficient mice also have an abnormal response after vaccination with an LCMV antigen (Badovinac *et al*, 2003). Vaccinated perforin-deficient mice have higher rates of death after challenge with LCMV compared with control mice. Perforin-deficient mice have marked expansions of antigen-specific $CD8^+$ T cells and elevated levels of IFN-γ and TNF-α. Depletion of $CD8^+$ T cells, or injection with antibodies to IFN-γ and TNF-α, reduced the rate of mortality after challenge with LCMV.

Injection of perforin-deficient mice with *Staphylococcus* enterotoxin B, a superantigen, results in expansion and persistence of $CD8^+$ T cells (Kagi *et al*, 1999). Perforin is critical for control of $CD8^+$ T-cell expansion in response to *L. monocytogenes* (Badovinac *et al*, 2000). Inoculation of perforin-deficient mice with *L. monocytogenes* results in three to fourfold increases in antigen-specific $CD8^+$ T cells compared with control mice. In addition, perforin-deficient mice injected with dendritic cells coated with a *L. monocytogenes* epitope have a threefold increase in antigen-specific $CD8^+$ T cells when compared with control mice. Taken together, these results indicate that perforin is critical for controlling the $CD8^+$ T-cell response to both infectious organisms and their antigens.

Jordan *et al* (2004) proposed a model for the pathophysiology of haemophagocytic lymphohistiocytosis in perforin-deficient mice. T cells stimulated by antigen-presenting cells (APCs) produce a variety of cytokines that can activate macrophages, including APCs. When APCs recognize foreign antigens, the cells promote presentation of the antigens, resulting in stimulation of $CD8^+$ T cells. Stimulated $CD8^+$ T cells produce cytokines, including IFN-γ, and begin to kill infected cells, including macrophages and APCs, by the perforin pathway. However, the pathway is impaired in perforin-deficient mice. The persistence of pathogen-infected cells results in sustained antigenic stimulation, T-cell and macrophage proliferation, and elevation of proinflammatory cytokines (Arnaout, 2000; Moretta *et al*, 2000). The