(Kogawa *et al*, 2002). Some, but not all parents, have impaired NK cell lytic function (Sullivan *et al*, 1998), but they are asymptomatic.

A variety of mutations, including stop codons, single amino acid mutations or deletions, have been reported throughout the perforin coding sequence in patients with FHL (Fig 1 and Table 1) (Stepp *et al*, 1999; Clementi *et al*, 2001, 2002; Goransdotter Ericson *et al*, 2001; Feldmann *et al*, 2002; Kogawa *et al*, 2002; Suga *et al*, 2002; Ueda *et al*, 2003; Busiello *et al*, 2004; Molleran Lee *et al*, 2004). These mutations result in decreased cytotoxic T-cell and NK cell cytotoxicity.

Certain mutations in perforin are seen in patients of different ethnic backgrounds. The most frequent mutations in Japanese patients are nucleotide changes that result in frameshift mutations at amino acid 69 (reported in 38% of Japanese patients) or amino acid 364 (reported in 63%) (Ueda *et al*, 2003). A stop codon at amino acid 374 was found in several Turkish patients (Stepp *et al*, 1999; Clementi *et al*, 2001, 2002; Goransdotter Ericson *et al*, 2001). A nucleotide deletion that results in a frameshift mutation at amino acid 17 was present in all 10 African-American patients studied in one series (Molleran Lee *et al*, 2004).

Mutations in the coding sequence of perforin are responsible for approximately 20–40% of cases of FHL among Japanese, European and Middle Eastern countries (Goransdotter Ericson *et al*, 2001; Suga *et al*, 2002), but mutations are reported in 58% of patients from North America (Molleran Lee *et al*, 2004). At least eight polymorphisms have been identified in the region of the perforin gene encoding the protein, with allelic frequencies ranging from 0·5% to 67% (Molleran Lee *et al*, 2004).

The impaired activity of perforin in patients with FHL may be the result of reduced expression, instability, or incorrect trafficking of the protein, failure of the protein to bind to target cells, or failure of the protein to lyse the cells. Voskoboinik *et al* (2004) showed that two mutations in a patient with haemophagocytic lymphohistiocytosis (Stepp *et al*, 1999; Feldmann *et al*, 2002) were responsible for a specific functional defect in perforin. Transfection of rat leukaemia cells with a vector expressing wild-type perforin results in lysis of target cells; however, transfection with perforin mutated at amino acid 225 (Arg > Trp) results in expression of a truncated protein that fails to traffic to granules and lyse target cells. Transfection of the cells with perforin mutated at amino acid 429 (Gly > Glu) results in expression of a full length protein that traffics to granules and is released, but is impaired for binding to target cells and has reduced cytotoxicity.

The severity of disease is often similar regardless of whether the perforin gene has a missense, nonsense, or frameshift mutation. However, patients presenting with FHL at a relatively later age were more likely to have missense mutations, compared with other mutations in two studies (Feldmann *et al*, 2002; Ueda *et al*, 2003). Patients with perforin mutations who expressed very low levels of the protein presented later in life (median age 54 months) than patients with no detectable protein (median age 3 months) (Molleran Lee *et al*, 2004). No significant difference, however, was noted in age of presentation when FHL patients with or without perforin mutations were compared.

Eight polymorphisms have been described in the perforin gene (Molleran Lee *et al*, 2004). These result in changes at nucleotides 272 (C to T), 368 (G to A), 435 (G to A), 462 (A to G), 519 (G to A), 755 (A to G), 822 (C to T) and 900 (C to T). Three of the polymorphisms result in amino acid changes: (A91V), (R123H) and (N252S). Two of these polymorphisms were reported to be present in patients with FHL (Stepp *et al*, 1999; Clementi *et al*, 2002; Feldmann *et al*, 2002; Busiello *et al*, 2004) (Table I); however, the allelic frequency of these nucleotide changes (3% for C272T and 2% for A755G) is probably too high for the rarity of FHL.

Most studies suggest that other genetic or environmental factors may influence the onset of the disease (Clementi *et al*, 2002). In one series, upper respiratory or gastrointestinal infections were present at the onset of disease, suggesting that these agents are triggering factors (Feldmann *et al*, 2002). These infections included respiratory syncytial virus, rotavirus, *Klebsiella pneumonia*, cytomegalovirus, and *Plasmodium falciparum*. Two patients with a family history of FHL were identified prior to the onset of disease. One infant was found to have perforin mutations, absent perforin expression, and no CTL activity; however, the patient was asymptomatic (Feldmann *et al*, 2002). A 30-month-old child was found to have impaired NK cell activity (Sullivan *et al*, 1998). At 40 months of age he developed pansinusitis and then presented with FHL. These cases suggest that infectious agents may be necessary to trigger the symptoms of FHL in patients with perforin deficiency.

Arico *et al* (2002) proposed a diagnostic algorithm for haemophagocytic lymphohistiocytosis that consists of measuring perforin expression by peripheral lymphocytes, 2B4 lymphocyte receptor activity, and NK cell activity. Seven patients with perforin mutations all had absent perforin expression. One patient with a mutation in SAP (responsible for X-linked lymphoproliferative disease) had impaired activity of the 2B4 receptor (which is located on NK cells and interacts with SAP). Eleven patients with haemophagocytic lymphohistiocytosis because of a genetic cause other than perforin mutations or sporadic disease (associated with infection) had reduced or normal NK cell activity, normal perforin expression, and normal 2B4 receptor function.

Identification of perforin mutations as a cause of FHL should allow prenatal diagnosis of the disorder. Unfortunately, a large number of mutations at different sites can cause the diagnosis, so that sequencing of the entire open reading frame is necessary. Gene therapy might be used in the future to treat the disease.

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Lack of Human Herpesvirus 8 Infection in Lungs of Japanese Patients with Primary Pulmonary Hypertension

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Samples of lung tissue, taken at autopsy, from 10 Japanese patients with primary pulmonary hypertension (PPH) and samples of lung tissue from 12 Japanese patients with secondary pulmonary hypertension were tested for the presence of human herpesvirus 8 (HHV-8). All samples from patients with PPH contained plexiform lesions around pulmonary arterial vessels, but immunohistochemistry failed to detect the HHV-8–encoded latency-associated nuclear antigen. HHV-8 DNA could not be amplified by polymerase chain reaction for the HHV-8–encoded K1 and $KS330_{23}$ genes in any sample. These data suggest that HHV-8 infection is not associated with PPH in Japanese patients.

Primary pulmonary hypertension (PPH) is a rare disease that leads to severe right heart failure, which is characterized histologically by vascular lesions in the lung and the proliferation of endothelial cells and smooth muscle cells in the pulmonary arterial walls; these conditions then induce luminal obstruction, resulting in elevation of pressure in the pulmonary arteries. Some cases of PPH are associated with genetic mutations in bone morphogenetic protein receptor 2 (BMPR2) [1]. Recently, human herpesvirus 8 (HHV-8)—also known as Kaposi sarcoma (KS)–associated herpesvirus—was identified, by polymerase chain reaction (PCR), in 10 of 16 samples of lung tissue from patients with PPH, and the expression of latency-associated nuclear antigen (LANA), encoded by HHV-8, was detected, by immunohistochemistry, in the vascular "plexiform" lesions in these patients' lungs, suggesting an association between HHV-8 and the pathogenesis of PPH [2]. Because only 2 of these 10 HHV-8–positive patients had BMPR2 mutations, HHV-8 infection did not correlate with BMPR2 mutations in these patients [2].

HHV-8 is categorized as a gamma herpesvirus [3], and the seroprevalence of HHV-8 varies geographically. HHV-8 has a high seroprevalence in the general population in African countries (40%) and in southern European countries (10%), but a low prevalence has been suggested in the United States (3%) and in Asian countries, including Japan (1.4%) [4]. HHV-8 has been detected in KS, primary effusion lymphoma (PEL), and some cases of multicentric Castleman disease (MCD) [3]. HHV-8–encoded LANA is always expressed in the cells of KS and PEL, suggesting an HHV-8 infection in the latent phase. In contrast, not only LANA but also other lytic antigens of HHV-8 are expressed in the cells of MCD, implying that it has a different pathogenesis than do KS and PEL [5]. LANA, however, plays an important role in the pathogenesis of KS and PEL [3]. The histological features of the plexiform lesions of PPH—proliferation of spindle-shaped cells with vascular slits—resemble the histological features of KS [2]. Although mutations of BMPR2 have been detected in some isolated cases of PPH and in some cases of familial PPH in Japan [6], the pathogenesis of most cases of PPH is still unknown. In the present study, we investigated the presence of HHV-8 in the lung tissue from 10 Japanese patients with PPH and from 12 Japanese patients with secondary pulmonary hypertension (SPH).

Subjects, materials, and methods. During 1981–2003, 10 Japanese patients with PPH underwent autopsy at Toho University Hospital in Tokyo, Japan, and samples of their lung tissue were taken for analysis; samples of lung tissue were also taken from 12 Japanese patients, living in the Tokyo area, who had SPH and were not infected with HIV (table 1). The mean age of the patients with PPH was 23.4 years (range, 0–51 years), and the mean age of the patients with SPH was 31.4 years (range, 0–83 years). Immunohistochemistry was performed to investigate the expression of LANA on cells of lung tissue, as described elsewhere [5]. A rabbit polyclonal antibody to LANA (dilution, 1:3000 [5]) and a rat monoclonal antibody to LANA (dilution, 1:3000; Advanced Biotechnologies) were used as primary antibodies. Samples of KS tissue obtained from additional patients were used as positive controls. For PCR analysis, DNA was extracted from samples of lung tissue that were fixed in formalin and embedded in paraffin. DNA from a sample of KS tissue obtained from an additional patient was used as a positive control, and DNA from a sample of healthy skin obtained from an additional patient was used as a negative control [5]. PCR

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Table 1. Characteristics of the study population and results of polymerase chain reaction (PCR) and immunohistochemistry (IHC).

Patient	Age, years	Sex	No. of paraffin blocks tested	Diagnosis	K1, by nested PCR	$KS330_{233}$, by PCR	β-globin, by PCR	No. of plexiform lesions	LANA, by IHC
1	29	M	3	PPH	–	–	+	124	–
2	41	M	3	PPH	–	–	+	50	–
3	0	F	3	PPH	–	–	+	103	–
4	39	F	3	PPH	–	–	+	81	–
5	0	F	1	PPH	–	–	+	18	–
6	21	F	5	PPH	–	–	+	119	–
7	16	F	3	PPH	–	–	+	24	–
8	24	M	3	PPH	–	–	+	110	–
9	13	F	2	PPH	–	–	+	41	–
10	51	F	2	PPH	–	–	+	42	–
11	61	M	1	SPH (ASD)	–	–	+	10	–
12	51	M	1	SPH (gastric cancer)	–	–	+	30	–
13	0	F	1	SPH (ECCD)	–	–	+	6	–
14	1	M	1	SPH (TGA)	–	–	+	20	–
15	1	F	2	SPH (DS, ASD, VSD)	–	–	+	39	–
16	8	M	1	SPH (ECCD)	–	–	+	0	–
17	0	F	1	SPH (DS, ASD, VSD)	–	–	+	0	–
18	83	F	1	SPH (RA)	–	–	+	21	–
19	47	F	2	SPH (ASD)	–	–	+	102	–
20	59	M	1	SPH (MI)	–	–	+	15	–
21	18	F	1	SPH (ASD, VSD)	–	–	+	6	–
22	48	M	1	SPH (ALS)	–	–	+	48	–

NOTE. For patients with secondary pulmonary hypertension (SPH), the primary condition (or related conditions) is listed in parentheses. ALS, amyotrophic lateral sclerosis; ASD, atrial septal defect; DS, Down syndrome; ECCD, endocardial cushion defect; LANA, latency-associated nuclear antigen; MI, myocardial infarction; PPH, primary pulmonary hypertension; RA, rheumatoid arthritis; TGA, transposition of great arteries; VSD, ventricular septal defect; –, not detected; +, detected.

was performed, as described elsewhere [7], to detect the $KS330_{233}$ gene of HHV-8 (HHV-8–encoded ORF26). Nested PCR was performed to detect the K1 gene of HHV-8. For the first round of nested PCR, the external primer pair K1SF (forward primer, 5′-TTGTGCCCTGGAGTGATT-3′) and K1SR (reverse primer, 5′-CAGCGTAAAATTATAGTA-3′) was used to amplify a 363-bp fragment of the K1 gene of HHV-8 [8]. The conditions for the first round of PCR were 1 cycle at 94°C for 4 min, followed by 35 cycles at 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min. For the second round of PCR, the inner primer pair K1VR1F1 (forward primer, 5′-TTGCCAATATCCTGGTATTGC-3′) and K1VR1R1 (reverse primer, 5′-CAAGGTTTGTAAGACAGGTTG-3′) was used to amplify a 162-bp fragment of the K1 gene; the same conditions as in the first round of PCR were used. The β-globin gene was amplified as a control, as described elsewhere [7].

Results. To investigate whether HHV-8 was present in the samples of lung tissue from patients with PPH, we first performed immunohistochemistry to detect LANA. Staining with hematoxylin-eosin revealed that all samples from patients with PPH had characteristic plexiform lesions in their pulmonary arteries (figure 1). In samples from patients with PPH, 18–124 plexiform lesions were tested (table 1). Some samples from patients with SPH also had plexiform lesions. Immunohistochemistry by use of 2 antibodies to LANA revealed that LANA was not present in any sample obtained from patients with either PPH or SPH (table 1), whereas LANA was detected as a dot-like nuclear staining pattern in samples of KS tissue obtained from control patients (figure 1). Although sclerosing lesions and proliferation of endothelial cells and smooth muscle cells around vessels were observed in the plexiform lesions, LANA was not present. To confirm the results of the immunohistochemistry, we extracted DNA from the samples of lung tissue and performed PCR. Both PCR amplification for the $KS330_{233}$ gene of HHV-8 and nested PCR amplification for the K1 gene of HHV-8 failed to detect HHV-8 DNA in all samples (table 1). The control gene β-globin was detected in all samples. These data and the results of the immunohistochemistry suggest that the patients with PPH did not have HHV-8 infection.

Discussion. In the present study, we have demonstrated that 10 Japanese patients with PPH and 12 Japanese patients with SPH did not have HHV-8 infection. Although we used testing procedures similar to those employed by Cool et al. [2, 9]—immunohistochemistry and PCR—our results were completely different from theirs.

Patients with PPH are found worldwide. Only 50% of patients

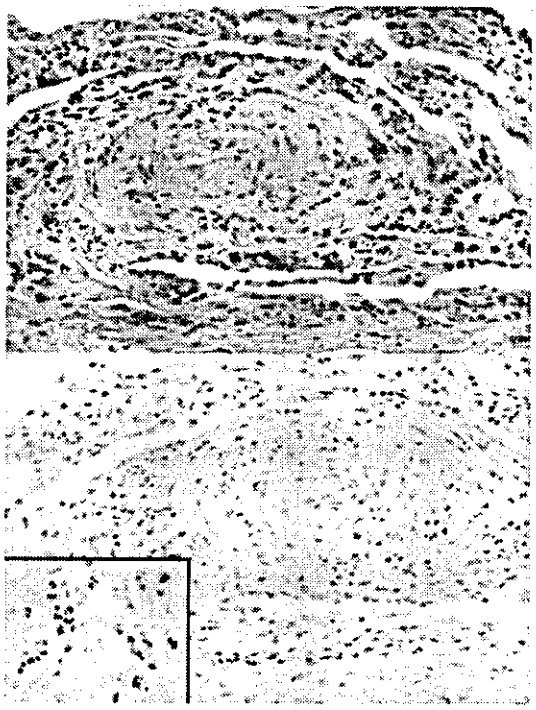

Figure 1. Plexiform lesions in lung tissue from a patient with primary pulmonary hypertension. *Top,* Lung tissue stained with hematoxylin-eosin. *Bottom,* Detection of latency-associated nuclear antigen (LANA) by immunohistochemistry. *Inset,* Expression of LANA (dot-like nuclear staining pattern) in Kaposi sarcoma from a positive control patient.

with familial PPH have BMPR2 mutations, and no BMPR2 mutations have been detected in patients with isolated cases of PPH. Because HHV-8 was not detected in 6 of the 16 patients with PPH whom Cool et al. studied, the authors suggested that BMPR2 mutations and HHV-8 infection were not correlated [2]. The present study has demonstrated that all 10 Japanese patients with PPH were negative for HHV-8 infection. Although we were unable to examine the seropositivity of the patients with PPH, in a study published elsewhere, we demonstrated that the seroprevalence of HHV-8 was low (1.4%) in the general population in Japan [4]. These data suggest that PPH might be induced by causative factors other than HHV-8 infection and BMPR2 mutations. Therefore, it is possible that the pathogenesis of PPH in Japan is different from that of PPH in the United States. Other genetic backgrounds, modifier genes, or other pathogens may be associated with cases of PPH in Japan.

The sensitivity and methods used in the present study, however, were different from those used by Cool et al. [2]. Our immunohistochemistry succeeded in detecting LANA in all cases of KS, regardless of the stage of disease or the patient's HIV infection status, and the results of immunohistochemistry correlated well with those of PCR [5]. Cool et al. detected LANA not only in the cells within plexiform lesions but also in bronchoepithelial cells and in inflammatory cells, including lymphocytes and macrophages [2, 9], but we were not able to detect LANA in any cells of the samples obtained from patients with PPH. LANA has been detected only in the nuclei of KS cells and not in surrounding cells, including epithelial cells, lymphocytes, and macrophages, even in samples of lung tissue from patients with KS [5]. To date, HHV-8 has been detected, by PCR, in patients with various diseases, but immunohistochemistry has yielded positive results only in samples from patients with KS, PEL, MCD, and some solid lymphomas [10, 11]. Recently, a low seroprevalence of antibodies to HHV-8 in patients with PPH in Germany was reported, suggesting that HHV-8 infection is rarely involved in the pathogenesis of PPH [12]. Further studies are required to clarify the strict association between HHV-8 infection and PPH.

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Viral Load of Human Herpesvirus 8 (HHV-8) in the Circulatory Blood Cells Correlates with Clinical Progression in a Patient with HHV-8-associated Solid Lymphoma with AIDS-associated Kaposi's Sarcoma

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We encountered a case of a rapidly progressive HHV-8-associated solid lymphoma with AIDS-associated Kaposi's sarcoma (KS). HHV-8 DNA load in whole blood cells was analyzed quantitatively by real-time PCR using amplification of the HHV-8-encoded ORF26 gene. Ours is the first observation that the rapid increase in the HHV-8 viral load (from 1.9×10^4 copies/μg to 1.6×10^6 copies/μg in 40 days) in conjunction with low CD4 + cell counts was accompanied by an accelerated clinical disease progression. The results indicate that the quantity of circulating HHV-8 is measurable with real-time PCR and can provide clinically useful information.

Keywords: HHV-8-associated solid lymphoma; HHV-8; Viral load; ORF26; Real-time PCR

INTRODUCTION

Human herpesvirus-8 (HHV-8) is etiologically linked to Kaposi's sarcoma (KS), the most common malignancy in patients with AIDS [1,2]. HHV-8 DNA is consistently found in KS tissues, and is detected in peripheral blood mononuclear cells (PBMC) of human immunodeficiency virus (HIV)-infected individuals [3,4]. Detection of HHV-8 DNA in PBMC from HIV type 1-infected persons is associated with an increased risk of subsequent development of KS [4,5] and with the clinical stage of KS [5,6]. AIDS-associated primary effusion lymphoma (PEL, body-cavity based lymphoma), a distinct subtype of non-Hodgkin's lymphoma (NHL), is another HHV-8-associated neoplasm, which is typically present as a malignant effusion without solid tumor masses in the body cavity of AIDS patients [7]. Recently, solid organ involvement of HHV-8-associated lymphomas has also been reported in some AIDS patients [8–11]. HHV-8-associated solid lymphomas were characterized by expressing CD30, exhibiting anaplastic large cell morphology and carrying clonal immunoglubulin gene rearrangement that indicates B-cell origin despite the usual presence of a non-specific immunophenotype. HHV-8 DNA and latency-associated nuclear antigen (LANA) have been detected in lymphoma cells from HHV-8-associated solid lymphoma patients. Epstein-barr virus (EBV) co-infection was also found in most of these patients. There have been no reports, however, of an elevated HHV-8 DNA load in serum nor in whole blood cells with HHV-8-associated solid lymphoma in AIDS patients. Yamamoto [12] reported a case of rapidly progressive HHV-8-associated solid lymphoma with anaplastic large cell morphology followed by systemic KS, which is complemented by our kinetic study of the HHV-8 DNA load and CD4 + cell counts in blood cells in the same patient. The quantitative real-time PCR (Taqman PCR) technique provides an accurate and reproducible measurement of the level of HHV-8 in the circulatory blood cells. We used this technique to quantify the HHV-8 DNA load in whole blood cells. Ours is the first report of the correlation between a rapid

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increase of HHV-8 viral load in conjunction with low CD4 + cell counts and disease progression.

CASE REPORT

Patient History

The clinical history, as well as the physical and laboratory findings including treatment modalities of this patient were described previously [12]. The patient was a 30-year-old Caucasian male with homosexual behavior and intravenous drug use history for 10 years. He had a 6-year HIV-1 infection history but did not contract any opportunistic infections until red spots were found on the skin of the arms, chest and abdomen and later on diagnosed as KS. Upon diagnosis with KS, the CD4 + cell count was 75/μl and HIV-1 plasma viral load was 431,992 copies/ml. He was transferred to the AIDS Clinical Center of the International Medical Center of Japan in Tokyo due to loss of consciousness, where Highly Active Antiretroviral Therapy (HAART) was stopped because he was unconscious. KS lesions were observed on his whole body. Liposomal doxorubicin (20 ng/m^2) was administered twice intravenously to treat KS. Whole blood cells were obtained before and after chemotherapy and the purified DNA was used for HHV-8 DNA quantification by means of real-time PCR. Biopsy results from the enlarged axillary and cervical lymph nodes showed large cell lymphoma morphology. HHV-8 and Epstein-Barr virus (EBV) producing proteins were detected in the lymphoma cells. Enzyme immunoassay (EIA) and immunofluorescence assay (IFA) were negative for the serum anti-HHV-8 antibody. The patient died 6 days after the induction of a CHOP regimen [12]. In autopsy, lymphoma cells were found not only in the cervical, mediastinal and inguinal lymph nodes, but also in the spleen, tonsils, gastrointestinal mucosa, lungs, adrenal glands and bone marrow. The lymphoma cells displayed anaplastic large blastic cell morphology and had an undeterminant phenotype. In immunohistochemistry, CD30-positive, CD43-positive, CD45RO-positive, CD45-positive, CD4-negative, CD5-negative, CD8-negative, CD15-negative and CD20-negative were detected in the lymphoma cells. Also, HHV-8-encoded LANA was expressed in most lymphoma cells. EBV-encoded small RNA-1 (EBER-1) was expressed in some lymphoma cells by *in situ* hybridization. KS was found in skin and peri-lymph node soft tissue in the inguinal region.

Establishment of Quantitative Real-time PCR Assay for HHV-8-Encoded ORF26 Gene

The amount of HHV-8 DNA purified from whole blood cells was determined by real-time PCR using amplification of the conserved region of the open reading frame (ORF) 26 gene. The primers and probe of ORF26 were conducted by the method described by White [13]. The amount of human genomic DNA present in the same sample was also determined by real-time PCR using amplification of the human GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene. The quantification of human GAPDH was used to normalize the target DNA. The primers and probes used to quantify ORF26 and GAPDH are shown in Table I. Quantitative real-time PCR was performed in duplicate with the aid of the Taqman Universal PCR Master Mix kit and the PE Biosystem 5700 sequence detector (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's protocol. Briefly, the reaction volume of 50 μl contained 25 μl of 2 × master buffer, 15 pmoles of 1 of primers, 10 pmoles of the dual-labeled probe, and 0.1 μg DNA of 1 of samples to be tested. All assays showed a linear relationship between the value of threshold cycle (Ct) for standards and the logarithm of the amount of ORF26 DNA added to the reaction. The PCR products were further examined by acrylamide gel electrophoresis for confirmation of specific HHV-8 DNA amplification.

Relationship Analysis of HHV-8 DNA Load, CD4 + cell Counts and Clinical Course

Real-time quantitative PCR of the HHV-8 ORF26 gene showed that the HHV-8 DNA load in whole blood cell was constitutively high (1.9×10^4 copies/μg) at the beginning of anti-KS therapy (Liposomal doxorubicin, 20 mg/m^2) (Fig. 1), while the CD4 + cell count was very low (32/μl) even though the patient had been treated with HAART. Systemic KS treatment with liposomal doxorubicin (20 ng/m^2) was administered twice intravenously

TABLE I Oligonucleotide primers used for real-time PCR assay

Name	Position[a]	Polarity	Sequence[b]
Taq26F	379 – 399	Sense	5'-CTCGAATCCAACGGATTTGAC-3'
Taq26R	434 – 452	Antisense	5'-TGCTGCAGAATAGCGTGCC-3'
Probe26	410 – 429	Sense	5'-F-CCATGGTCGTGCCGCAGCA-T-3'
GapdhF	6 – 24	Sense	5'-GAAGGTGAAGGTCGGAGTC-3'
GapdhR	212 – 231	Antisense	5'-GAAGATGGTGATGGGATTTC-3'
GapdhP	183 – 202	Antisense	5'-J-CAAGCTTCCCGTTCTCAGCC-T-3'

[a]Numbers indicate nucleotide position of the ORF26 and GAPDH sequence.
[b]F, FAM reporter dye; T, TAMRA quencher dye; J, Joe reporter dye.

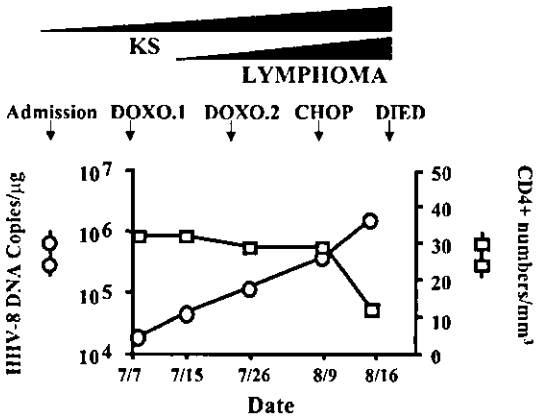

FIGURE 1 The relationship between HHV-8 viral load in whole blood cells and CD4 + cell counts on the one hand, and clinical course on the other in an HHV-8-associated solid lymphoma patient with AIDS-KS. DOXO.1: first administration of Liposomal Doxorubicin. DOXO.2: second administration of Liposomal Doxorubicin.

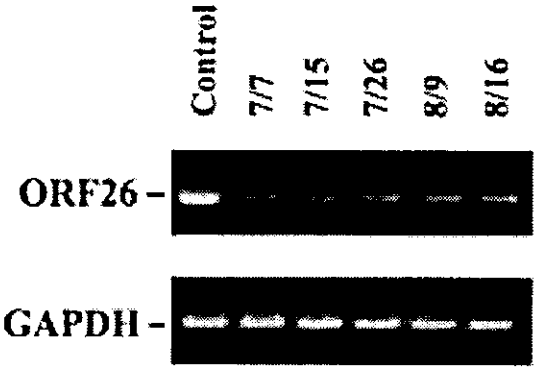

FIGURE 2 Specific amplification of real-time PCR products corresponding to HHV-8-encoded ORF26 gene and GAPDH internal control was confirmed by gel electrophoresis. The control was BCBL-1 (a B cell line derived from body-cavity-based lymphoma, which is latently infected with HHV-8) derived DNA. 7/7, 7/15, 7/26, 8/9 and 8/16 represent dates when whole blood cells were obtained from the patient.

with a 12-day interval. During treatment, right axillary and left cervical lymph nodes grew to 2 and 3–5 cm in diameter, respectively. The HHV-8 viral load was increased to 4.7×10^4 copies/μg shortly after the first course of treatment. Examination of biopsy material from these lymph nodes showed large cell lymphoma morphology, and the lymphoma spread rapidly to the eyelids, neck and arms, which appeared as growing lymphadenopathy. The HHV-8 viral load increased to 1.3×10^5 copies/μg during the first week after the second treatment course. Since the rapidly progressive lymphoma did not respond to liposomal doxorubicin, a CHOP regimen consisting of prednisolone (120 mg), doxorubicin (30 mg; reduced by 30% due to the co-existing thrombocytopenia), vincristine (2 mg, reduced by 7.4%) and cyclophosphamide (750 mg, reduced by 50%) was administered. However, the level of HHV-8 continued to increase to 3.9×10^5 copies/μg in a blood sample obtained a few days after CHOP administration, while the CD4 + cell counts remained low (29/μl). The patient died 6 days after the induction of the CHOP regimen. In the final sample obtained 2 days before the patient died, the HHV-8 viral load was 1.6×10^6 copies/μg, and the CD4 + cell counts were reduced to 12/μl. In addition, neither effusion lymphoma nor lymphocytic leukemia was detected in this patient during the whole clinical course. Overall, the HHV-8 DNA load in the whole blood cells increased by a factor of about 100 despite the therapy for KS, while the CD4 + cell counts stayed low during the progression of the lymphoma (Fig. 1). All of the real-time PCR products were confirmed as specific and the amplified product was verified as correct by acrylamide gel electrophoresis (Fig. 2).

Immunohistochemical Studies

Immunohistochemistry was performed as described previously [14]. After endogenous peroxidase was blocked with methanol-0.6% H_2O_2 for 30 min at room temperature, the anti-ORF50 or anti-ORF59 polyclonal antibodies were allowed to react at 4°C. Immunohistochemical staining showed that lymphoma cells expressing HHV-8 encoded ORF50 and ORF59 (Fig. 3a,b) antigens in the cells' nuclei. This finding suggests that HHV-8 replication did occur in the tumor mass of this patient.

DISCUSSION

Our observation of a nearly 100-fold increase of the HHV-8 DNA load in whole blood cells within 40 days and the detection of HHV-8 lytic protein in the lymphoma cells indicate that HHV-8 replicates in HHV-8-associated solid lymphoma with AIDS-associated KS. HHV-8 is etiologically associated with KS, primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD). The HHV-8-associated solid lymphoma has recently been proposed as a new type of lymphoma. It is a solid lymphoma and is often complicated with other HHV-8-associated diseases such as KS [8]. HHV-8 usually establishes latent infection in the natural host cells. Activation of HHV-8 replication in the latently infected cells, reflecting an increase in HHV-8 DNA load, is responsible for viral spread and presumed to contribute to the development of HHV-8-associated diseases [15]. It has been reported that detection of HHV-8 DNA in PBMC from HIV type 1-infected persons is associated with an increased risk of subsequent development of KS [4,5] and with KS disease progression [5,6]. Some studies have suggested quantification of the HHV-8 viral load might be useful for monitoring the therapeutic response of patients with HHV-8-associated diseases [16]. Poor prognosis is common among cases of HHV-8-associated lymphoma. Liposomal doxorubicin and the CHOP regimen are first-line agents for the treatment of KS

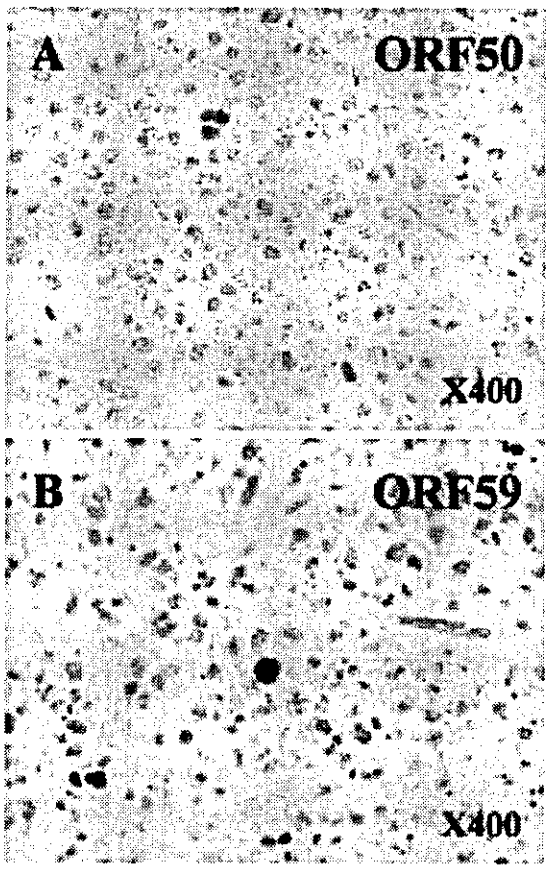

FIGURE 3 Immunohistochemical staining showed that the lymphoma cells of this patient expressed HHV-8 encoded ORF50 (A) and ORF59 (B) antigens in the cells' nuclei. Specimens were obtained at autopsy.

and lymphoma, but were not able to suppress the progression of the disease in our patient, as evidenced by the nearly 100-fold increase in the HHV-8 viral load during the 40 days of chemotherapy, and by the rapid lymphoma progression. The clinical deterioration seen in our patient accompanied by the increase in the HHV-8 viral load suggests that HHV-8 replication had occurred in this patient, and that HHV-8 had been involved in lymphoma progression. Our finding that detection of high load HHV-8 DNA is associated with lower CD4 + cell counts is consistent with data reported by others [4], providing further evidence that advanced immunosuppression is responsible for HHV-8 replication and development of lymphoma as well as KS. Co-infection with HIV may also affect HHV-8 replication and HHV-8-associated disorders through cytokine production and HIV-1 Tat protein secretion from HIV-infected cells [17–19]. Interferon γ (IFNγ) and oncostatin M (OSM) reportedly induce HHV-8 replication [20–21]. We have found that IL-6 activates HHV-8 replication in PEL-derived BCBL-1 cells [15]. In addition, EBV coinfection reported in most cases of HHV-8-associated solid lymphoma suggests HHV-8 may act in conjunction with EBV in the progression of lymphoma. However, our previous publication of this case suggested that HHV-8, rather than EBV, is the main viral agent associated with the pathogenesis of lymphoma in the patient [12]. EBV co-infection found in our patient probably contributed to the progression of HIV infection and the loss of functional immune responses.

In summary, our results suggest that the HHV-8 viral load in whole blood cells measured by real-time quantitative PCR may be useful for monitoring the response to therapies used to treat HHV-8-associated diseases. Further investigation is needed to identify the precise role of HHV-8 as well as inflammatory cytokines in HHV-8-associated solid lymphoma.

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Simvastatin induces apoptosis of Epstein–Barr virus (EBV)-transformed lymphoblastoid cell lines and delays development of EBV lymphomas

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Simvastatin and pravastatin are inhibitors of 3-hydroxy-3-methylglutaryl CoA reductase, and are used as antihypercholesterolemia drugs. Simvastatin, but not pravastatin, binds to the inserted domain of leukocyte function antigen (LFA)-1 and inhibits the function of LFA-1, including adhesion and costimulation of lymphocytes. Epstein–Barr virus (EBV)-transformed lymphoblastoid cell lines (LCLs) express high levels of LFA-1 on their surface and grow in tight clumps. Here we show that simvastatin (2 μM) inhibits clump formation and induces apoptosis of EBV-transformed LCLs. The apoptosis-inducing effect of simvastatin depends on binding to the inserted domain of LFA-1. Simvastatin, but not pravastatin, dissociates EBV latent membrane protein 1 from lipid rafts of LCLs, resulting in down-regulation of nuclear factor κB activity and induction of apoptosis. Analysis of multiple EBV-positive and -negative cell lines indicated that both LFA-1 and EBV latent membrane protein 1 expression were required for simvastatin's effects. Administration of simvastatin to severe combined immunodeficiency mice followed by inoculation with LCLs resulted in delayed development of EBV lymphomas and prolonged survival of animals. To our knowledge, this is the first report in which a drug that targets LFA-1 has been used to treat B cell lymphoma. These data suggest that simvastatin may have promise for treatment or prevention of EBV-associated lymphomas that occur in immunocompromised persons.

Infection of primary B cells with Epstein–Barr virus (EBV) results in transformation with growth of the cells in tight clumps and immortalization of the cells. These immortalized B cells have an immunoblastic morphology and express each of the EBV nuclear antigens (EBNAs) and latent membrane proteins (LMPs) (1, 2). EBNA-2 is a transactivator that up-regulates expression of cellular genes and LMPs. LMP-1 is an oncoprotein that constitutively activates nuclear factor κB (NF-κB) to induce B cell proliferation (3). LMP-1 also induces expression of adhesion molecules leukocyte function antigen (LFA)-1, LFA-3, and intercellular adhesion molecule 1 (ICAM-1) on the surface of EBV-transformed B cells (4, 5). The high level expression of adhesion molecules contributes to clumping of EBV-infected B cells *in vitro* (6).

EBV-associated immunoblastic lymphomas occur in immunocompromised patients such as those with AIDS or transplant recipients (7, 8). Because these EBV-associated immunoblastic lymphomas express each of the EBNAs and LMPs (8) that induce proliferation of B cells, the virus is thought to be directly responsible for the pathogenesis of these tumors (3). LMP-1 in EBV-associated lymphoma cells binds to tumor necrosis factor receptor-associated factors, and the tumors show activation of NF-κB (9). Many immunocompromised patients with EBV-associated immunoblastic lymphoma have tumors at extranodal sites such as the brain, lung, or gastrointestinal tract. The high-level expression of LFA-1 and other cellular adhesion molecules in these tumors may contribute to their extranodal location (10). The prognosis of these lymphomas is often poor for patients with irreversible immunosuppression, and treatment options are limited.

Simvastatin is a member of the statin family of drugs that inhibit 3-hydroxy-3-methylglutaryl CoA reductase (11). Statins lower plasma cholesterol levels, resulting in reduction of the risk of cardiovascular disease (12). Weitz-Schmidt *et al.* (13) demonstrated that certain statins, including simvastatin and lovastatin, bind to the I (inserted) domain of LFA-1 and inhibit its function (13). In contrast, other statins such as pravastatin do not bind to LFA-1. LFA-1 is expressed on the surface of various leukocytes and plays an important role in cell adhesion and costimulation of T cells. The I domain of LFA-1 is the binding site for ICAM-1, a ligand of LFA-1 (14, 15). The binding of simvastatin or lovastatin to the LFA-1 I domain induces a conformational change in LFA-1, resulting in inhibition of the interaction of LFA-1 with ICAM-1 (13). As a result of their binding to LFA-1, these statins inhibit the costimulatory activity of LFA-1 and suppress the inflammatory response in a murine model of peritonitis (13).

Here, we investigate the ability of simvastatin to inhibit EBV-positive B cell proliferation. Because simvastatin binds to and inhibits the function of LFA-1, we postulated that the drug would inhibit the growth of these cells both *in vitro* and *in vivo*. Inoculation of EBV-transformed lymphoblastoid cell lines (LCLs) into severe combined immunodeficiency (SCID) mice results in the formation of EBV-associated immunoblastic lymphomas that contain EBV genomes and express EBNAs, LMPs, and adhesion molecules including LFA-1 (16, 17). In the present study, we treated EBV-transformed LCLs *in vitro* with simvastatin, and administered the drug and inoculated SCID mice with LCLs to assess development of B cell lymphomas.

Materials and Methods

Cell Culture and Viability Assay. Three EBV-transformed LCLs, 12A1, 6B10, and 295H, EBV-positive Burkitt lymphoma cell lines [P3HR-1 (18), Akata (19), Mutu-1 (20), and Mutu-3 (20)], a human herpesvirus-8-positive EBV-negative primary effusion lymphoma cell line (BCBL-1) (21), an EBV-negative Burkitt lymphoma cell line (BJAB) (22), and EBV-negative T cell lines [Jurkat (23) and II-23 (24) cells; obtained from Carl Ware, La Jolla Institute for Allergy and Immunology, San Diego] were tested. For cell proliferation and viability assays, 2×10^4 cells per ml were cultured in 12- or 24-well plates for 5–7 days. Cell viability was assessed with XTT (Cell Proliferation Kit II, Roche Molecular Biochemicals), trypan blue, or propidium iodide (PI) staining. For PI staining, cells were washed with PBS, PI (5 μg/ml) was added, cells were washed, and fluorescent intensity was assessed with flow cytometry. Percent cell death was determined by the ratio of PI-positive cells to all gated cells.

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Abbreviations: EBV, Epstein–Barr virus; ICAM-1, intercellular adhesion molecule 1; I domain, inserted domain; LCL, lymphoblastoid cell line; LFA, leukocyte function antigen; LMP, latent membrane protein; PI, propidium iodide; SCID, severe combined immunodeficiency.

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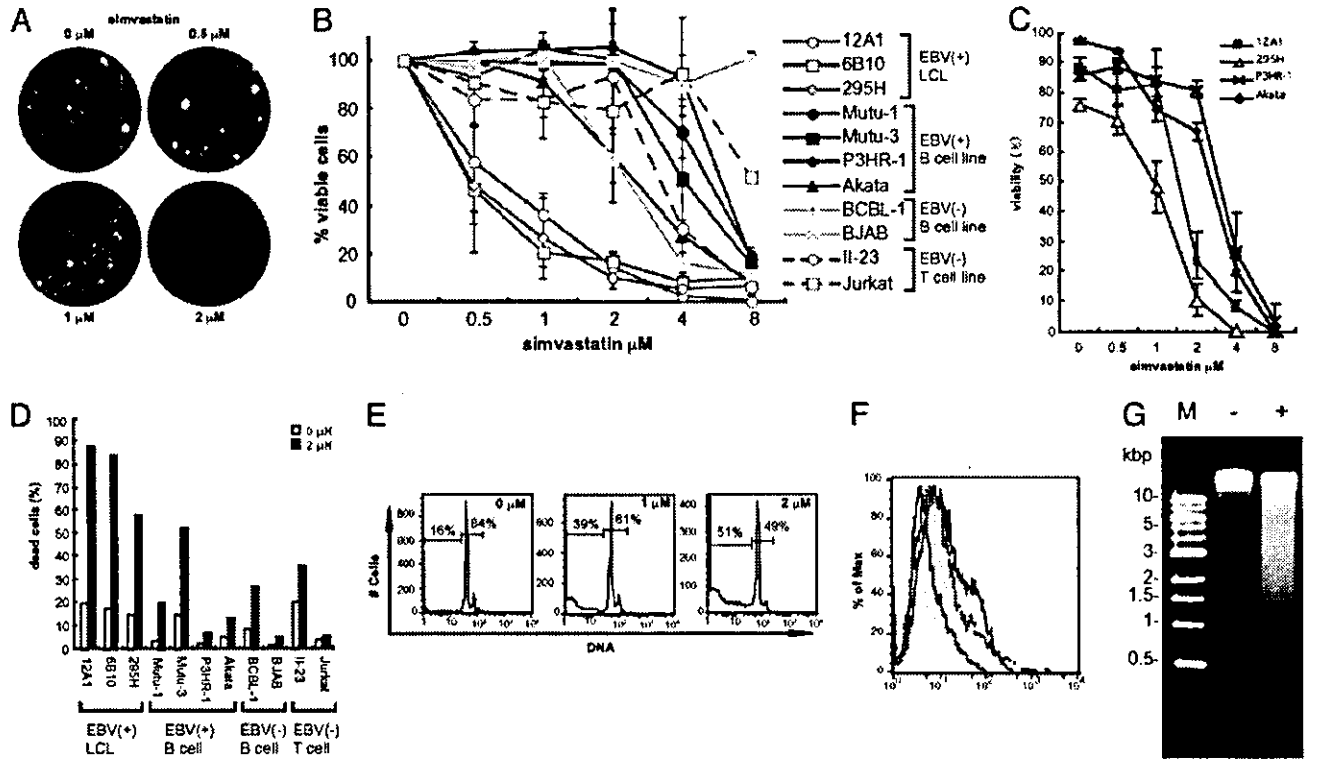

Fig. 1. Simvastatin inhibits clump formation and induces apoptosis in LCLs. (*A*) Cell clumping of LCLs was observed after simvastatin was added at 0–2 μM for 5 days. (*B*) XTT cell proliferation assay was performed after addition of simvastatin (0–8 μM) to various cells lines for 7 days. Error bars indicate standard deviations for four independent experiments. (*C*) Cell viability was assayed by trypan blue staining after cell lines were cultured with 0–8 μM simvastatin for 7 days. Error bars indicate standard deviations for three separate experiments. (*D*) Percentage of dead cells in the absence (open bars) or presence (filled bars) of 2 μM simvastatin for 7 days as determined by PI staining and flow cytometry. PI-positive cells were counted as dead cells. (*E*) PI staining. Cells were treated with or without simvastatin for 5 days. Cell populations in sub-G_0–G_1 and G_0–G_1–S–M phase are indicated. (*F*) TUNEL assays were performed for cells treated with 2 μM simvastatin for 5 days (gray area with solid line), no simvastatin for 5 days (white area with solid line), or serum starvation for 72 h (white area with dotted line). (*G*) DNA ladder formation for cells cultured with (+) or without (−) 2 μM simvastatin for 5 days.

Reagents and Antibodies. Simvastatin and pravastatin (Calbiochem) were converted to their open acid forms before use *in vitro* (25). Soluble ICAM-1 was purchased from R & D Systems. LFA-1 antibodies TS1/22 (American Type Culture Collection) (26) and G25.2 (BD Pharmingen) were used as primary antibodies, and fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ fragment of goat anti-mouse Ig (Caltag, Burlingame, CA) was used as the secondary antibody for immunofluorescence and flow cytometry. TS1/22 antibody was obtained from hybridoma cells.

Lipid Raft Studies. Detergent extraction and flotation assay for lipid rafts were performed as described (27). Immunoblotting was performed by using anti-LMP-1 monoclonal antibody (S-12, BD Pharmingen), anti-CD71 monoclonal antibody (Zymed), and anti-Lyn monoclonal antibody (Santa Cruz Biotechnology).

Table 1. Protein expression, activation of NF-κB, and cell death induced by simvastatin in cell lines

Cell line	Description	Clumping formation	LFA-1*	ICAM-1*	EBV†	LMP-1‡	NF-κB‡	Cell death by simvastatin§
12A1	LCL	++	++	++	+	+++	++	+
6B10	LCL	++	++	++	+	+++	+++	+
295H	LCL	+	+	++	+	+++	+++	+
Mutu-1	B cell line (BL)	−	−	−	+	−	−	−
Mutu-3	B cell line (BL)	+	+	++	+	+	+	+
P3HR-1	B cell line (BL)	−	++	++	+	−	+	−
Akata	B cell line (BL)	−	−	−	+	−	+	−
BCBL-1	HHV-8-positive cell	−	−	+	−	−	−	−
BJAB	B cell line (BL)	−	+	++	−	−	−	−
II-23	T cell line	−	++	+	−	−	+	−
Jurkat	T cell line	−	−	+	−	−	−	−

*Expression levels of LFA-1 and ICAM-1 were determined with flow cytometry (Fig. 5).
†EBV-positive cells lines are indicated in refs. 18–24.
‡Expression level of LMP-1 and constitutive activation of NF-κB were determined by immunoblot and gel shift assay, respectively (Fig. 5).
§Cell death induced by 2 μM simvastatin is defined as >50% cell death by PI staining (Fig. 1*D*). BL, Burkitt lymphoma; HHV-8, human herpesvirus 8.

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.

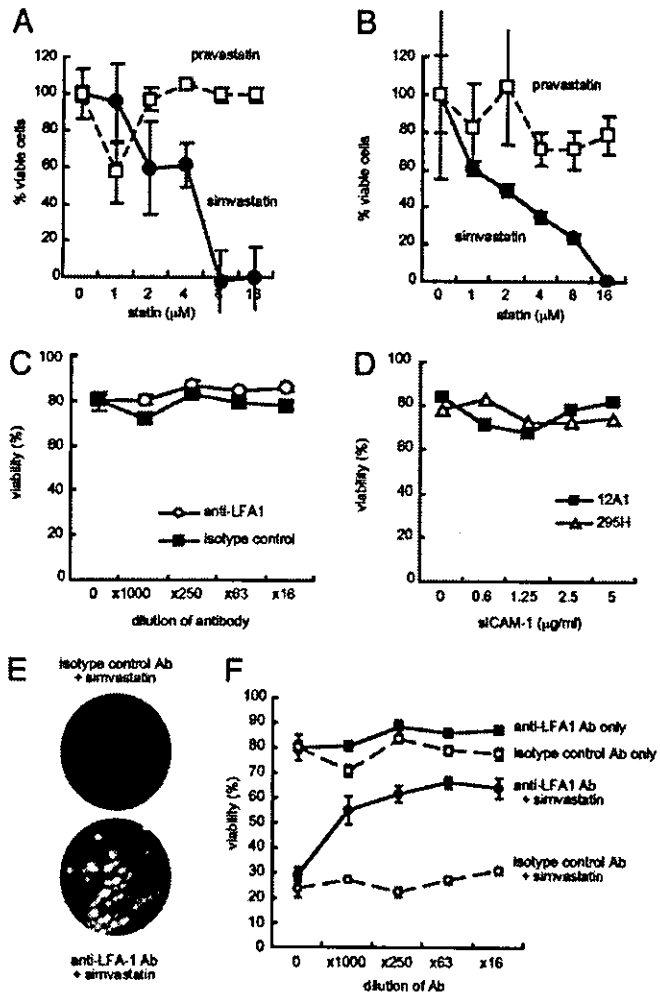

Fig. 2. Apoptosis-inducing effect of simvastatin depends on binding to the I domain of LFA-1. (*A* and *B*) XTT cell proliferation assay was performed for LCLs 12A1 (*A*) and 6B10 (*B*) in the presence of simvastatin or pravastatin after 5 days. (*C*) Cell viability was assayed by trypan blue staining 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.

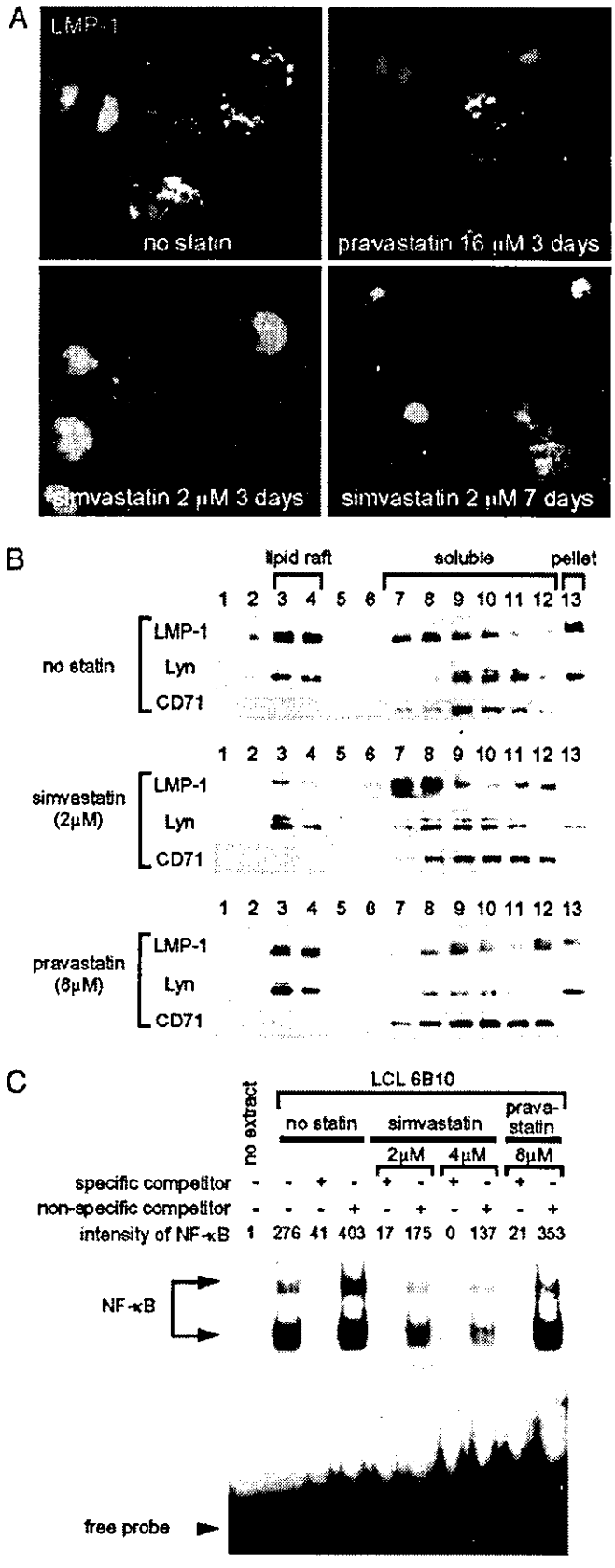

Fig. 3. Simvastatin alters the localization of LMP-1, displaces LMP-1 from lipid rafts, and inhibits NF-κB activation in LCLs. (*A*) Immunofluorescence of LMP-1 in LCL 6B10 in the absence or presence of simvastatin or pravastatin. Cells were fixed, permeabilized, and incubated with anti-LMP-1 antibody (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

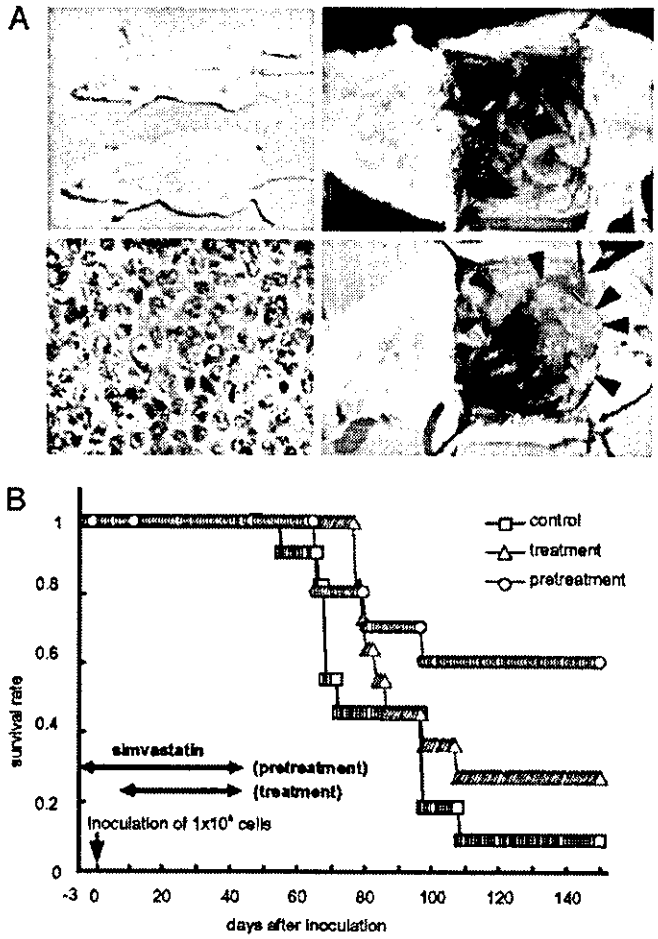

Fig. 4. Simvastatin prolongs survival of SCID mice inoculated with LCLs. (*A*) Appearance of mice at 45 days after inoculation. The mouse that did not receive simvastatin (*Upper Left*, lower mouse) has ascites, but the simvastatin-treated mouse (upper mouse) has no ascites. The simvastatin-treated mouse (*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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