impairment in cytotoxicity of $CD8^+$ T cells allows the APCs to continue to present antigens, which in turn increases the proliferation of these T cells. This results in uncontrolled production of IFN-γ as well as other cytokines, and leads to the characteristic histologic and clinical features of haemophagocytic lymphohistiocytosis, including haemophagocytosis.

In the absence of challenge with an infectious pathogen or its antigens, abnormal immune responses can be seen in animals with both perforin deficiency and an additional genetic disease. When perforin-deficient mice are bred on the background of lupus-prone mice, they are more prone to autoimmune disease, and develop autoantibodies and immune complex formation in various organs (Peng *et al*, 1998). Perforin-deficient severe combined immunodeficiency (SCID) mice have more expansion of $CD8^+$ T cells than control mice in a GvHD model (Spaner *et al*, 1999). Perforin is important for preventing autoimmunity by the elimination of autoreactive B cells and antigen-specific T cells (Shustov *et al*, 2000). Perforin-deficient mice are more susceptible to autoimmune encephalitis than control mice (Malipiero *et al*, 1997). Perforin-deficient mice that also lack Fas ligand develop severe pancreatitis with macrophage proliferation when compared with mice that lack Fas ligand alone (Spielman *et al*, 1998).

The role of perforin in humans

Perforin in normal adults and children

Perforin expression is confined to NK, $CD8^+$ T, $CD56^+$ T, γ-δ T, and activated $CD4^+$ T cells (Kogawa *et al*, 2002). Perforin expression in NK, $CD56^+$ T, and $CD8^+$ T cells is age-dependent, with higher levels of perforin in adults compared with children. Healthy infants have very low levels (0–3%) of $CD8^+$ T cells that express perforin, compared with adults (8–28%). In contrast, infants have frequencies of NK cells (91–97%) and $CD56^+$ T cells (4–30%) expressing perforin that are more similar to the frequencies of NK cells (86–98%) and $CD56^+$ T cells (30–77%) expressing perforin in adults.

Perforin in human infections

Perforin is important for the control of viral diseases in humans. Lysis of human immunodeficiency virus (HIV)-infected cells by CTLs requires granule exocytosis and perforin (Shankar *et al*, 1999). Perforin is important for the control of HIV in patients. Long-term non-progressors (who maintain very low levels of HIV replication for years) have high frequencies of HIV-specific CD8 T cells that express perforin, and the level of expression of perforin within these cells is high. In contrast, the vast majority of patients chronically infected with HIV have progressive disease with high levels of HIV replication, and HIV-specific T cells that express little or no perforin (Migueles *et al*, 2002; Zhang *et al*, 2003). However, these patients have large numbers of CD8 T cells that do express perforin, but are not specific for HIV (Appay *et al*, 2000).

Virus-specific CD8 T cells from healthy patients chronically infected with other persistent viruses, including EBV and cytomegalovirus, express very low levels of perforin and are impaired for cytotoxicity against autologous cells incubated with viral peptides (Zhang *et al*, 2003). In contrast, cell lines expressing higher levels of perforin are cytotoxic for target cells pulsed with these peptides. Furthermore, the levels of perforin in $CD8^+$ T cells from lymphoid tissue of HIV-infected persons early in their infection are significantly higher than during the chronic phase of infection (Andersson *et al*, 1999). Taken together these results suggest that perforin is important for controlling the early phase of these infections, but once a chronic infection is established, the numbers of perforin positive virus-specific T cells, and their level of perforin expression, declines and limits the chance of eradicating the virus.

Perforin is important for cytotoxicity of EBV-specific $CD8^+$ T cells in patients with EBV lymphoproliferative disease (Yoshimi *et al*, 2002). Cytotoxic T cell activity from a patient with EBV-lymphoproliferative disease was blocked with concanamycin A, which inhibits perforin, but not Fas-mediated killing (Yoshimi *et al*, 2002). The perforin pathway is the major mechanism used by $CD4^+$ T cells to lyse EBV-infected B cells (Sun *et al*, 1999; Khanolkar *et al*, 2001).

Familial haemophagocytic lymphohistiocytosis: a disease caused by perforin deficiency

Clinical features

Familial haemophagocytic lymphohistiocytosis (FHL) is an autosomal recessive trait in which infants and young children who are usually healthy at birth, present with dysregulated cellular immune responses. Most patients present with symptoms during the first year of life (Arico *et al*, 1996), but some present as late at the third decade of life (Clementi *et al*, 2002). Signs and symptoms include fever, splenomegaly, hepatomegaly, anaemia, thrombocytopenia, neutropenia, hypertriglyceridaemia, hypofibrinogenaemia, and cerebrospinal fluid pleocytosis in over 50% of patients (Henter *et al*, 1998). Lymphadenopathy, rash, neurological abnormalities, leucopenia, elevated liver function tests, elevated ferritin, and hyponatraemia may also be present. Neurological abnormalities range from irritability and hypotonia to seizures, cranial nerve deficits and ataxia. The inciting event is thought to be initiated by infection. Bone marrow biopsy late in the disease usually shows hypoplasia or aplasia. Patients who are not treated, or who do not respond to therapy, usually die within a few months of the onset of symptoms. Most deaths are the result of infection, disseminated intravascular coagulopathy with uncontrolled bleeding, or central nervous system disease.

Haemophagocytosis is a prominent feature of the disease. Haemophagocytosis occurs when activated macrophages (histiocytes) ingest erythrocytes and sometimes platelets and leucocytes (Fig 3). The bone marrow is the most common site of haemophagocytosis, although the spleen, liver and

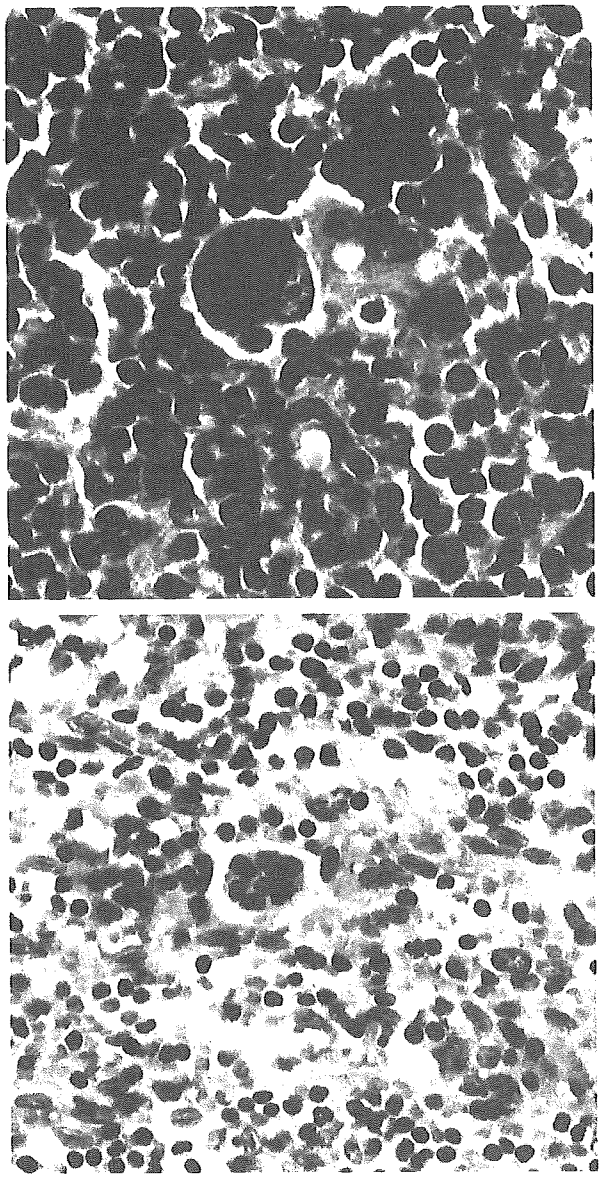

Fig 3. Histiocytosis with erythrophagocytosis (A) and phagocytosis of leucocytes (B) in the spleen of a patient with perforin mutations. Original magnification ×400. Photomicrograph courtesy of Dr Elaine Jaff.

lymph nodes are also frequently affected. In the early stage of the disease, histiocytes are present in focal areas of the bone marrow; in later stages of the disease they are distributed diffusely throughout the bone marrow. When haemophagocytosis is prominent in the bone marrow, there are decreased numbers of normal precursors, which is associated with pancytopenia. In the lymph nodes, histiocytes are increased in the sinuses and T-cell areas, and lymphocyte depletion is often observed. In the spleen, histiocytes are distributed diffusely and erythrophagocytosis is frequently present.

NK cell and CTL cytotoxicity are severely impaired. Uncontrolled immune responses result in infiltration and destruction of tissues by activated macrophages ($CD68^+$), $CD8^+$ T cells (MHC class I positive). Activated macrophages and T cells are often present in the bone marrow, spleen, lymph nodes, liver and central nervous system; other organs including the lungs, heart, intestine, thymus, kidney and pancreas may also be infiltrated with these cells. Uncontrolled activated macrophages and T cells release pro-inflammatory cytokines, which result in elevated serum levels of IFN-γ, TNF-α, IL-1 receptor antagonist, soluble IL-2 receptor, IL-6, IL-10, IL-12, IL-18, IP-10, monokine induced by IFN-γ (MIG) and M-CSF (Komp *et al*, 1989; Henter *et al*, 1991a; Akashi *et al*, 1994; Osugi *et al*, 1997; Takada *et al*, 1999, 2003). High levels of these T-helper type 1 and type 2 cytokines contribute to haemophagocytosis, cellular infiltration and organ damage. Lipoprotein lipase deficiency is associated with elevated serum triglycerides (Henter *et al*, 1991b). The level of spontaneously activated caspases in IL-2-stimulated lymphocytes from patients with FHL is reduced, which may contribute to the large numbers of activated T cells (Fadeel *et al*, 1999).

The diagnosis of FHL generally requires (i) a positive family history of the disease, (ii) fever, (iii) splenomegaly, (iv) cytopenia (affecting two of three cell lineages), (v) hypertriglyceridaemia and/or hypofibrinogenaemia, and (vi) haemophagocytosis in the spleen, bone marrow, or lymph nodes without underlying malignancy (Henter *et al*, 1991c). Some cases may not fit all the criteria.

The differential diagnosis of FHL includes both inherited and acquired disorders. Haemophagocytic syndrome is associated with certain infections, malignancies (including T and B-cell lymphomas, acute non-lymphocytic leukaemia and multiple myeloma), and rheumatic disease (including systemic lupus erythematosis and rheumatoid arthritis) (Reiner & Spivak, 1988; Janka *et al*, 1998).

Infection-associated haemophagocytic syndrome has been most frequently associated with EBV. EBV-encoded RNA and DNA are present in the infiltrating T or, less commonly, in NK cells (Kawaguchi *et al*, 1993; Imashuku, 2002). Most cases occur in otherwise immunocompetent children and adolescents. Patients with EBV-associated haemophagocytic syndrome have increased numbers of $CD8^+$ T cells expressing perforin (Kogawa *et al*, 2002). Perforin is detectable and perforin mutations are absent in patients with EBV-associated haemophagocytic syndrome (Ma *et al*, 2001; Ueda *et al*, 2003).

Haemophagocytic syndrome is also seen in patients with EBV-associated peripheral T or nasal T/NK cell lymphoma, EBV-associated NK cell leukaemia, and chronic active EBV disease (CAEBV). CAEBV infection is a rare disease in the US, but relatively common in Asian countries. Patients present with prolonged fever, lymphadenopathy, hepatosplenomegaly with liver dysfunction, and occasionally with haemophagocytic syndrome (Schooley *et al*, 1986; Straus, 1988; Kimura *et al*, 2001). Patients may have lymphadenitis, meningoencephalitis, pneumonitis, uveitis or persistent hepatitis. Biopsies show EBV

DNA, RNA or proteins in the affected tissues. Patients with CAEBV often develop progressive cellular and humoral immunodeficiency with pancytopenia and hypogammaglobulinaemia that renders them susceptible to opportunistic infections or B or T-cell lymphoproliferative disease (Okano, 2002). One patient with CAEBV was found to have mutations in both alleles of the perforin gene resulting in an inability to express the mature (60 kDa) form of the protein (Katano *et al*, 2004). The amount of the native form of perforin in the patient's peripheral blood mononuclear cells was extremely low and these cells were impaired for Fas-independent cytotoxicity. The patient had no family history of FHL, and he developed T-cell lymphoproliferative disease with haemophagocytic lymphohistiocytosis and died at 18 years of age, 10 years after the onset of CAEBV.

Infection with other viruses including cytomegalovirus, human herpesvirus 6, parvovirus, adenovirus and HIV has also been associated with haemophagocytic syndrome (Reiner & Spivak, 1988; Hoang *et al*, 1998). Infection with bacteria (especially enteric Gram-negative rods and brucella), fungi (including histoplasma and candida), and protozoa (e.g. leishmania) have also been reported to be associated with haemophagocytic syndrome.

At least three genetic diseases other than FHL have been associated with haemophagocytic syndrome. Patients with X-linked lymphoproliferative disease have mutations in SLAM (signalling lymphocytic activation molecule)-associated protein (SAP, also referred to as SH2D1A and DSHP) (Coffey *et al*, 1998; Nichols *et al*, 1998; Sayos *et al*, 1998). SAP is important for the down-regulation of IFN-γ production by T cells (Latour *et al*, 2001) and for NK cell cytotoxicity (Nakajima *et al*, 2000). When patients with the X-linked lymphoproliferative disease are infected with EBV they usually develop fulminant infectious mononucleosis with haemophagocytosis. A patient with X-linked lymphoproliferative disease was reported to have increased numbers of $CD8^+$ T cells expressing perforin (Kogawa *et al*, 2002). SAP-deficient mice show no overt phenotype and have normal lymphocyte development, however, infection with LCMV or *T. gondii* results in increased numbers of IFN-γ-producing T cells (Czar *et al*, 2001; Wu *et al*, 2001). SAP-deficient mice also have impaired long-term humoral immunity (Crotty *et al*, 2003).

Patients with Chediak–Higashi disease have mutations in *CHS1* and have large intracellular granules in their CTLs, NK cells, neutrophils and other lysosome-containing cells (Barbosa *et al*, 1996; Nagle *et al*, 1996). These patients have partial albinism and are predisposed to bacterial infections. Their cells have impaired chemotaxis, impaired phagocytosis, and their NK cells and neutrophils show reduced cytotoxicity. Some patients develop fatal haemophagocytic syndrome. A patient with Chediak–Higashi disease was reported to have normal levels of perforin in his NK cells (Kogawa *et al*, 2002). Human CHS1, known as the lysosomal trafficking regulator (LYST) is important for the packaging and sorting of lysosomal proteins, including perforin and granzymes, in the cell (Baetz *et al*, 1995; Barbosa *et al*, 1996; Nagle *et al*, 1996). Beige mice with a mutation on the beige (Lyst) gene, which corresponds to the *CHS1/LYST* gene in humans, have severe immunodeficiency and reduced function of T cells and NK cells because of impairment of granule exocytosis (Biron *et al*, 1987).

Patients with Griscelli syndrome type II have mutations in Rab27a and may also present with haemophagocytosis (Menasche *et al*, 2000). Rab27a regulates the trafficking of vesicles, and mutations in the protein impair granule exocytosis and cause disease in mice (Haddad *et al*, 2001). These patients have partial albinism, large clumps of pigment in their hair shafts, and frequent bacterial infections associated with neutropenia. Ashen mice with mutations in Rab27a are considered an animal model for Griscelli syndrome. Rab27a plays an important role in transporting melanosomes, and ashen mice have a light coat colour because of an abnormal perinuclear distribution of pigment granules in melanosomes (Wilson *et al*, 2000). T cells in ashen mice have a defect in granule exocytosis, resulting in impairment of the perforin pathway (Haddad *et al*, 2001).

Treatment of FHL (reviewed in Janka & Schneider, 2004) includes an inductive regimen of etoposide and corticosteroids followed by ciclosporin maintenance therapy with alternating pulses of etoposide and corticosteroids until stem transplantation can be performed. A new protocol is being tested in which ciclosporin will be given as part of the initial therapy. Stem cell transplantation is the only curative treatment for patients with FHL at present.

Genetics

Linkage analysis studies showed that FHL is linked to three loci: 9q21-22 [FHL1 (Ohadi *et al*, 1999)], 10q21 (FHL2), and at least a third unidentified locus. Estimates suggest that 20–40% of cases are linked to chromosome 10, 10% to chromosome 9, while most cases are to other mutations (Goransdotter Ericson *et al*, 2001). While the gene responsible for FHL1 is unknown, perforin mutations are responsible for FHL2 (Stepp *et al*, 1999), and Munc 13-4 mutations result in FHL3 (Feldmann *et al*, 2003). Munc 13-4 is located on chromosome 17q25 and is important for fusion of the granule membrane with the cell membrane. Patients with Munc 13-4 mutations present with typical symptoms of FHL and their cells have defective CTL activity.

Stepp *et al* (1999) first showed that the perforin gene, located in 10q21, is responsible for FHL in several patients. These patients have homozygous mutations in both alleles, or heterozygous mutations in different alleles of perforin that result in expression of very little or no perforin in their cytotoxic cells and impaired cytotoxicity in a Fas-independent killing assay. In contrast, levels of granzyme B, another protein in cytotoxic cells, are normal.

Parents of patients with perforin mutations, who are asymptomatic carriers and heterozygous for the mutation, have reduced levels of perforin in NK, $CD8^+$, or $CD56^+$ T cells

Table I. Mutations reported in the perforin gene in patients with familial haemophagocytic lymphohistiocytosis.

Mutation		
Nucleotide	Amino acid	Reference
A1G	M1V	Ueda *et al* (2003)
G3A	M1I	Feldmann *et al* (2002); Molleran Lee *et al* (2004)
T50del	L17frameshift	Stepp *et al* (1999); Clementi *et al* (2001); Feldmann *et al* (2002); Kogawa *et al* (2002); Molleran Lee *et al* (2004)
C116A	P39H	Kogawa *et al* (2002); Molleran Lee *et al* (2004)
G133A	G45R	Kogawa *et al* (2002); Molleran Lee *et al* (2004)
G134A	G45E	Molleran Lee *et al* (2004)
G148A	V50M	Goransdotter Ericson *et al* (2001); Molleran Lee *et al* (2004)
C160T	R54C	Kogawa *et al* (2002); Molleran Lee *et al* (2004)
C190T	Q64stop	Stepp *et al* (1999); Feldmann *et al* (2002)
C207del	P69frameshift	Suga *et al* (2002); Ueda *et al* (2003)
G208T	D70Y	Molleran Lee *et al* (2004)
T217C	C73R	Molleran Lee *et al* (2004)
C272T†	A91V	Clementi *et al* (2002); Busiello *et al* (2004)
T283C	W94R	Clementi *et al* (2001)
G445A	G149S	Kogawa *et al* (2002); Molleran Lee *et al* (2004)
C449A	S150stop	Molleran Lee *et al* (2004)
T469G	F157V	Molleran Lee *et al* (2004)
T548G	V183G	Stepp *et al* (1999); Feldmann *et al* (2002)
T577C*	F193L	Katano *et al* (2004)
C657A	Y219stop	Clementi *et al* (2001); Goransdotter Ericson *et al* (2001)
G658A	G220S	Clementi *et al* (2001); Feldmann *et al* (2002)
C662T	T221I	Clementi *et al* (2001)
A665G	H222R	Molleran Lee *et al* (2004)
C666A	H222Q	Molleran Lee *et al* (2004)
T671A	I224D	Goransdotter Ericson *et al* (2001)
C673T	R225W	Stepp *et al* (1999); Clementi *et al* (2001); Feldmann *et al* (2002); Molleran Lee *et al* (2004)
C694T	R232C	Clementi *et al* (2001)
G695A	R232H	Feldmann *et al* (2002); Busiello *et al* (2004)
A755G†	N252S	Stepp *et al* (1999); Feldmann *et al* (2002)
G781A	E261K	Feldmann *et al* (2002)
G836A	C279Y	Stepp *et al* (1999); Feldmann *et al* (2002)
AAG853-855del	K285del	Goransdotter Ericson *et al* (2001)
C895T	R299C	Molleran Lee *et al* (2004)
A938T	D313V	Molleran Lee *et al* (2004)
G949A	G317R	Ueda *et al* (2003)
C1034T	P345L	Stepp *et al* (1999); Feldmann *et al* (2002)
A1081T	R361W	Molleran Lee *et al* (2004)
G1083del	R361frameshift	Suga *et al* (2002); Ueda *et al* (2003)
CT1090-1091del	L364frameshift	Suga *et al* (2002); Ueda *et al* (2003)
G1122A	W374stop	Stepp *et al* (1999); Clementi *et al* (2001, 2002); Goransdotter Ericson *et al* (2001); Feldmann *et al* (2002); Suga *et al* (2002); Ueda *et al* (2003)
1182Tinsert	G394frameshift	Clementi *et al* (2001)
G1229C*	R410P	Katano *et al* (2004)
C1246T	Q416stop	Ueda *et al* (2003)
G1286A	G429E	Stepp *et al* (1999); Feldmann *et al* (2002)
A1442C	Q481P	Molleran Lee *et al* (2004)
T1491A	C497stop	Suga *et al* (2002); Ueda *et al* (2003)
1628Tinsert	L542frameshift	Molleran Lee *et al* (2004)
C1636 del	E545frameshift	Molleran Lee *et al* (2004)

*This patient (with mutations at nucleotides 577 and 1229) had chronic active EBV with many of the features of FHL, but no family history.
†Changes at these nucleotides have been reported to be polymorphisms rather than mutations (Molleran Lee *et al*, 2004). See text for details.

(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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References

Akashi, K., Hayashi, S., Gondo, H., Mizuno, S., Harada, M., Tamura, K., Yamasaki, K., Shibuya, T., Uike, N., Okamura, T., Miyamoto, T. & Niho, Y. (1994) Involvement of interferon-gamma and macrophage colony-stimulating factor in pathogenesis of haemophagocytic lymphohistiocytosis in adults. *British Journal of Haematology*, **87,** 243–250.

Andersson, J., Behbahani, H., Lieberman, J., Connick, E., Landay, A., Patterson, B., Sonnerborg, A., Lore, K., Uccini, S. & Fehniger, T.E. (1999) Perforin is not co-expressed with granzyme A within cytotoxic granules in CD8 T lymphocytes present in lymphoid tissue during chronic HIV infection. *AIDS*, **13,** 1295–1303.

Appay, V., Nixon, D.F., Donahoe, S.M., Gillespie, G.M., Dong, T., King, A., Ogg, G.S., Spiegel, H.M., Conlon, C., Spina, C.A., Havlir, D.V., Richman, D.D., Waters, A., Easterbrook, P., McMichael, A.J. & Rowland-Jones, S.L. (2000) HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function. *Journal of Experimental Medicine*, **192,** 63–75.

Arico, M., Janka, G., Fischer, A., Henter, J.I., Blanche, S., Elinder, G., Martinetti, M. & Rusca, M.P. for the FHL Study Group of the Histiocyte Society (1996) Haemophagocytic lymphohistiocytosis: report of 122 children from the International Registry. *Leukemia*, **10,** 197–203.

Arico, M., Allen, M., Brusa, S., Clementi, R., Pende, D., Maccario, R., Moretta, L. & Danesino, C. (2002) Haemophagocytic lymphohistiocytosis: proposal of a diagnostic algorithm based on perforin expression. *British Journal of Haematology*, **119,** 180–188.

Arnaout, R.A. (2000) Perforin deficiency: fighting unarmed? *Immunology Today*, **21,** 52.

Badovinac, V.P., Tvinnereim, A.R. & Harty, J.T. (2000) Regulation of antigen-specific CD8+ T cell homeostasis by perforin and interferon-gamma. *Science*, **290,** 1354–1358.

Badovinac, V.P., Hamilton, S.E. & Harty, J.T. (2003) Viral infection results in massive CD8+ T cell expansion and mortality in vaccinated perforin-deficient mice. *Immunity*, **18,** 463–474.

Baetz, A., Isaaz, S. & Griffiths, G.M. (1995) Loss of cytotoxic T lymphocyte function in Chediak-Higashi syndrome arise from a secretory defect that prevents lytic granule exoctyosis. *Journal of Immunology*, **154,** 6122–6131.

Balaji, K.N., Schaschke, N., Machleidt, W., Catalfamo, M. & Henkart, P.A. (2002) Surface cathepsin B protects cytotoxic lymphocytes from self-destruction after degranulation. *Journal of Experimental Medicine*, **196,** 493–503.

Barbosa, M.D., Nguyen, Q.A., Tchernev, V.T., Ashley, J.A., Detter, J.C., Blaydes, S.M., Brandt, S.J., Chotai, D., Hodgman, C., Solari, R.C., Lovett, M. & Kingsmore, S.F. (1996) Identification of the homologous beige and Chediak-Higashi syndrome genes. *Nature*, **382,** 262–265.

Barry, M., Heibein, J.A., Pinkoski, M.J., Lee, S.F., Moyer, R.W., Green, D.R. & Bleackley, R.C. (2000) Granzyme B short-circuits the need for caspase 8 activity during granule-mediated cytotoxic T-lymphocyte killing by directly cleaving Bid. *Molecular and Cellular Biology*, **20,** 3781–3794.

Binder, D., van den Broek, M.F., Kagi, D., Bluethmann, H., Fehr, J., Hengartner, H. & Zinkernagel, R.M. (1998) Aplastic anemia rescued by exhaustion of cytokine-secreting CD8+ T cells in persistent infection with lymphocytic choriomeningitis virus. *Journal of Experimental Medicine*, **187,** 1903–1920.

Biron, C.A., Pedersen, K.F. & Welsh, R.M. (1987) Aberrant T cells in beige mutant mice. *Journal of Immunology*, **138,** 2050–2056.

van den Broek, M.F., Kagi, D., Zinkernagel, R.M. & Hengartner, H. (1995) Perforin dependence of natural killer cell-mediated tumor control in vivo. *European Journal of Immunology*, **25,** 3514–3516.

Browne, K.A., Blink, E., Sutton, V.R., Froelich, C.J., Jans, D.A. & Trapani, J.A. (1999) Cytosolic delivery of granzyme B by bacterial toxins: evidence that endosomal disruption, in addition to transmembrane pore formation, is an important function of perforin. *Molecular and Cellular Biology*, **19,** 8604–8615.

Busiello, R., Adriani, M., Locatelli, F., Galgani, M., Fimiani, G., Clementi, R., Ursini, M.V., Racioppi, L. & Pignata, C. (2004) Atypical features of familial hemophagocytic lymphohistiocytosis. *Blood*, **103,** 4610–4612.

Clementi, R., zur Stadt, U., Savoldi, G., Varotto, S., Conter, V., De Fusco, C., Notarangelo, L.D., Schnedier, M., Klersy, C., Janka, G., Danesino, C. & Arico, M. (2001) Six novel mutations in the PRF1 gene in children with haemophagocytic lymphohistiocytosis. *Journal of Medical Genetics*, **38,** 643–646.

Clementi, R., Emmi, L., Maccario, R., Liotta, F., Moretta, L., Danesino, C. & Arico, M. (2002) Adult onset and atypical presentation of hemophagocytic lymphohistiocytosis in siblings carrying PRF1 mutations. *Blood*, **100,** 2266–2267.

Coffey, A.J., Brooksbank, R.A., Brandau, O., Oohashi, T., Howell, G.R., Bye, J.M., Cahn, A.P., Durham, J., Heath, P., Wray, P., Pavitt, R., Wilkinson, J., Leversha, M., Huckle, E., Shaw-Smith, C.J., Dunham, A., Rhodes, S., Shuster, V., Porta, G., Yin, L., Serafini, P., Sylla, B., Zollo, M., Franco, B., Bolino, A., Seri, M., Lanyi, A., Davis, J.R., Webster, D., Harris, A., Lenior, G., de St. Basile, G., Jones, A., Behloradsky, B.H., Achatz, H., Nurken, J., Fassler, R., Sumegi, J., Romeo, G., Vaudin, M., Ross, M.T., Meindl, A. & Bentley, D.R. (1998) Host response to EBV infection in X-linked lymphoproliferative disease results from mutations in an SH2-domain encoding gene. *Nature Genetics*, **20,** 129–135.

Crotty, S., Kersh, E.N., Cannons, J., Schwartzberg, P.L. & Ahmed, R. (2003) SAP is required for generating long-term humoral immunity. *Nature*, **421,** 282–287.

Czar, M.J., Kersh, E.N., Mijares, L.A., Lanier, G., Lewis, J., Yap, G., Chen, A., Sher, A., Duckett, C.S., Ahmed, R. & Schwartzberg, P.L. (2001) Altered lymphocyte responses and cytokine production in mice deficient in the X-linked lymphoproliferative disease gene SH2D1A/DSHP/SAP. *Proceedings of the National Academy of Sciences of the United States of America*, **98,** 7449–7454.

Darmon, A.J., Nicholson, D.W. & Bleackley, R.C. (1995) Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B. *Nature*, **377,** 446–448.

Denkers, E.Y., Yap, G., Scharton-Kersten, T., Charest, H., Butcher, B.A., Caspar, P., Heiny, S. & Sher, A. (1997) Perforin-mediated cytolysis plays a limited role in host resistance to *Toxoplasma gondii*. *Journal of Immunology*, **159,** 1903–1908.

Fadeel, B., Orrenius, S. & Henter, J.I. (1999) Induction of apoptosis and caspase activation in cells obtained from familial haemophagocytic lymphohistiocytosis patients. *British Journal of Haematology*, **106,** 406–415.

Feldmann, J., Le Deist, F., Ouachee-Chardin, M., Certain, S., Alexander, S., Quartier, P., Haddad, E., Wulffraat, N., Casanova, J.L., Blanche, S., Fischer, A. & de Saint Basile, G. (2002) Functional consequences of perforin gene mutations in 22 patients with familial haemophagocytic lymphohistiocytosis. *British Journal of Haematology*, **117,** 956–972.

Feldmann, J., Callebaut, I., Raposo, G., Certain, S., Bacq, D., Dumont, C., Lambert, N., Ouachee-Chardin, M., Chedeville, G., Tamary, H., Minard-Colin, V., Vilmer, E., Blanche, S., Le Deist, F., Fischer, A. & de Saint Basile, G. (2003) Munc13-4 is essential for cytolytic granules fusion and is mutated in a form of familial hemophagocytic lymphohistiocytosis (FHL3). *Cell*, **115,** 461–473.

Franco, M.A., Tin, C., Rott, L.S., van Cott, J.L., McGhee, J.R. & Greenberg, H.B. (1997) Evidence for CD8+ T-cell immunity to murine rotavirus in the absence of perforin, Fas, and gamma-interferon. *Journal of Virology*, **71,** 479–486.

Froelich, C.J., Orth, K., Turbov, J., Seth, P., Gottlieb, R., Babior, B., Shah, G.M., Bleackley, R.C., Dixit, V.M. & Hanna, W. (1996) New paradigm for lymphocyte granule-mediated cytotoxicity. Target cells bind and internalize granzyme B, but an endosomolytic agent is necessary for cytosolic delivery and subsequent apoptosis. *Journal of Biological Chemistry*, **271,** 29073–29079.

Gebhard, J.R., Perry, C.M., Harkins, S., Lane, T., Mena, I., Asenio, V.C., Campbell, I.L. & Whitton, J.L. (1998) Coxsackievirus B3-induced myocarditis: perforin exacerbates disease, but plays no detectable role in virus clearance. *American Journal of Pathology*, **153,** 417–428.

Ghiasi, H., Cai, S., Perng, G-C., Nesburn, A.B. & Wechsler, S.L. (1999) Perforin pathway is essential for protection of mice against lethal ocular HSV-1 challenge but not corneal scarring. *Virus Research*, **65,** 97–101.

Goransdotter Ericson, K., Fadeel, B., Nilsson-Ardnor, S., Soderhall, C., Samuelsson, A., Janka, G., Schneider, M., Gurgey, A., Yalman, N., Revesz, T., Egeler, R., Jahnukainen, K., Storm-Mathiesen, I., Haraldsson, A., Poole, J., de Saint Basile, G., Nordenskjold, M. & Henter, J. (2001) Spectrum of perforin gene muations in familial hemophagocytic lymphohistiocytosis. *American Journal of Human Genetics*, **68,** 590–597.

Graubert, T.A., DiPersio, J.F., Russell, J.H. & Ley, T.J. (1997) Perforin/granzyme-dependent and independent mechanisms are both important for the development of graft-versus-host disease after murine bone marrow transplantation. *Journal of Clinical Investigation*, **100,** 904–911.

Haddad, E.K., Wu, X., Hammer, Jr, J.A. & Henkart, P.A. (2001) Defective granule exocytosis in Rab27a-deficient lymphocytes from ashen mice. *Journal of Cell Biology*, **152,** 835–842.

Henkart, P.A. (2000) Overview of cytotoxic lymphocyte effector mechanisms. In: *Cytotoxic Cells: Basic Mechanisms* (ed. by Sitkovsky, M. & P.A. Henkart), pp. 111–145. Lippincott, Williams & Wilkins, Philadelphia, PA.

Henkart, P.A., Williams, M.S., Zacharchuk, C.M. & Sarin, A. (1997) Do CTL kill target cells by inducing apoptosis? *Seminars in Immunology*, **9,** 135–144.

Henter, J.I., Ekinder, G., Soder, O., Hansson, M., Andersson, B. & Andersson, U. (1991a) Hypercytokinemia in familial hemophagocytic lymphohistiocytosis. *Blood*, **78,** 2918–2922.

Henter, J.I., Carlson, L.A., Soder, O., Nilsson-Ehle, P. & Elinder, G. (1991b) Lipoprotein alterations and plasma lipoprotein lipase reduction in familial hemophagocytic lymphohistiocytosis. *Acta Pediatrica Scandinavica*, **80,** 675–681.

Henter, J.I., Elinder, G., Ost, A. & the FHL Study Group of the Histiocyte Society (1991c) Diagnostic guidelines for hemophagocytic lymphohistiocytosis. *Seminars in Oncology*, **18,** 29–33.

Henter, J.I., Arico, M., Elinder, G., Imashuku, S. & Janka, G. (1998) Familial hemophagocytic lymphohistiocytosis. *Hematology/Oncology Clinics of North America*, **12,** 417–433.

Hoang, M.P., Dawson, B., Rogers, Z.R., Scheuermann, R.H. & Rogers, B.B. (1998) Polymerase chain reaction amplification of archival material for Epstein-Barr virus, cytomegalovirus, human herpesvirus 6, and parvovirus B19 in children with bone marrow hemophagocytosis. *Human Pathology*, **29,** 1074–1077.

Imashuku, S. (2002) Clinical features and treatment strategies of Epstein–Barr virus-associated hemophagocytic lymphohistiocytosis. *Critical Reviews in Oncology/Hematology*, **44,** 259–272.

Janka, G.E. & Schneider, E.M. (2004) Modern management of children with haemophagocytic lymphohistiocytosis. *British Journal of Haematology*, **124,** 4–14.

Janka, G., Imashuku, S., Elinder, G., Schneider, M. & Henter, J.I. (1998) Infection- and malignancy-associated hemophagocytic syndromes: secondary hemophagocytic lymphohistiocytosis. *Hematology/Oncology Clinics of North America*, **12,** 435–444.

Jiang, Z., Podack, E. & Levy, R.B. (2001) Major histocompatibility complex-mismatched allogeneic bone marrow transplantation using perforin and/or Fas ligand double-defective CD4+ donor T cells: involvement of cytotoxic function by donor lymphocytes prior to graft-versus-host disease pathogenesis. *Blood*, **98,** 390–397.

Jordan, M.B., Hildeman, D., Kappler, J. & Marrack, P. (2004) An animal model of hemophagocytic lymphohistiocytosis (HLH): CD8+ T cells and interferon gamma are essential for the disorder. *Blood*, **104,** 735–743.

Kagi, D., Ledermann, B., Burkl, K., Seiler, P., Odermatt, B., Olsen, K.J., Podack, E.R., Zinkernagel, R.M. & Hengartner, H. (1994a) Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature*, **369,** 31–37.

Kagi, D., Ledermann, B., Burki, K., Hengartner, H. & Zinkernagel, R.M. (1994b) CD8+ T cell-mediated protection against an intracellular bacterium by perforin-dependent cytotoxicity. *European Journal of Immunology*, **24,** 3068–3072.

Kagi, D., Seiler, P., Pavlovic, J., Ledermann, B., Burki, K., Zinkernagel, R.M. & Hengartner, H. (1995) The roles of perforin- and Fas-dependent cytotoxicity in protection against cytopathic and non-cytopathic viruses. *European Journal of Immunology*, **25,** 3256–3262.

Kagi, D., Odermatt, B. & Mak, T.W. (1999) Homeostatic regulation of CD8+ T cells by perforin. *European Journal of Immunology*, **29,** 3262–3272.

Katano, H., Ali, M.A., Patera, A.C., Catalfamo, M., Jaffe, E.S., Kimura, H., Dale, J.K., Straus, S.E. & Cohen, J.I. (2004) Chronic active Epstein-Barr virus infection associated with mutations in perforin that impair its maturation. *Blood*, **103,** 1244–1252.

Kawaguchi, H., Miyashita, T., Herbst, H., Niedobitek, G., Asada, M., Tsuchida, M., Hanada, R., Kinoshita, A., Sakurai, M., Kobauashi, N. & Mizutami, S. (1993) Epstein-Barr virus-infected T lymphocytes in Epstein-Barr virus-associated hemophagocytic syndrome. *Journal of Clinical Investigation*, **92,** 1444–1450.

Khanolkar, A., Yagita, H. & Cannon, M.J. (2001) Preferential utilization of the perforin/granzyme pathway for lysis of Epstein-Barr virus-transformed lymphoblastoid cells by virus-specific CD4+ T cells. *Virology*, **287,** 79–88.

Kimura, H., Hoshino, Y., Kanegane, H., Tsuge, I., Okamura, T., Kawa, K. & Morishima, T. (2001) Clinical and virologic characteristics of chronic active Epstein-Barr virus infection. *Blood*, **98,** 280–286.

Kogawa, K., Lee, S.M., Villanueva, J., Marmer, D., Sumegi, J. & Filipovich, A.H. (2002) Perforin expression in cytotoxic lymphocytes from patients with hemophagocytic lymphohistocytosis and their family members. *Blood*, **99,** 61–66.

Kojima, H., Shinohara, N., Hanaoka, S., Someya-Shirota, Y., Takagaki, Y., Ohno, H., Saito, T., Katayama, T., Yagita, H., Okumura, K., Shinkai, Y., Alt, F.W., Matsuzawa, A., Yonehara, S. & Takayama, H. (1994) Two distinct pathways of specific killing revealed by perforin mutant cytotoxic T lymphocytes. *Immunity*, **1,** 357–364.

Komp, D.M., McNamara, J. & Buckley, P. (1989) Elevated soluble interleukin-2 receptor in childhood hemophagocytic histiocytic syndromes. *Blood*, **73,** 2128.

Latour, S., Gish, G., Helgason, C.D., Humphries, R.K., Pawson, T. & Veillette, A. (2001) Regulation of SLAM-mediated signal transduction by SAP, the X-linked lymphoproliferative gene product. *Nature Immunology*, **2,** 681–690.

Lichtenheld, M.G., Olsen, K.J., Lu, P., Lowrey, D.M., Hameed, A., Hengartner, H. & Podack, E.R. (1988) Structure and function of human perforin. *Nature*, **335,** 448–451.

Lieberman, J. (2003) The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. *Nature Reviews in Immunology*, **3,** 361–370.

Lowin, B., Peitsch, M.C. & Tschopp, J. (1995) Perforin and granzymes: crucial effector molecules in cytolytic T lymphocyte and natural killer cell-mediated cytotoxicity. *Current Topics in Microbiology and Immunology*, **198,** 1–24.

Ma, X., Okamura, A., Yosioka, M., Ishiguro, N., Kikuta, H. & Kobayashi, K. (2001) No mutations of SAP/SH2D1A/DSHP and perforin genes in patients with Epstein-Barr virus-associated hemophagocytic syndrome in Japan. *Journal of Medical Virology*, **65,** 358–361.

Malipiero, U., Frei, K., Spanaus, K.S., Agresti, C., Lassman, H., Hahne, M., Tschopp, J., Eugster, H.P. & Fontana, A. (1997) Myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis is chronic/relapsing in perforin knockout mice, but monophasic in Fas- and Fas ligand-deficient lpr and gld mice. *European Journal of Immunology*, **27,** 3151–3160.

Martin, P.J., Akatsuka, Y., Hahne, M. & Sale, G. (1998) Involvement of donor T-cell cytotoxic effector mechanisms in preventing allogeneic marrow graft rejection. *Blood*, **92,** 2177–2181.

Masson, D., Peters, P.J., Geuze, H.J., Borst, J. & Tschopp, J. (1990) Interaction of chondroitin sulfate with perforin and granzymes of cytolytic T-cells is dependent on pH. *Biochemistry*, **29,** 11229–11235.

Matloubian, M., Suresh, M., Glass, A., Galvan, M., Chow, K., Whitmore, J.K., Walsh, C.M., Clark, W.R. & Ahmed, R. (1999) A role for perforin in downregulating T-cell responses during chronic viral infection. *Journal of Virology*, **73,** 2527–2536.

Menasche, G., Pastural, E., Feldmann, J., Certain, S., Ersoy, F., Dupuis, S., Wulffraat, N., Bianchi, D., Fischer, A., Le Deist, F. & de Saint Basile, G. (2000) Mutations in RAB27A cause Griscelli syndrome associated with haemophagocytic syndrome. *Nature Genetics*, **25,** 173–176.

Metkar, S.S., Wang, B., Aguilar-Santelises, M., Raja, S.M., Uhlin-Hansen, L., Podack, E., Trapani, J.A. & Froelich, C.J. (2002) Cytotoxic cell granule-mediated apoptosis: perforin delivers granzyme B-serglycin complexes into target cells without plasma membrane pore formation. *Immunity*, **16,** 417–428.

Migueles, S.A., Laborico, A.C., Shupert, W.L., Sabbaghian, M.S., Rabin, R., Hallahan, C.W., Van Baarle, D., Kostense, S., Miedema, F., McLaughlin, M., Ehler, L., Metcalf, J., Liu, S. & Connors, M. (2002) HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. *Nature Immunology*, **3,** 1061–1068.

Molleran Lee, S., Villanueva, J., Sumegi, J., Zhang, K., Kogawa, K., Davis, J. & Filipovich, A.H. (2004) Characterisation of diverse PRF1 mutations leading to decreased killer cell activity in North American families with haemophagocytic lymphohistiocytosis. *Journal of Medical Genetics*, **41,** 137–144.

Moretta, L., Moretta, A., Hengartner, H. & Zinkernagel, R.M. (2000) On the pathogenesis of perforin defects and related immunodeficiencies. *Immunology Today*, **21,** 593–594.

Mullbacher, A., Hla, R.T., Museteanu, C. & Simon, M.M. (1999) Perforin is essential for control of ectromelia virus but not related poxviruses in mice. *Journal of Virology*, **73,** 1665–1667.

Nagle, D.L., Karim, M.A., Woolf, E.A., Holmgren, L., Bork, P., Misumi, D.J., McGrail, S.H., Dussault, Jr, B.J., , Perou, C.M., Boissy, R.E., Duyk, G.M., Spritz, R.A. & Moore, K.J. (1996) Identification and mutation analysis of the complete gene for Chediak-Higashi syndrome. *Nature Genetics*, **14,** 307–311.

Nakajima, H., Park, H.L. & Henkart, P.A. (1995) Synergistic roles of granzymes A and B in mediating target cell death by rat basophilic leukemia mast cell tumors also expressing cytolysin/perforin. *Journal of Experimental Medicine*, **181,** 1037–1046.

Nakajima, H., Cella, M., Bouchon, A., Grierson, H.L., Lewis, J., Duckett, C., Cohen, J.I. & Colonna, M. (2000) Patients with X-linked lymphoproliferative disease have a defect in 2B4 receptor-mediated NK cell cytotoxicity. *European Journal of Immunology*, **30,** 3309–3318.

Nichols, K.E., Harkin, D.P., Levitz, S., Krainer, M., Kolquist, K.A., Genovese, C., Bernard, A., Ferguson, M., Zuo, L., Snyder, E., Buckler, A.J., Wise, C., Ashley, J., Lovett, M., Valentine, M.B., Look, A.T., Gerld, W., Housman, D.E. & Haber, D.A. (1998) Inactivating mutations in an SH2 domain-encoding gene in X-linked lymphoproliferative syndrome. *Proceedings of the National Academy of Sciences of the United States of America*, **95,** 13765–13770.

Ohadi, M., Lalloz, M.R., Sham, P., Zhao, J., Dearlove, A.M., Shiach, C., Kinsey, S., Rhodes, M. & Layton, D.M. (1999) Localization of a gene for familial hemophagocytic lymphohistiocytosis at chromosome 9q21.3–22 by homozygosity mapping. *American Journal of Human Genetics*, **64,** 165–171.

Okano, M. (2002) Overview and problematic standpoints of severe chronic active Epstein-Barr virus infection syndrome. *Critical Reviews in Oncology and Hematology*, **44,** 273–282.

Osugi, Y., Hara, J. & Tagawa, S. (1997) Cytokine production regulating Th1 and Th2 cytokines in hemophagocytic lymphohistiocytosis. *Blood*, **89,** 4100–4103.

Peng, S.L., Moslehi, J., Robert, M.E. & Craft, J. (1998) Perforin protects against autoimmunity in lupus-prone mice. *Journal of Immunology*, **160,** 652–660.

Perry, L.L., Feilzer, K., Hughes, S. & Caldwell, H.D. (1999) Clearance of *Chlamydia trachomatis* from the murine genital mucosa does not require perforin-mediated cytolysis or Fas-mediated apoptosis. *Infection and Immunity*, **67,** 1379–1385.

Reiner, A.P. & Spivak, J.L. (1988) Hemophagocytic histiocytosis: a report of 23 new patients and a review of the literature. *Medicine*, **67,** 369–388.

Rossi, C.P., McAllister, A., Tanguy, M., Kagi, D. & Brahic, M. (1998) Theiler's virus infection of perforin-dependent mice. *Journal of Virology*, **72,** 4515–4519.

Rottenberg, M.E., Rothfuchs, G., Gigliotti, D., Svanholm, C., Bandholtz, L. & Wigzell, H. (1999) Role of innate and adaptive immunity in the outcome of primary infection with *Chlamydia pneumoniae*, as analyzed in genetically modified mice. *Journal of Immunology*, **162,** 2829–2836.

Russell, J.H. & Ley, T.J. (2002) Lymphocyte-mediated cytotoxicty. *Annual Reviews of Immunology*, **20,** 323–370.

Sayos, J., Wu, C., Morra, M., Wang, N., Zhang, X., Allen, D., van Schaik, S., Notarangelo, L., Geha, R., Roncarolo, M.G., Oettgen, H., de Vries, J.E., Aversa, G. & Terhorst, C. (1998) The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM. *Nature*, **395,** 462–469.

Schooley, R.T., Carey, R.W., Miller, G., Henle, W., Eastman, R., Mark, E.J., Kenyon, K., Wheeler, E.O. & Rubin, R.H. (1986) Chronic Epstein-Barr virus infection associated with fever and interstitial pneumonitis. Clinical and serologic features and response to antiviral chemotherapy. *Annals of Internal Medicine*, **104,** 636–643.

Shankar, P., Xu, Z. & Liberman, J. (1999) Viral-specific cytotoxic T lymphocytes lyse HIV-infected primary T lymphocytes by the granule exocytosis pathway. *Blood*, **94,** 3084–3093.

Shi, L., Kraut, R.P., Aebersold, R. & Greenberg, A.H. (1992) A natural killer cell granule protein that induces DNA fragmentation and apoptosis. *Journal of Experimental Medicine*, **175,** 553–566.

Shiver, J.W., Su, L. & Henkart, P.A. (1992) Cytotoxicity with target DNA breakdown by rat basophilic leukemia cells expressing both cytolysin and granzyme A. *Cell*, **71,** 315–322.

Shustov, A., Luzina, I., Nguyen, P., Papadimitriou, J.C., Handwerger, B., Elkon, K.B. & Via, C.S. (2000) Role of perforin in controlling B-cell hyperactivity and humoral autoimmunity. *Journal of Clinical Investigation*, **106,** R39–R47.

Smyth, M.J., Thia, K.Y., Street, S.E.A., MacGregor, D., Godfrey, D.I. & Trapani, J.A. (2000) Perforin-mediated cytotoxicity is critical for surveillance of spontaneous lymphoma. *Journal of Experimental Medicine*, **192,** 755–760.

Spaner, D., Raju, K., Rabinovich, B. & Miller, R.G. (1999) A role for perforin in activation-induced T cell death in vivo: increased expansion of allogeneic perforin-deficient T cells in SCID mice. *Journal of Immunology*, **162,** 1192–1199.

Spielman, J., Leek, R.K. & Podack, E.R. (1998) Perforin/Fas-ligand double deficiency is associated with macrophage-expansion and severe pancreatitis. *Journal of Immunology*, **161,** 7063–7070.

Stepp, S.E., Dufourcq-Lagelouse, R., Le Deist, F., Bhawan, S., Certain, S., Mathew, P.A., Henter, J.I., Bennett, M., Fischer, A., de Saint Basile, G. & Kumar, V. (1999) Perforin gene defects in familial hemophagocytic lymphohistiocytosis. *Science*, **286,** 1957–1959.

Straus, S.E. (1988) The chronic mononucleosis syndrome. *Journal of Infectious Diseases*, **157,** 405–412.

Street, S.E., Cretney, E. & Smyth, M.J. (2001) Perforin and interferon-gamma activities independently control tumor initiation, growth, and metastases. *Blood*, **97,** 192–197.

Suga, N., Takada, H., Nomura, A., Ohga, S., Ishii, E., Ihara, K., Ohshima, K. & Hara, T. (2002) Perforin defects of primary haemophagocytic lymphohistiocytosis in Japan. *British Journal of Hematology*, **116,** 346–349,

Sullivan, K., Delaat, C.A., Douglas, S.D. & Filipovich, A.H. (1998) Defective natural killer cell function in patients with hemophagocytic lymphohistiocytosis and in first degree relatives. *Pediatric Research*, **44,** 465–468.

Sun, Q., Burton, R.L., Pollok, K.E., Emanuel, D.J. & Lucas, K.G. (1999) CD4(+) Epstein-Barr virus-specific cytotoxic T-lymphocytes from human umbilical cord blood. *Cellular Immunology*, **195,** 81–88.

Takada, H., Ohga, S., Mizuno, Y., Suminor, A., Matsuzaki, A., Ihara, K., Kinukawa, N., Oshima, K., Kohno, K., Kurimoto, M. & Hara, T. (1999) Oversecretion of IL-18 in haemophagocytic lymphohistiocytosis: a novel marker of disease activity. *British Journal of Haematology*, **106,** 182–189.

Takada, H., Takahata, Y., Nomura, A., Ohga, S., Mizuno, Y. & Hara, T. (2003) Increased serum levels of interferon-gamma-inducible protein 10 and monokine induced by gamma interferon in patients with haemophagocytic lymphohistiocytosis. *Clinical Experimental Immunology*, **133,** 448–453.

Ueda, I., Morimoto, A., Inaba, T., Yagi, T., Hibi, S., Sugimoto, T., Sako, M., Yani, F., Fukushima, T., Nakayama, M., Ishii, E. & Imashuku, S. (2003) Characteristic peforin gene mutations of haemophagocytic lymphohistiocytosis in Japan. *British Journal of Haematology*, **121,** 503–510.

Uellner, R., Zvelebil, M.J., Hopkins, J., Jones, J., MacDougall, L.K., Morgan, B.P., Podack, E., Waterfield, M.D. & Griffiths, G.M. (1997) Perforin is activated by a proteolytic cleavage during biosynthesis which reveals a phospholipid-binding C2 domain. *EMBO Journal*, **16,** 7287–7296.

Usherwood, E.J., Brooks, J.W., Sarawar, S.R., Cardin, R.D., Young, W.D., Allen, D.J., Doherty, P.C. & Nash, A.A. (1997) Immunological control of murine gammaherpesvirus infection is independent of perforin. *Journal of General Virology*, **78,** 2025–2030.

Voskoboinik, I., Thia, M-C., De Bono, A., Browne, K., Cretney, E., Jackson, J.T., Darcy, P.K., Jane, S.M., Smyth, M.J. & Trapani, J.A. (2004) The functional basis for hemophagocytic lymphohistiocytosis in a patient with co-inherited missense mutations in the perforin (PFN1) gene. *Journal of Experimental Medicine*, **200,** 811–816.

Walsh, C.M., Matloubian, M., Liu, C.C., Ueda, R., Kurahara, C.G., Christensen, J.L., Huang, M.T.F., Young, J.D.E., Ahmed, R. & Clark, W.R. (1994) Immune function in mice lacking the perforin gene. *Proceedings of the National Academy of Sciences of the United States of America*, **91,** 10854–10858.

Wilson, S.M., Yip, R., Swing, D.A., O'Sullivan, T.N., Zhang, Y., Novak, E.K., Swank, R.T., Russell, L.B., Copeland, N.G. & Jenkins, N.A. (2000) A mutation in Rab27a causes the vesicle transport defects observed in ashen mice. *Proceedings of the National Academy of Sciences United States of America*, **97,** 7933–7938.

Wu, C., Nguyen, K.B., Pien, G.C., Wang, N., Gullo, C., Duncan, H., Sosa, M.R., Edwards, M.J., Borrow, P., Satoskar, A.R., Sharpe, A.H., Biron, C.A. & Terhorst, C. (2001) SAP controls T cell responses to virus and terminal differentiation of T(H)2 cells. *Nature Immunology*, **2,** 410–414.

Yoshimi, A., Tsuge, I., Namizaki, H., Hoshino, Y., Kimura, H., Takahashi, Y., Watanabe, N., Kuzushima, K. & Kojima, S. (2002) Epstein-Barr virus-specific T-cell cytotoxicity is mediated through the perforin pathway in patients with lymphoproliferative disorders after allogeneic bone marrow transplantation. *British Journal of Hematology*, **116,** 710–715.

Zhang, D., Shankar, P., Xu, Z., Harnisch, B., Chen, G., Lange, C., Lee, S.J., Valdez, H., Lederman, M.M. & Lieberman, J. (2003) Most antiviral CD8 T cells during chronic viral infection do not express high levels of perforin and are not directly cytotoxic. *Blood*, **101,** 226–235.

Zhou, P., Freidag, B.L., Caldwell, C.C. & Seder, R.A. (2001) Perforin is required for primary immunity to *Histoplasma capsulatum*. *Journal of Immunology*, **166,** 1968–1974.

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 $KS330_{233}$ 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

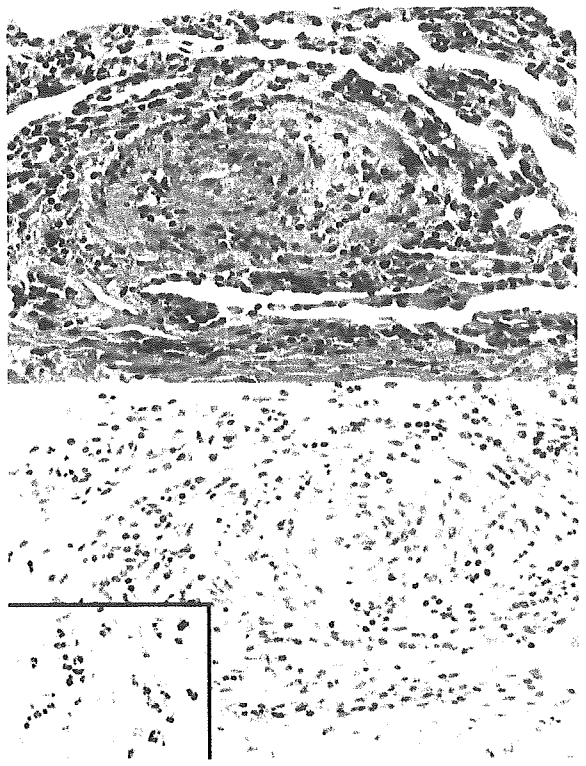

Figure 1. Plexiform lesions in lung tissue from a patient with primary pulmonary hypertension. *Top,* Lung tissue stained with hematoxylin-eosin. *Bottom,* Detection of latency-associated nuclear antigen (LANA) by immunohistochemistry. *Inset,* Expression of LANA (dot-like nuclear staining pattern) in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

References

1. Deng Z, Morse JH, Slager SL, et al. Familial primary pulmonary hypertension (gene PPH1) is caused by mutations in the bone morphogenetic protein receptor–II gene. Am J Hum Genet **2000**; 67:737–44.
2. Cool CD, Rai PR, Yeager ME, et al. Expression of human herpesvirus 8 in primary pulmonary hypertension. N Engl J Med **2003**; 349: 1113–22.
3. Moore PS, Chang Y. Kaposi's sarcoma–associated herpesvirus. In: Knipe DM, Howley PM, eds. Fields virology. 4th ed. Vol. 2. Philadelphia: Lippincott Williams & Wilkins, **2001**:2803-33.
4. Katano H, Iwasaki T, Baba N, et al. Identification of antigenic proteins encoded by human herpesvirus 8 and seroprevalence in the general population and among patients with and without Kaposi's sarcoma. J Virol **2000**; 74:3478–85.
5. Katano H, Sato Y, Kurata T, Mori S, Sata T. High expression of HHV-8–encoded ORF73 protein in spindle-shaped cells of Kaposi's sarcoma. Am J Pathol **1999**; 155:47–52.
6. Uehara R, Suzuki H, Kurokawa N, et al. Novel nonsense mutation of the BMPR-II gene in a Japanese patient with familial primary pulmonary hypertension. Pediatr Int **2002**; 44:433–5.
7. Katano H, Sato Y, Sata T. Expression of p53 and human herpesvirus 8 (HHV-8)–encoded latency-associated nuclear antigen (LANA) with inhibition of apoptosis in HHV-8–associated malignancies. Cancer **2001**; 92:3076–84.
8. Zong JC, Ciufo DM, Alcendor DJ, et al. High-level variability in the ORF-K1 membrane protein gene at the left end of the Kaposi's sarcoma–associated herpesvirus genome defines four major virus subtypes and multiple variants or clades in different human populations. J Virol **1999**; 73:4156–70.
9. Cool CD, Rai PR, Voelkel NF. HHV-8 in pulmonary hypertension. N Engl J Med **2004**; 350:195.
10. Katano H, Sato Y, Kurata T, Mori S, Sata T. Expression and localization of human herpesvirus 8–encoded proteins in primary effusion lymphoma, Kaposi's sarcoma, and multicentric Castleman's disease. Virology **2000**; 269:335–44.
11. Katano H, Suda T, Morishita Y, et al. Human herpesvirus 8–associated solid lymphomas that occur in AIDS patients take anaplastic large cell morphology. Mod Pathol **2000**; 13:77–85.
12. Henke-Gendo C, Schulz TF, Hoeper MM. HHV-8 in pulmonary hypertension. N Engl J Med **2004**; 350:194–5.

References

1. Forastiere F, Stafoggia M, Picciotto S, Bellander T, D'Ippoliti D, Lanki T, von Klot S, Nyberg F, Paatero P, Peters A, *et al.* A case-crossover analysis of out-of-hospital coronary deaths and air pollution in Rome, Italy. *Am J Respir Crit Care Med* 2005;172:1549–1555.
2. Schwartz J, Park SK, O'Neill MS, Vokonas PS, Sparrow D, Weiss S, Kelsey K. Glutathione-S-transferase M1, obesity, statins, and autonomic effects of particles: gene-by-drug-by-environment interaction. *Am J Respir Crit Care Med* 2005;172:1529–1533.
3. Romieu I, Téllez-Rojo MM, Lazo M, Manzano-Patiño A, Cortez-Lugo M, Julian P, Bélanger MC, Hernandez-Avila M, Holguin F. Omega-3 fatty acid prevents heart rate variability reductions associated with particulate matter. *Am J Respir Crit Care Med* 2005;172:1534–1540.
4. Cascio WE. Cardiopulmonary health effects of air pollution: is a mechanism emerging? *Am J Respir Crit Care Med* 2005;172:1482–1484.
5. Brunekreef B, Holgate ST. Air pollution and health. *Lancet* 2002;360:1233–1242.
6. Pope CA III, Burnett RT, Thurston GD, Manzano-Patiño A, Cortez-Lugo M, Julien P, Bélanger MC, Hernandez-Avila M, Holguin F. Cardiovascular mortality and long-term exposure to particulate air pollution. Epidemiological evidence of general pathophysiological pathways of disease. *Circulation* 2004;109:71–77.
7. Brook RD, Franklin B, Cascio W, Hong Y, Howard G, Lipsett M, Luepker R, Mittleman M, Samet J, Smith SC Jr, *et al.* Air pollution and cardiovascular disease: a statement for healthcare professionals from the Expert Panel on Population and Prevention Science of the American Heart Association. *Circulation* 2004;109:2655–2671.

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Herpesvirus-associated Pulmonary Hypertension?

Herpesviruses have brilliantly adapted to survive in their host of choice, employing specialized gene products that facilitate their transmission, replication (through cell lysis), and persistence (through latency). Unfortunately, the actions of many of these viral gene products negatively impact infected cells (1), ultimately leading to malignancies and lymphoproliferative diseases, particularly in immunodeficient individuals (2). In 1994, a novel γ herpesvirus homologous to Epstein-Barr virus was detected in Kaposi's sarcoma (KS) lesions from patients with AIDS, and was named Kaposi's sarcoma–associated herpesvirus (KSHV or human herpesvirus 8, HHV-8) (3). This initial finding immediately sparked an intense investigation into the manner in which a herpesvirus could trigger this neoplasm, which was composed of transformed spindle cells with potent angioproliferative properties. The causal role of KSHV has also been established in other malignancies, such as primary effusion lymphoma, and in benign diseases, such as multicentric Castleman's disease, a lymphadenopathy with polyclonal hypergammaglobulinemia (4).

In 2003, Cool and colleagues demonstrated the presence of KSHV in primary pulmonary hypertension (PPH), a disease of the pulmonary arterial wall that is histologically characterized by a lumen-occluding vascular or plexiform lesion containing endothelial and smooth muscle cells (5). Clinically, PPH is characterized by an elevation in pulmonary arterial pressure and eventual heart failure. Although genetic mutations in bone morphogenic protein receptor 2 have been detected in familial cases of PPH (6), no other genetic mutation had been uniformly detected in PPH, leading these investigators to speculate that an infectious agent, plausibly KSHV, may play a causative role in PPH. Their evidence for KSHV infection in PPH stemmed from an initial screen of laser-capture microdissected (LCM) plexiform lesions in which they detected open reading frame (ORF) 26 from KSHV in 4 of 15 patients. Further polymerase chain reaction (PCR) and immunohistochemical analysis by this group revealed the presence of KSHV genome (v-cyclin encoded by ORF72) and KSHV-encoded latency-associated nuclear antigen-1 (LANA-1), respectively, in plexiform lesions and cells outside these lesions (5). However, these results generated immediate controversy as Henke-Gendo and colleagues (7) reported that KSHV infectivity (based on plasma seropositivity) did not differ between the PPH and healthy control groups they studied. Cool and colleagues (7) countered with previously published data showing that nearly 20% of serum samples are negative in patients with KS. However, three independent research groups, using sophisticated immunohistochemical and PCR techniques, have subsequently failed to consistently show the presence of KSHV in PPH lung lesions (8–10). In this issue of the *Journal* (pp. 1581–1585) Henke-Gendo and colleagues (11) call into further question whether KSHV is associated with PPH since their sensitive PCR techniques failed to detect KSHV genome in formalin-fixed lung sections despite evidence of LANA-1 positivity in approximately 62% of these samples.

Conflicting results are common in biomedical research, and differential findings are often simplistically explained by sampling and/or analysis differences. Nevertheless, detection of KSHV genome and protein products is complicated by a number of technical issues relating to the complex lytic and latent phases of this herpesvirus, the status of the tissue analyzed (i.e., fresh vs. fixed; LCM plexiform lesions vs. whole tissues), and the relative abundance of KSHV-infected cells in the analyzed tissue. Immunohistochemical localization of LANA-1 has proven to be a reliable diagnostic tool in screening for the presence of KSHV in KS lesions. The KSHV genome has been detected by PCR in various diseases, including multiple myeloma, Bowen disease, sarcoidosis, and idiopathic pulmonary fibrosis, but LANA-1 staining in tissues associated with these diseases has proven to be elusive, leading many to question the presence and role of KSHV in each (2). Surprisingly, both Cool and coworkers (5) and Henke-Gendo and colleagues (11) showed strong LANA-1 staining in PPH lesions. In both studies, the LANA-1 staining observed in PPH plexiform lesions was suggestive of the "speckled" nuclear pattern classically observed in KS lesions. However, Henke-Gendo and colleagues (11) conclude that the LANA-1 staining they observed was a false-positive finding since they failed to detect KSHV genome by PCR in their lung samples.

Although the bulk of the published data now indicates that it very unlikely that KSHV is present in PPH, it is too soon to rule out the presence of and a putative role for other herpesviruses (or some yet-to-be-discovered virus) in PPH for several reasons. First, there is a striking similarity between the plexiform lesions observed in PPH and cutaneous KS; both exhibit slitlike vascular spaces with sheets of endothelial cells expressing factor 8–related antigen and vascular endothelial growth factor. Second, PPH is a heterogeneous disease, which involves a complex interplay of several genetic and environmental factors. Third, the murine viral equivalent of KSHV has been shown to profoundly remodel the lung (12, 13). Finally, novel antiviral approaches may be in order for the treatment of PPH given the beneficial effects of valacyclovir in idiopathic pulmonary fibrosis (14) and sirolimus in renal-transplant recipients (15). At the very least, clarification of the presence of KSHV in PPH will benefit from the implementation of novel genomic and proteomic detection techniques to LCM plexiform lesions.

Conflict of Interest Statement: Neither of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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References

1. Hong YK, Foreman K, Shin JW, Hirakawa S, Curry CL, Sage DR, Libermann T, Dezube BJ, Fingeroth JD, Detmar M. Lymphatic reprogramming of blood vascular endothelium by Kaposi sarcoma-associated herpesvirus. *Nat Genet* 2004;36:683–685.
2. Moore PS, Chang Y. Kaposi's sarcoma-associated herpesvirus. In: Knipe DM, Howley PM, editors. Fields virology, 4th ed. Philadelphia: Lippincott, Williams, & Wilkins; 2001.
3. Chang Y, Cesarman E, Pessin MS, Lee F, Culpepper J, Knowles DM, Moore PS. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science* 1994;266:1865–1869.
4. Katano H, Sato Y, Kurata T, Mori S, Sata T. Expression and localization of human herpesvirus 8-encoded proteins in primary effusion lymphoma, Kaposi's sarcoma, and multicentric Castleman's disease. *Virology* 2000;269:335–344.
5. Cool CD, Rai PR, Yeager ME, Hernandez-Saavedra D, Serls AE, Bull TM, Geraci MW, Brown KK, Routes JM, Tuder RM, *et al.* Expression of human herpesvirus 8 in primary pulmonary hypertension. *N Engl J Med* 2003;349:1113–1122.
6. Deng Z, Morse JH, Slager SL, Cuervo N, Moore KJ, Venetos G, Kalachikov S, Cayanis E, Fischer SG, Barst RJ, *et al.* Familial primary pulmonary hypertension (gene PPH1) is caused by mutations in the bone morphogenetic protein receptor-II gene. *Am J Hum Genet* 2000;67:737–744.
7. Henke-Gendo C, Schulz TF, Hoeper MM. HHV-8 in pulmonary hypertension. *N Engl J Med* 2004;350(2):194–195; author reply 195.
8. Laney AS, De Marco T, Peters JS, Malloy M, Teehankee C, Moore PS, Chang Y. Kaposi sarcoma-associated herpesvirus and primary and secondary pulmonary hypertension. *Chest* 2005;127:762–767.
9. Katano H, Ito K, Shibuya K, Saji T, Sato Y, Sata T. Lack of human herpesvirus 8 infection in lungs of Japanese patients with primary pulmonary hypertension. *J Infect Dis* 2005;191:743–745.
10. Daibata M, Miyoshi I, Taguchi H, Matsubara H, Date H, Shimizu N, Ohtsuki Y. Absence of human herpesvirus 8 in lung tissues from Japanese patients with primary pulmonary hypertension. *Respir Med* 2004;98:1231–1232.
11. Henke-Gendo C, Mengel M, Hoeper MM, Alkharsah K, Schulz TF. Absence of Kaposi's sarcoma-associated herpesvirus in patients with pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2005;172:1581–1585.
12. Lok SS, Haider Y, Howell D, Stewart JP, Hasleton PS, Egan JJ. Murine gammaherpes virus as a cofactor in the development of pulmonary fibrosis in bleomycin resistant mice. *Eur Respir J* 2002;20:1228–1232.
13. Dunne DW, Butterworth AE, Fulford AJ, Kariuki HC, Langley JG, Ouma JH, Capron A, Pierce RJ, Sturrock RF. Immunity after treatment of human schistosomiasis: association between IgE antibodies to adult worm antigens and resistance to reinfection. *Eur J Immunol* 1992;22:1483–1494.
14. Tang YW, Johnson JE, Browning PJ, Cruz-Gervis RA, Davis A, Graham BS, Brigham KL, Oates JA Jr, Loyd JE, Stecenko AA. Herpesvirus DNA is consistently detected in lungs of patients with idiopathic pulmonary fibrosis. *J Clin Microbiol* 2003;41:2633–2640.
15. Stallone G, Schena A, Infante B, Di Paolo S, Loverre A, Maggio G, Ranieri E, Gesualdo L, Schena FP, Grandaliano G. Sirolimus for Kaposi's sarcoma in renal-transplant recipients. *N Engl J Med* 2005;352:1317–1323.

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Seroprevalence of Human Herpesvirus 8 in the Vanuatu Islands in Eastern Melanesia

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Human herpesvirus 8 (HHV-8), also known as Kaposi's sarcoma (KS)-associated herpesvirus (KSHV), is a lymphotropic oncogenic gammaherpesvirus that is closely related to Epstein-Barr virus (EBV). It was first identified in the KS lesions of acquired immunodeficiency syndrome (AIDS) patients (1). The HHV-8 DNA sequence has been demonstrated in all forms of KS in patients with or without AIDS (2). It was also shown that the virus is encoded with several genes homologous to cell cycle-associated genes and cytokines, suggesting that HHV-8 infection is necessary for the development of KS (3). The worldwide geographic distribution of HHV-8 infection appears to vary greatly. HHV-8 infection is uncommon in the general population of Japan (seroprevalence: 0.2 - 1.4%) (4,5) and western countries, such as the United States (5.2%) (6) and Britain (1.7%) (7), but is more common in some Mediterranean countries, including Italy (13.8%) (8) and Greece (16.7%) (9), and is widespread in some parts of Africa (38.7% in Uganda, 37.5% in Zambia, and 41.9% in Ghana) (6) and China (46.6% in Xinjiang area) (10).

Vanuatu consists of 80 islands in Melanesia, in the South-

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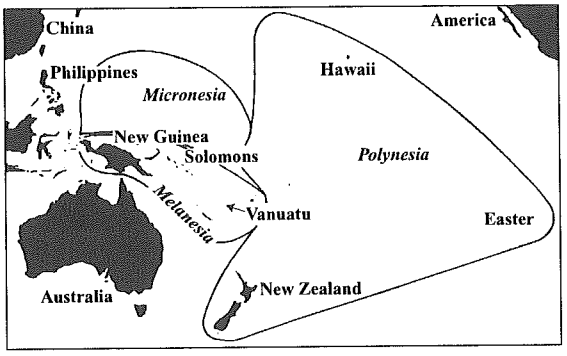

Fig. 1. Map showing the location of Vanuatu.

west Pacific (Fig. 1). No information is available about the distribution of HHV-8 infection in indigenous populations of this area. To verify whether HHV-8 infection is endemic in Vanuatu and to determine seroprevalence rates, we studied sera from residents of four representative islands in Vanuatu: Aneityum Island, Etafe Island, Pantecost Island, and Santo Island, obtained between 1998 and 1999. Subject ages (mean ± SD) were 15.6 ± 15.3 (65 males) and 20.0 ± 15.9 (75 females) on Aneityum Island, 9.77 ± 0.91 (26 males) and 9.63 ± 0.77 (24 females) on Etafe Island, 18.9 ± 20.0 (31 males) and 20.2 ± 17.1 (39 females) on Pantecost Island, and 7.32 ± 2.89 (37 males) and 6.52 ± 2.82 (33 females) on Santo Island. Informed consent was obtained from all individuals or their guardians prior to participation in the study. A finger prick blood sample was collected on chromatography filter paper (ET31CHR; Whatman Ltd., Kent, UK), then stored at −20°C until analysis. Serum was extracted from the filter papers containing drops of whole blood, as described by Evengard et al. (11). In brief, a paper punch with a diameter of about 6 mm was used for punching out discs from the filter paper. Discs were placed singly in tubes containing phosphate-buffered saline (PBS) with 0.05% Tween 20 and incubated for 2 h at room temperature. The serum was withdrawn by a pasteur pipette. All sera were heat inactivated at 56°C for 30 min before use. The presence of anti-HHV-8 antibodies was determined by a mixed-antigen enzyme-linked immuno-sorbent assay (using K 8.1, ORF 59, ORF 65, and ORF 73 proteins as mixed antigens) and an indirect immunofluorescence assay using acetone-fixed TY-1 cells (HHV-8-positive primary effusion lymphoma cell line) (5,12).

Only one positive female was found among 70 individuals (1.43%) on Santo Island, and there were no positive findings on the other three islands. This seropositivity rate was found to be much lower than that (30.4% in Bensbach and 21.3% in Port Moresby) of the population of Papua New Guinea (PNG) in the west of Melanesia (13). The islands of Vanuatu were settled less than 4,000 years ago during a rapid population expansion from Island Southeast Asia that continued into western Polynesia (14). PNG had then already been occupied for at least 35,000 years (15). There continues to be discussion about possible interaction between these two populations when the second group colonized. Diamond (16) believed that this interaction was limited and called it "Express Train". In contrast Terrell (17) believed it was multiple and called it "Entangled Bank". Our results may suggest that HHV-8 prevalence was low in the founder population of Vanuatu, which was distinct from the PNG population, or that the latter was infected with HHV-8 rather recently. No definite differences in social or behavioral activities that may have affected the prevalence of HHV-8 infection have been observed between the population of Vanuatu and PNG.

In conclusion, HHV-8 infection appears to be uncommon in the populations of Vanuatu. However, the reason for the different prevalence of seropositivity compared with that of PNG remains unclear.

REFERENCES

1. Chang, Y., Cesarman, E., Pessin, M. S., Lee, F., Culpepper, J., Knowles, D. M. and Moore, P. S. (1994): Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science, 266, 1865-1869.
2. Moore, P. S. and Chang, Y. (1995): Detection of herpesvirus-like DNA sequences in Kaposi's sarcoma in patients with and without HIV infection. N. Engl. J. Med., 332, 1181-1185.
3. Jaffe, H. W. and Pellette, P. E. (1999): Human herpesvirus 8 and Kaposi's sarcoma. N. Engl. J. Med., 340, 1912-1913.
4. Fujii, T., Taguchi, H., Katano, H., Mori, S., Nakamura, T., Nojiri, N., Nakajima, K., Tadokoro, K., Juji, T. and Iwamoto, A. (1999): Seroprevalence of human herpesvirus-8 in human immunodeficiency virus 1-positive and human immunodeficiency virus 1-negative populations in Japan. J. Med. Virol., 57, 159-162.
5. Katano, H., Iwasaki, T., Baba, N., Terai, M., Mori, S., Iwamoto, A., Kurata, T. and Sata, T. (2000): Identification of antigenic proteins encoded by human herpesvirus 8 and seroprevalence in the general population and among patients with and without Kaposi's sarcoma. J. Virol., 74, 3478-3485.
6. Ablashi, D., Chatlynne, L., Cooper, H., Thomas, D., Yadav, M., Norhanom, A. W., Chandana, A. K., Churdboonchart, V., Kurpradist, S. A. R., Patnaik, M., Liegmann, K., Masood, R., Reitz, M., Cleghorn, F., Manns, A., Levine, P. H., Rabkin, C., Biggar, R., Jensen, F., Gill, P., Jack, N., Edwards, J., Whitman, J. and Boshoff, C. (1999): Seroprevalence of human herpesvirus-8 (HHV-8) in countries of Southeast Asia compared to the USA, the Caribbean and Africa. Br. J. Cancer, 81, 893-897.
7. Simpson, G. R., Schulz, T. F., Whitby, D., Cook, P. M., Boshoff, C., Rainbow, L., Howard, M. R., Gao, S. J., Bohenzky, R. A., Simmonds, P., Lee, C., de Ruiter, A., Hatzakis, A., Tedder, R. S., Weller, I. V., Weiss, R. A. and Moore, P. S. (1996): Prevalence of Kaposi's sarcoma associated herpesvirus infection measured by antibodies to recombinant capsid protein and latent immunofluorescence antigen. Lancet, 348, 1133-1138.
8. Whitby, D., Luppi, M., Barozzi, P., Boshoff, C., Weiss, R. A. and Torelli, G. (1998): Human herpesvirus 8 seroprevalence in blood donors and patients with lymphoma from different regions of Italy. J. Nat. Cancer Inst., 90, 395-397.
9. Gazouli, M., Papaconstantinou, I., Zavos, G., Metaxa-Mariatou, V., Nasioulas, G., Boletis, J., Arapadoni-Dadioti, P., Giaslakiotis, K., Zografidis, A. and Kostakis, A. (2004): Human herpesvirus type 8 genotypes in iatrogenic, classic and AIDS-associated Kaposi's sarcoma from Greece. Anticancer Res., 24, 1597-1602.
10. Dilnur, P., Katano, H., Wang, Z. H., Osakabe, Y., Kudo, M., Sata, T. and Ebihara, Y. (2001): Classic type of Kaposi's sarcoma and human herpesvirus 8 infection in

Xinjiang, China. Pathol. Int., 51, 845-852.
11. Evengard, B. B., Linder, E. and Lundbergh, P. (1988): Standardization of a filter-paper technique for blood sampling. Ann. Trop. Med. Parasitol., 82, 295-303.
12. Katano, H. and Sata, T. (2000): Human herpesvirus 8-virology, epidemiology and related diseases. Jpn. J. Infect. Dis., 53, 137-155.
13. Rezza, G., Danaya, R. T., Wagner, T. M., Sarmati, L., Owen, I. L., Monini, P., Andreoni, M., Suligoi, B., Ensoli, B. and Pozio, E. (2001): Human herpesvirus-8 and other viral infections, Papua New Guinea. Emerg. Infect. Dis., 7, 893-895.
14. Kirch, P. V. and Hunt, T. L. (1988): Radiocarbon dates from the Mussau Islands and the Lapita colonization of the Southwest Pacific. Radiocarbon, 30, 161-169.
15. Groube, L., Chappell, J., Muke, J. and Price, D. (1986): A 40,000 year-old human occupation site at Huon Peninsula, Papua New Guinea. Nature, 324, 453-455.
16. Diamond, J. M. (1988): Express train to Polynesia. Nature, 336, 307-308.
17. Terrell, J. (1988): History as a family tree, history as an entangled bank: constructing images and interpretations of prehistory in the South Pacific. Antiquity, 62, 642-657.

MAJOR ARTICLE

Quantitative Analysis of Kaposi Sarcoma–Associated Herpesvirus (KSHV) in KSHV-Associated Diseases

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Background. Accurate numbers of copies of Kaposi sarcoma–associated herpesvirus (KSHV) and numbers of virus-infected cells in lesions caused by KSHV-associated diseases are unknown.

Methods. Quantitative polymerase chain reaction (PCR) and computerized imaging of immunohistochemical analysis were performed on pathologic sections of samples from persons with KSHV-associated diseases.

Results. Real-time PCR and semiquantitative PCR–Southern blotting demonstrated that DNA extracted from biopsy samples of KS lesions contained ~1–2 viral copies/cell. KSHV-associated lymphoma contained 10–50 viral copies/cell. Computerized-image analysis demonstrated that ~49% of cells expressed KSHV-encoded latency-associated nuclear antigen in KS biopsy samples. On the basis of results of real-time PCR and computerized-image analysis, the predicted number of viral copies was 3.2 viral copies/cell in KS lesions. Computerized-image analysis also revealed that the expression of open-reading frame (ORF)–50 protein, an immediate early protein of KSHV, was very rare in KS lesions, which implies that they were mainly composed of proliferating cells latently infected with KSHV. In multicentric Castleman disease lesions, 25% of virus-infected cells expressed ORF50 protein, which suggests the frequent lytic replication of KSHV.

Conclusions. Numbers of viral copies and of virus-positive cells vary among KSHV-associated diseases, which suggests different mechanisms of viral pathogenesis. The combination of real-time PCR and computerized-image analysis provides a useful tool for the assessment of the number of viral copies in KSHV-associated diseases.

Kaposi sarcoma–associated herpesvirus (KSHV, also called human herpesvirus [HHV]–8) has been detected by polymerase chain reaction (PCR) and immunohistochemical analysis in almost all cases of KS, regardless of HIV infection status [1–6]. Primary effusion lymphoma (PEL) is also a KSHV-associated disease [7], and KSHV-associated solid lymphoma has been reported to be a variant of PEL that forms solid tumors [8, 9]. Some, but not all, cases of multicentric Castleman disease (MCD) are also KSHV positive [10, 11].

Similarly to other herpesviruses, KSHV has 2 phases of infection: lytic and latent [1, 12]. During the lytic phase, KSHV replicates in infected cells, which results in cell lysis. However, the virus does not replicate in latently infected cells, although they harbor viral episomes and express several KSHV-encoded latency-associated proteins, such as latency-associated nuclear antigen (LANA) and LANA2 [1, 13, 14]. Although latent infection predominates in KSHV-infected PEL cell lines, phorbol ester stimulation can induce lytic infection in these cells [12]. Gene expression during the lytic phase is classified into immediate early, early, and late expression [12]. Open-reading frame (ORF)–50 was identified as an immediate early protein that was required for the lytic replication of KSHV [12, 15, 16]. Immunohistochemical studies demonstrated that KS cells expressed LANA; however, the expression of lytic proteins was very rare in KS lesions, which suggests that latent infection predominates in KS cells [5, 6, 17]. Lytic proteins are expressed by some B cells in the mantle zone of MCD, which suggests that lytic replication frequently occurs in MCD lesions [6, 17].

Numbers of KSHV copies in KSHV-associated diseases have been investigated by several groups [18–26]. An early study that used conventional PCR and South-

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Table 1. No. of copies of Kaposi sarcoma–associated herpesvirus (KSHV), determined by real-time polymerase chain reaction (PCR) and PCR–Southern blot.

Patient (disease)	Real-time PCR		PCR–Southern blot	
	Mean copies/cell	Average (SD)	No.	Mean copies/cell
1 (PEL)	82.01	82.01	1	50
2 (KSHV-associated solid lymphoma)	14.26	10.75 (4.97)	...	...
3 (KSHV-associated solid lymphoma)	7.23		2	2
4 (AIDS-associated patch KS)	0.09	0.13 (0.11)	...	...
5 (AIDS-associated plaque KS)	0.04		...	...
6 (AIDS-associated patch KS)	0.25		...	...
7 (AIDS-associated nodular KS)	0.67	1.72 (1.51)	3	2
8 (AIDS-associated nodular KS)	1.02		4	0.4
9 (AIDS-associated nodular KS)	0.53		...	...
10 (AIDS-associated nodular KS)	3.13		5	0.4
11 (AIDS-associated nodular KS)	3.93		6	2
12 (AIDS-associated nodular KS)	0.14		...	...
13 (AIDS-associated nodular KS)	3.41		...	...
14 (AIDS-associated nodular KS)	0.91		...	...
15 (classic patch KS)	0.16	2.60 (2.70)	...	...
16 (classic patch KS)	5.50		7	0.08
17 (classic patch KS)	2.13		...	...
18 (classic nodular KS)	0.00	1.64 (2.63)	...	...
19 (classic nodular KS)	4.67		8	2
20 (classic nodular KS)	0.25		...	...
21 (MCD)	0.27	0.27	9	1
22 (control, BCBL-1)	78.01	87.08 (12.83)	...	...
23 (control, TY-1)	96.15		...	...

NOTE. MCD, multicentric Castleman disease; PEL, primary effusion lymphoma.

ern blot analysis demonstrated that a PEL cell contained ~50 copies of KSHV genome, whereas the KSHV genome was detected at a rate of ~1 viral copy/cell in KS lesions [18]. Recently, real-time PCR was used to detect KSHV, and several reports have described numbers of viral copies in peripheral blood mononuclear cells (PBMCs) derived from patients with KS [19–26]. Studies using real-time PCR have demonstrated that numbers of viral copies in PBMCs varied among diseases and disease stages [21–24]. However, to our knowledge, there has been no report that has compared numbers of viral copies in KS, PEL, or MCD lesions using real-time PCR. Therefore, the aim of the present study was to determine numbers of viral copies in lesions of KSHV-associated diseases using pathologic samples. Pathologic tissue samples—such as biopsy samples—frequently contain both virus-infected cells and noninfected cells. Thus, results from real-time PCR do not solely represent numbers of viral copies in virus-infected cells. To solve this problem, we combined real-time PCR with computerized-image analysis that allowed an assessment of numbers of virus-infected cells in immunostained sections. Using these methods, we identified numbers of both viral copies and virus-infected cells in appropriate sections. Thus, we obtained relatively accurate numbers of viral copies in histologic sections of KSHV-associated disease lesions.

PATIENTS, MATERIALS, AND METHODS

Patients and samples. All patients provided informed consent for specimens to be obtained. For PCR analysis, 21 clinical samples were collected (table 1). For immunohistochemical analysis, 27 histopathologic specimens from KSHV-infected patients (table 2) were collected from 1995 to 2004. All KS specimens were categorized into groups according to the clinical stage of KS (patch, plaque, or nodular) on the basis of clinical and histologic data. DNA extracted from 2 KSHV-positive cell lines (BCBL-1 and TY-1), a KSHV-negative Epstein-Barr virus–positive Burkitt lymphoma cell line (Raji), and human umbilical vascular endothelial cells (HUVECs) was used as a control for PCR studies [27, 28].

Preparation of DNA. DNA was extracted from fresh-frozen clinical materials or from formalin-fixed, paraffin-embedded tissue samples from 21 biopsies of KSHV-infected patients (table 1). For fresh-frozen materials, the DNeasy Tissue Kit (Qiagen) was used in accordance with the manufacturer's instructions. For the isolation of DNA from formalin-fixed, paraffin-embedded biopsy samples, 5-μm sections (n = 3–4) were deparaffinized with xylene, digested with proteinase K, and processed for phenol/chloroform extraction with sodium acetate/ethanol precipitation.

Table 2. Percentage of latency-associated nuclear antigen (LANA)– or open-reading frame (ORF)–50–positive cells in immunohistochemical analysis (IHC) with computerized-image analysis.

IHC, samples	Cases, no.	Minimum/maximum (average), %
LANA		
AIDS-associated patch/plaque KS	11	12/95 (48)
AIDS-associated nodular KS	4	28/72 (57)
AIDS-associated KS involving LN	2	28/31 (30)
AIDS-associated KS involving GI tract	2	6/7 (7)
Classic patch/plaque KS	2	25/47 (36)
MCD	4	5/21 (8)
KSHV-associated solid lymphoma	2	79/80 (80)
ORF50		
AIDS-associated patch/plaque KS	5	0
AIDS-associated nodular KS	4	0
Classic KS, patch/plaque	2	0
MCD	5	1/3 (2)
KSHV-associated solid lymphoma	2	0/5 (3)

NOTE. GI, gastrointestinal; KSHV, Kaposi sarcoma–associated herpesvirus; LN, lymph node; MCD, multicentric Castleman disease.

Real-time quantitative PCR. Amounts of KSHV DNA were determined by quantitative real-time (TaqMan) PCR using the ABI Prism 7900HT sequence detection system (Applied Biosystems), which amplified segments within the KSHV LANA gene (one of the latent proteins coded on ORF73). Sequences and usage parameters of primers and probes have been described elsewhere [19]. We also determined the amounts of human genomic DNA that were present in DNA extracted from each specimen. Primers and probes for the gene encoding human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed, using Primer Express software (Applied Biosystems), to obtain a 104-bp amplicon. Forward and reverse primer sequences were 5′-GCTCCCTCTTTCTTTGCAGCAAT-3′ and 5′-TACCATGAGTCCTTCCACGATAC-3′, respectively. The fluorogenic TaqMan probe was 5′-(FAM)TCCTGCACCACCAACTGCTTAGCACC(TAMRA)-3′. PCR amplification was performed in 25-μL reaction mixtures using QuantiTect probe PCR Master Mix (Qiagen), 0.4 μmol/L each primer, 0.2 μmol/L TaqMan probe, and 2 μL of isolated DNA. PCR conditions were 15 min at 95°C, followed by 45 cycles of 15 s at 94°C and 1 min at 60°C. Quantitative results were obtained by generating standard curves for pGEM-T plasmids (Promega) that contained each KSHV (ORF73) and cellular target (GAPDH) amplicon. The number of viral copies per cell was calculated by dividing the number of ORF73 copies by one-half of the number of GAPDH copies, because there are 2 alleles of GAPDH in each cell.

Detection of KSHV by semiquantitative PCR–Southern blotting analysis. Semiquantitative PCR–Southern blotting was performed to determine copy numbers of KSHV in DNA samples after PCR amplification of $KS330_{233}$ [11, 29]. The β-globin gene was simultaneously amplified as described elsewhere [11]. For PCR–Southern blot analysis, digoxygenin (DIG)–labeled $KS330_{233}$ and a 110-bp DNA fragment of the β-globin gene, whose sequences were confirmed by sequencing, were used as probes [11]. Procedures for Southern blot analysis and the detection of DIG were those of the manufacturer (Roche Diagnostic). Copy numbers of KSHV were determined by comparing results for $KS330_{233}$ and β-globin, on the basis of the information that each cell has 2 copies of the β-globin genome.

Histologic and immunohistochemical analyses. Serial sections were prepared and stained with hematoxylin-eosin (HE) for light microscopy or were subjected to immunohistochemical staining with antiserum against LANA or ORF50 proteins (lytic antigens) [4, 30]. Immunohistochemical staining was visualized using the avidin-streptavidin-peroxidase method with 3-3′diaminobenzidine as the chromogen, as described elsewhere [4, 30]. For double immunohistochemical staining of vascular endothelial cell growth factor receptor–3 (VEGFR-3) and LANA, an anti-LANA rabbit polyclonal antibody, a peroxidase-conjugated anti-rabbit goat antibody (Envision; Dako Cytomation), and aminoethylcarbazole (AEC; Nichirei) were used as the primary antibody, secondary antibody, and chromogen, respectively. After the color development of AEC, slides were washed with PBS and processed for VEGFR-3 staining. Anti–VEGFR-3 mouse monoclonal antibody (D2-40; Nichirei) and alkaline phosphatase–conjugated anti–mouse IgG goat antibody (Envision; Dako Cytomation) were used, and a positive signal was detected with Fast Blue BB (Sigma-Aldrich). Slides were mounted with a glycerol-based mounting solution.