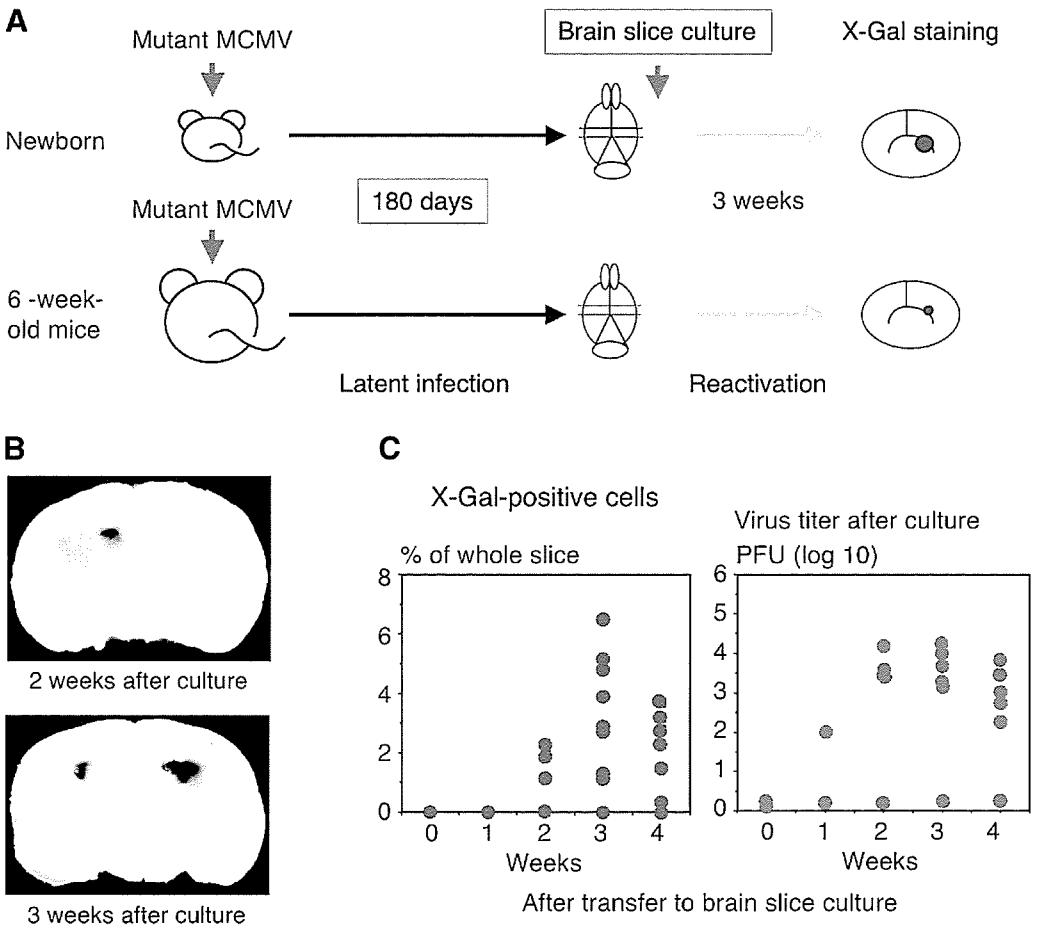

Figure 7. Reactivation of latent MCMV infection in mouse brain cells after transfer to brain slice culture. (A) For neonatal infection, BALB/c mice were injected intracerebrally with mutant MCMV (RM461) (5×10^2 PFU) within 24 h after birth. For young adult infection, 6-week-old mice were injected with RM461 (5×10^4 PFU). These two groups of mice were maintained for more than 6 months. For the uninfected control groups, neonatal and young adult mice were injected with MEM and maintained for the same period. Then, the brains were taken out and transferred to brain slice cultures until 4 weeks. (B) The slices were stained with X-Gal staining for β-galactosidase expression. (C) The proportion of stained viral infected cells in whole slices (left) and viral titers in slices (right) were measured every week

infection in the brains occurs not only in congenital infected individuals but also in immunocompromised adults. Although the mechanism of reactivation in the brain slice culture is not known, the basic mechanism may be similar to explant reactivation, in which latent cells are reactivated by co-culture of cell suspensions with susceptible fibroblasts [38,115,116]. It is not known whether there is a correlation between the susceptibility of neural progenitor cells to CMV infection and their tendency to be a preferential site of CMV latency. At this moment, it is difficult to distinguish neural stem cells from neural progenitor cells using neural markers. Neural progenitor cells are transient cells in the process of differentiation from neural stem cell to mature cells. It is not possible that these transient cells might be sites of latency. It is hypothesised that latent CMV infection may occur in neural stem cells. Once latently infected stem cells are committed to differentiate to progenitor cells by some kind of stimulation, latent virus is reactivated in these cells, in which a lot of factors for viral gene expression are induced, eventually leading to permissive infection. It is possible that neural stem cells might have strong suppressors for expression of the IE promoter [117] or might be deficient in factors necessary for activation of the promoter. At

present, it is difficult to demonstrate latent virus genome directly in neural stem cells, presumably because of the rarity of these cells and the difficulty in isolating these cells without induction of reactivation. Interestingly, our hypothesis that neural stem/progenitor cells may be the sites of latent infection in the brain may be related to the observation that hematopoietic progenitor cells are similarly sites of latent infection [111,118]. It was reported that reactivation occurred when these cells were stimulated to differentiate by certain factors, such as allogeneic stimulation [110,119].

CONCLUSION: IMPLICATION OF NEUROPATHOGENESIS IN CMV INFECTION

Early embryos, including ES cells, are not susceptible to MCMV. In contrast, embryonic brains are highly susceptible to MCMV infection (Figure 8, left upper). The cells responsible for the susceptibility are neural stem/progenitor cells in the ventricular walls. During differentiation, acute infection tends to occur in immature glial cells, consequently the lytic infection may cause disorders of brain development (Figure 8, right upper). CMV also infects neuronal cells, although lytic infection is difficult to observe. The possibility of persistent MCMV infection in cerebral neurons is indicated by the following specific neuronal features of MCMV infection: prolonged expression of the viral antigen and very low production of infectious virus, evasion of innate immune responses mediated by natural killer cells and macrophages, and decrease in the susceptibility of MCMV-infected neurons to excitotoxic cell death, in addition to neuron-specific activation of the MCMV e1-promoter. The possible persistent infection in neurons may influence neuronal functions, resulting in neuronal disorders (Figure 8, right lower). Human infants

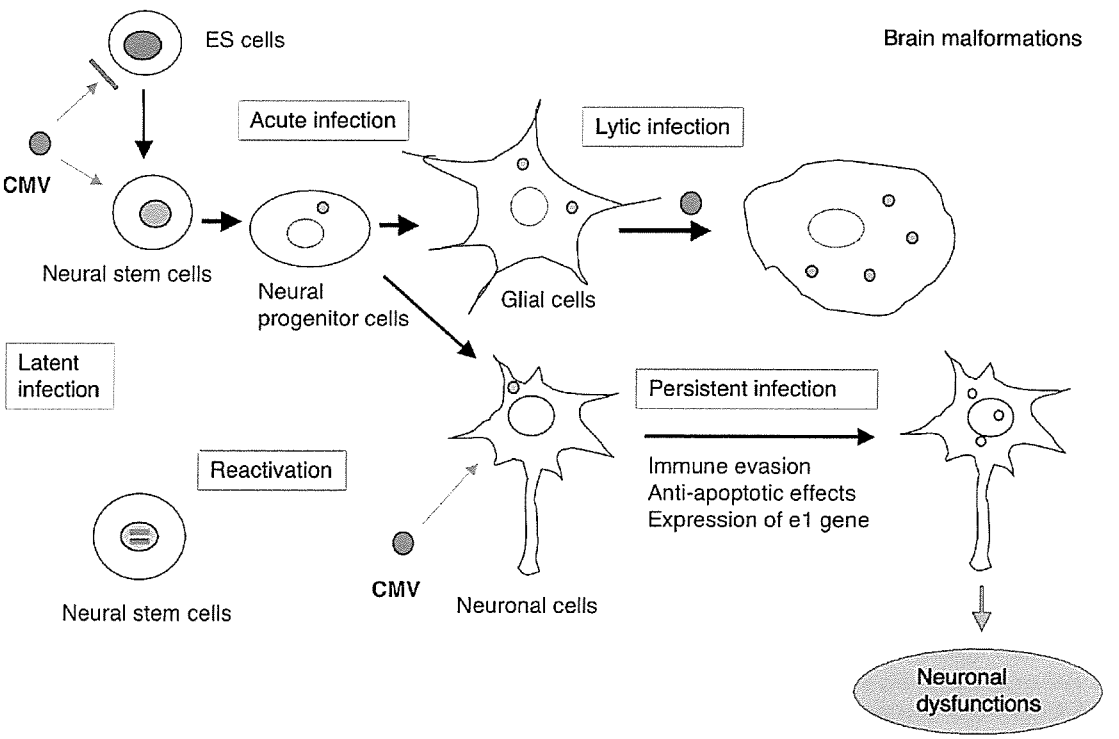

Figure 8. Schematic illustration of hypothetical neuropathogenesis of the brain disorders caused by CMV infection in the developing brains. In the early embryonic stage, ES cells are not susceptible to MCMV, but neural stem/progenitor cells in the embryonic brains are highly susceptible to CMV infection. In acute infection, immature glial cells undergo lytic infection, resulting in brain malformation. Infection of neuronal cells with CMV tends become persistent by evasion of innate immunity, anti-apoptotic effects, and neuron-specific activation of the e1 gene, causing neuronal dysfunctions. On the other hand, infection of embryonic brains with CMV may become latent in neural stem cells. After a long time period, the latent infection may be reactivated and become lytic in neural progenitor cells or glial cells

with symptomatic congenital CMV infection at birth frequently develop severe developmental disorders such as microcephaly, while infants with subclinical congenital infection subsequently have functional brain disorders such as mental retardation. The different infectious dynamics in the developing mouse brains may offer explanations for the pathogenesis of two conditions of congenital CMV infection in humans. On the other hand, CMV infection in the developing brain may become latent in neural stem cells. After a long time period, the latent infection may be reactivated by some stimulation such as immune suppression and lytic infection occurs in neural progenitor cells or glial cells (Figure 8, left lower), causing ventricular encephalitis or neuronal dysfunction by affecting the expression of neuronal genes such as NMDA receptor.

In perspective, it may be one of the most attractive subjects to study the mechanisms by which latent viruses in the brain affect the behavior and thought processes of human beings.

ACKNOWLEDGEMENTS

We thank Dr E. S. Mocarski, Department of Microbiology and Immunology, Stanford University School of Medicine, for providing the recombinant MCMV (RM461). We also thank Dr Richard D. Palmiter, University of Washington, Seattle, WA, for providing the pnlacF vector. This work was supported in part the grant (15390126) from the Ministry of Education, Culture, Science, and Technology, and the grant (16140201) from the Ministry of Health, Labor and Welfare, in Japan.

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Short Communication

Intervirology

Intervirology 2005;48:201–206
DOI: 10.1159/000081749

Received: February 9, 2004
Accepted after revision: May 24, 2004

Analysis of Human Cytomegalovirus UL144 Variability in Low-Passage Clinical Isolates in Japan

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Key Words
Cytomegalovirus · Genotypes · UL144 · gB · Congenital infection

Abstract
To explore a possible role for viral genes as determinants of virulence, portions of the UL144 tumor necrosis factor-like receptor gene and the UL55 envelope glycoprotein B gene from 42 patients with congenital human cytomegalovirus (HCMV) infection or other diseases were sequenced. Of the 42 patients, 16 (38%) had UL144 group 1 [group 1A, 15 of 16 (94%); group 1B, 1 of 16 (6%); group 1C, 0 of 16 (0%)], 5 patients (12%) had UL144 group 2, and 21 patients (50%) had UL144 group 3. Although group 1C was not found in Japan strains (0%), it was found in USA strains (22%). Other HCMV polymorphisms should be further evaluated for their potential relevance to neonatal infection, and acquired immunodeficiency syndrome-associated HCMV diseases.

Introduction

Human cytomegalovirus (HCMV) is a widespread human pathogen that has a minor clinical impact on healthy individuals, but causes various organ diseases in immunosuppressed patients and neural damage in fetuses infected in utero [1], and presents as a lifelong latent infection. However, latently infected HCMV is frequently activated in immunocompromised individuals such as patients with AIDS or organ and bone marrow transplants, thereby causing severe morbidity and eventual mortality [2]. However, the mechanisms of the viral pathogenesis have not yet been well understood.

The diversity of organs and cell types infected by HCMV in vivo has led to the hypothesis that HCMV disease and tissue tropism may be related to sequence variations among strains [3, 4]. Strain-dependent biological behavior depending on genetic polymorphisms has been examined most frequently in the region encoding UL55 envelope glycoprotein B (gB) [5], which was found to have a high level of sequence variability among clinical isolates [4, 6, 7]. gB is essential for HCMV infection both in vivo and in vitro. It is a target of neutralizing antibodies and mediates direct cell-to-cell transmission as well as virus entry from the extracellular environment [8]. A number of studies have attempted to reveal correlations of gB types

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with specific disease manifestations or sites of infection [4, 5, 7, 9]. However, a definitive association has not been established between gB types and HCMV diseases, leaving open the possibility that other variant HCMV-encoded products may play a role in viral pathogenesis, possibly in combination with gB.

HCMV pathogenesis has been investigated using well-characterized laboratory strains such as AD169 and Towne. These strains were derived from clinical isolates [10, 11], but have been extensively passaged in in vitro cell culture. A lower passage clinical strain, Toledo, was found to have a set of nineteen new open reading frames (ORFs), which were not present in the laboratory strains, but were present in several other low-passage clinical isolates [12]. Thus, the products encoded by these ORFs cannot be essential for HCMV replication in cell culture. However, the fact that the clinical isolates still retain these ORFs suggests that the predicted gene products may be essential for viral infection in vivo.

The possibility that these new ORFs may provide genetic markers for HCMV pathogenesis prompted us to investigate these genes in HCMV clinical isolates. Lurain et al. [13] analyzed the sequence variability of one of these ORFs, UL144, which encodes a protein homologous to the herpes simplex virus entry mediator [14]. Moreover, the UL144 ORF is a prime candidate for a pathogenesis marker because it is a member of the tumor necrosis factor receptor superfamily [15, 16]. Tumor necrosis factor receptor superfamily members play several roles in natural defense and adaptive immune responses that are critical for defense against viral pathogens such as HCMV [17].

In this report, we describe the significant strain-specific sequence variability for UL144 and gB in a large group of clinical isolates in Japan.

Materials and Methods

Cells and Specimens

All virus strains were propagated in human embryonic lung fibroblasts (HEL) in Eagle's minimum essential medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (Z. L. Bocknek Lab, Ontario, Canada), kanamycin (60 μg/ml), and 0.12% $NaHCO_3$. Forty-two HCMV clinical strains were isolated from 26 children with congenital infections (16 children in Miyazaki Prefecture, 8 in Kanazawa Prefecture, and 2 in Kagoshima Prefecture) and 16 adults recruited after organ transplantation or with other diseases (4 adults in Miyazaki Prefecture, 7 in Kanazawa Prefecture, and 5 in Kagoshima Prefecture).

Virus Isolation

Urine and blood samples and a throat swab were collected from the patients for HCMV isolation between 1989 and 1999. An informed consent was obtained from each patient prior to the sample collection. HCMV clinical strains were isolated by inoculating the fresh samples onto confluent monolayers of human embryonic lung fibroblast in 24-well plates (IWAKI, Scitech Div, Asahi Techno Glass, Chiba, Japan). Viral isolation was confirmed by detecting the specific cytopathic effect characteristic of HCMV. All the clinical HCMV strains were stored in liquid nitrogen as infected cells until use.

PCR Amplification

The clinical isolates were passaged in vitro less than five times prior to DNA extraction. Viral DNA was prepared from the infected cells by Hirt's method [18]. DNA samples were diluted in sterile distilled water to optimal concentrations for use as templates and stored at 4° until use. Two regions of the HCMV genome, gB and UL144, were analyzed using individual PCR primers for each. A 410-bp fragment encompassing the proteolytic cleavage site (nucleotides 1072–1482) of the HCMV gB gene (EMBL Accession No. X04606) was amplified with the following primers: forward, 5′-TCCGAAGCCGAAGACTCGTA-3′ and reverse, 5′-GATGTAACCGCGCAACGTGT-3′. The UL144 region was amplified using the UL144-B primer set described by Lurain et al. [13]. This primer pair, forward 5′-TCGTATTACAAACCGCGGAGAGGAT-3′ and reverse 5′-ACTCAGACACGGTTCCGTAA-3′, yield a 737-bp product corresponding to the complete coding sequence of UL144. Amplification was carried out with a programmable temperature control system, PC-700 (Astec Co. Fukuoka, Japan). The conditions for amplification with all primer sets were 94° for 5 min followed by 35 cycles of 94° for 1 min, 55° for 1 min, and 72° for 1 min. The 35 cycles were followed by a single extension at 72° for 7 min.

The PCR products were subjected to electrophoresis in 2% agarose gels. For the DNA sequencing, the amplified DNA was purified from the agarose gels using a QIAquik PCR purification kit (QIAGEN, Valencia, Calif., USA) according to the manufacturer's instructions.

DNA Sequencing

The purified PCR products were sequenced directly using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems Japan, Tokyo). Sequencing reactions were performed at 96° for 10 s, 50° for 5 s, and 60° for 4 min for a total of 30 cycles. The sequencing products were analyzed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Amino acid sequences were derived from the UL144 DNA sequence data using a web-based translation program (URL: www.ebi.ac.uk/translate).

Phylogenetic Analysis

Sequence alignment was accomplished using the web-based Clustal W alignment programs (www/ebi.ac.uk/clustalW). Unrooted phylogenetic trees were constructed for the UL144 DNA sequence data using the above website (www/ebi.ac.uk/clustalW). The final tree was rendered with Tree View [19]. Amino acid sequences were aligned with a web-based translation program (URL: www.ebi.ac.uk/translate). Protein homolog of the predicted UL144 product were identified by a BLAST search of protein databases [20].

Nucleotide Sequence Accession Numbers

The UL144 sequences of the clinical strains have been assigned GenBank Accession No. AF084976–AF085005 and AF179196–AF179210.

Results and Discussion

Profiles of Clinical Strains

Cha et al. [12] suggested that the nineteen newly described ORFs could be responsible for HCMV virulence and tissue tropism, based on the Toledo strain and only five additional clinical isolates. However, it is necessary to demonstrate that these ORFs are consistently present in a large number of strains, such as our 42 clinical HCMV isolates (table 1). To confirm whether Japanese clinical isolates have nineteen ORFs as reported by Cha et al. [12], we examined UL144 gene variability of 42 clinical HCMV isolates in Japan.

A total of 42 clinical HCMV strains were collected from three different prefectures, Miyazaki (M series), Kagoshima (KG series) and Kanazawa (KS series), over the past 10 years. The clinical symptoms or clinical conditions were as follows: 4 patients with CMV retinitis, 2 with CMV skin disease, 4 with CMV pneumonitis, 3 with a kidney transplant, 3 with cancer, one with Hirschsprung disease, one pregnant woman, and 24 with congenital infection. The sample source of each strain is also listed in table 1. Since both UL55 (gB) and UL144 genes are polymorphic among HCMV strains [6, 13, 21–23], both of these genes were sequenced and compared among the 42 clinical HCMV isolates in Japan.

UL144 Variability

All the HCMV isolates were successfully amplified with the UL144B primer set, sequenced, and analyzed phylogenetically (fig. 1, table 2). All the HCMV strains could be categorized according to the schema of Lurain et al. [13].

This schema segregates the strains into five major groups as follows: group 1, 16 of 42 (38.1%), group 2, 5 of 42 (11.9%); and group 3, 21 of 42 (50%). The group 1 strains were further divided into groups 1A, 15 of 16 (93.7%), 1B, 1 of 16 (6.3%) and 1C, 0 of 16 (0%), with 1A being most closely related to the Toledo HCMV strain. The nucleotide polymorphisms conferred substantial amino acid substitutions when compared with Toledo (fig. 2). Only five of the current strains had sequences similar to those of the Toledo strain.

We also compared the nucleotide sequence data and clade classification according to the geographic sources of the HCMV strains. Although modest differences, only for the inside of group 1 strains, in the percentages of isolates conforming to each of the five UL144 clades were observed, the overall distribution of the UL144 grouping differed substantially between the USA and Japan strains (table 2).

Table 1. HCMV clinical strains

No.	Strain	Clinical history	Source	UL144 group	gB type
1	M8	Skin disease	Urine	1A	1
2	M9	Skin disease	Urine	1A	1
3	M14	Congenital infection	Urine	1A	1
4	M15	Congenital infection	Urine	1A	3
5	M16	Congenital infection	Urine	1A	1
6	M18	Congenital infection	Urine	1A	1
7	M19	Retinitis	Urine	1A	3
8	KG1	Congenital infection	Urine	1A	1
9	KG2	Congenital infection	Urine	1A	1
10	KG4	Hirschsprung disease	Urine	1A	3
11	KG5	Pneumonitis	Throat swab	1A	3
12	KG7	Pneumonitis	Blood	1A	1
13	KS2	Carcinoma (fever)	Saliva	1A	1
14	KS4	Leukemia (fever)	Urine	1A	1
15	KS8	Congenital infection	Urine	1A	1
16	KS12	Kidney transplant	Urine	1B	1
17	M4	Congenital infection	Urine	2	3
18	M12	Congenital infection	Urine	2	3
19	KS1	Retinitis	Tears	2	1
20	KS9	Congenital infection	Urine	2	1
21	KS10	Congenital infection	Urine	2	1
22	M1	Congenital infection	Urine	3	3
23	M2	Congenital infection	Urine	3	1
24	M3	Congenital infection	Urine	3	1
25	M5	Congenital infection	Urine	3	1
26	M6	Congenital infection	Urine	3	1
27	M7	Congenital infection	Urine	3	1
28	M10	Congenital infection	Urine	3	1
29	M11	Congenital infection	Urine	3	1
30	M13	Congenital infection	Urine	3	1
31	M17	Congenital infection	Urine	3	1
32	M20	Retinitis-encephalitis	Urine	3	1
33	KG3	Pneumonitis	Urine	3	3
34	KG6	Pneumonitis	Throat swab	3	1
35	KS3	Congenital infection	Urine	3	1
36	KS5	Retinitis	Tears	3	1
37	KS11	Kidney transplant	Urine	3	1
38	KS12	Congenital infection	Urine	3	1
39	KS14	Congenital infection	Urine	3	3
40	KS15	Kidney transplant	Urine	3	1
41	KS16	Leukemia (fever)	Urine	3	1
42	KS17	Pregnant women	Urine	3	1

UL55 Variability

The sequence analysis of UL55 (gB) in the 42 samples obtained from 24 congenital CMV infections and other patients revealed no statistical difference between the two groups for the presence of the gB gene (tables 1, 3). Sequence analysis of UL55 (gB) in the 42 samples revealed that 33 of 42 (79%) were gB type 1 (Towne type)

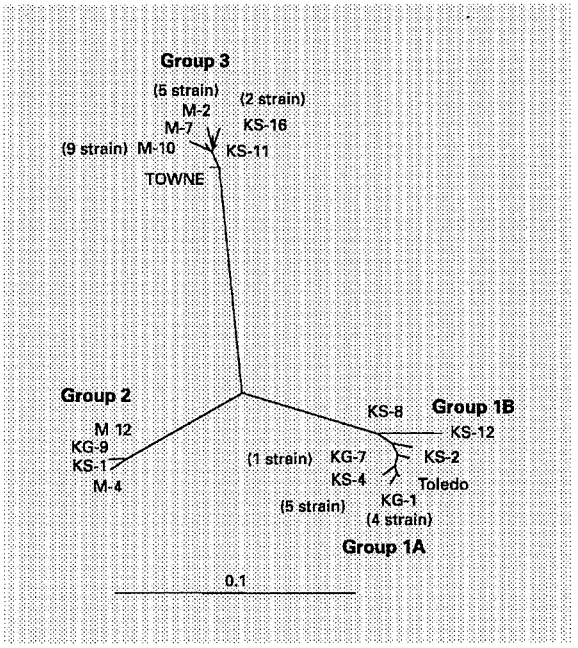


Fig. 1. Results of the phylogenetic analysis of the UL144 DNA sequences. An unrooted phylogenetic dendrogram showing the relationships of the HCMV strains according to the sequence polymorphisms of the UL144 gene. Group designations conform to the schema proposed by Lurain et al. [13]. Numbers indicate individual strains. M = Miyazaki strains; KS = Kanazawa strains; KG = Kagoshima strains.

Table 2. Comparison of UL144 typing of clinical CMV isolates

	Group 1	Group 2	Group 3	Total
USA [12]	18 (40%) 1A 10 (56%) 1B 4 (22%) 1C 4 (22%)	4 (9%)	23 (51%)	45
Japan	16 (38%) 1A 15 (94%) 1B 1 (6%) 1C 0 (0%)	5 (12%)	21 (50%)	42

USA = Chicago, Maywood; Japan = Kanazawa, Miyazaki, Kagoshima.

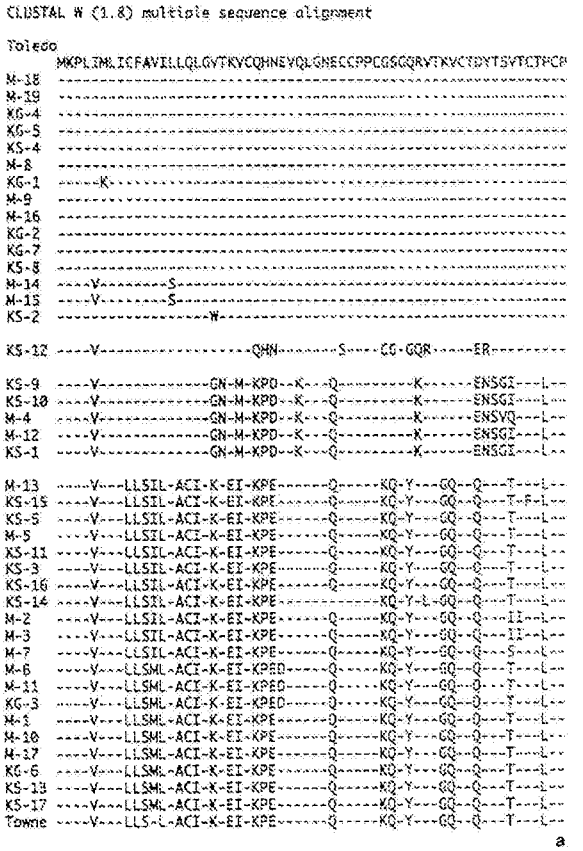
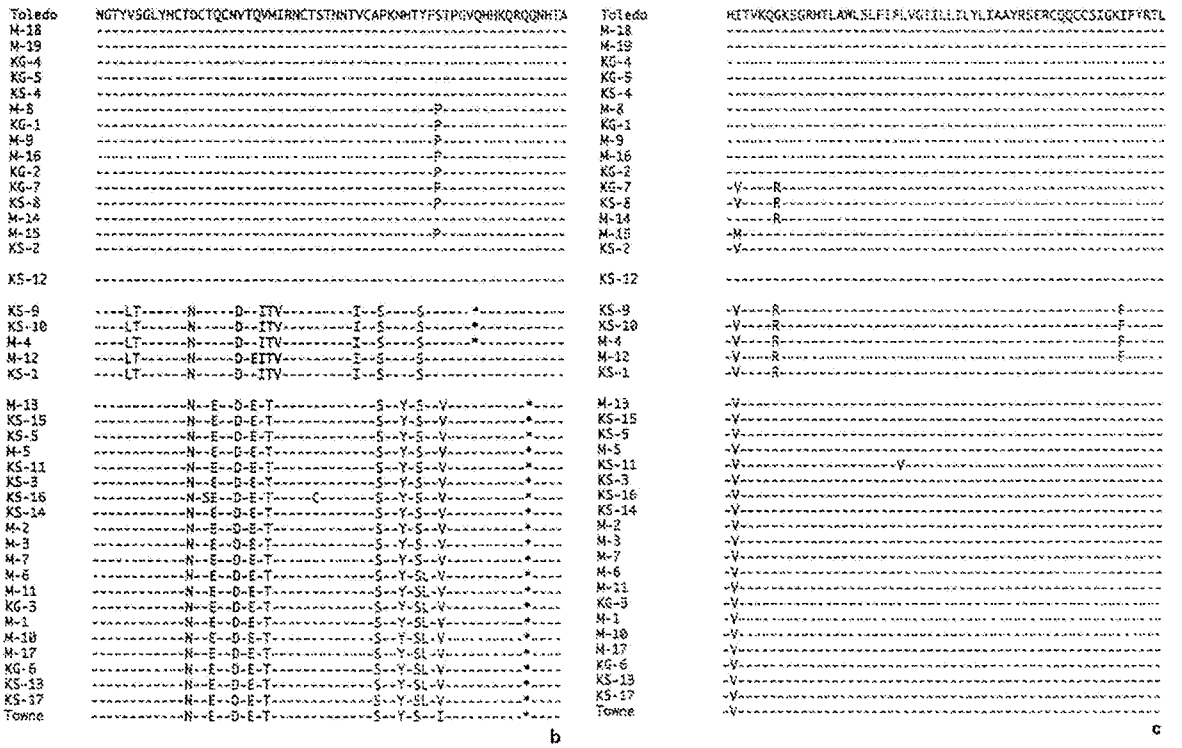


Fig. 2a–c. Alignment of the amino acid sequences of the 42 clinical strains with the Toledo and Towne sequences as references. Numbers above the sequences represent amino acid residues. Hyphens indicate identity. Stars indicate deletions. Strains are listed according to sequence groups determined by phylogenetic analysis of the DNA sequences.

Table 3. Comparison of gB typing of clinical CMV isolates

	Type 1	Type 2	Type 3	Type 4	Total
Wada et al. [24]	4 (20%)	15 (75%)	1 (5%)	0	20
Our data	33 (79%)	0	9 (21%)	0	42
Congenital	19 (58%)	5 (56%)			
Other	14 (42%)	4 (44%)			



and 9 (21%) were type 3 (table 3). None of the samples were types 2 or 4. In addition, no specific correlation of UL144 with the gB type was found statistically.

The Toledo and Davis laboratory-adapted strains showed gB type 3 and type 1 genotypes, respectively. On the other hand, Wada et al. [24] reported that, among 20 Japanese bone marrow transplant recipients, 20% had gB type 1, 75% had type 2, and 5% had type 3. This discrepancy is possibly due to a regional difference in the source of the patients who were analyzed.

One gene variation may contribute to differences in HCMV pathogenicity depending on the function of its product. In the case of HCMV, a gB polymorphism has been correlated with cell tropism and virulence [25]. In conclusion, precise definition of the genotypes of single viral proteins present in clinical strains, their immunological properties and their role in virus cell tropism might be extremely useful for understanding the virus pathogenicity. The comparison between UL144 and gB variability shown in this study suggests that gB and UL144 sequence clusters are not phylogenetically linked, as has been reported recently for the UL144 and UL4 genotypes [9, 13, 22, 26].

There was no correlation between the UL144 or the UL55 (gB) type and the mortality from HCMV infection in our study with only a small number of patients. However, a large-scale study with multivariant analysis is necessary to determine whether or not the virulence of each UL144 or gB type is different.

Acknowledgments

We would like to thank N. Nakanishi and Y. Minakami for their technical assistance. This work was supported in part by grants-in-aid from the Ministry of Education, Culture and Science in Japan, by the research fund of Institute of Kampo Medicine (Japan), and by grants-in-aid of Kagoshima University.

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Higher prevalence of human herpesvirus 8 DNA sequence and specific IgG antibodies in patients with pemphigus in China

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Background: Environmental factors, including virus infection, may play a role in the onset and/or development of pemphigus. However, it is controversial whether human herpesvirus (HHV)-8 is involved in pathogenesis of pemphigus.

Objective: The possible association of pemphigus with HHV-8 was investigated.

Methods: A total of 36 lesional skin and 13 peripheral blood mononuclear cell specimens from 58 patients with pemphigus, and 18 normal skin and 230 peripheral blood mononuclear cell specimens from healthy individuals, were tested for HHV-8 DNA sequence by a nested polymerase chain reaction assay. In all, 29 sera from the patients and 109 sera from healthy individuals were tested for HHV-8-specific IgG antibodies by enzyme-linked immunosorbent assays using HHV-8-specific oligopeptides as antigens.

Results: Prevalence of both HHV-8 DNA sequence (36.1% and 30.8% in lesional skin and in peripheral blood mononuclear cells, respectively) and HHV-8-specific IgG antibodies (34.5%) for patients with pemphigus was statistically higher than that of control subjects (<8% in both assays). There was no significant difference in HHV-8 prevalence among different types of pemphigus.

Conclusion: HHV-8 infection might be a contributing factor in the development of pemphigus. (J Am Acad Dermatol 2005;52:460-7.)

Pemphigus is an autoimmune blistering skin disease mediated by autoantibodies against desmoglein 1 and 3. Genetic and environmental factors may play a role in its pathogenesis.[1] As genetic factors, HLA antigen class II, HLA-DR4, HLA-DR14, HLA-DQ1, and HLA-DQ3 were reported to be strongly associated with pemphigus vulgaris (PV),[2-7] and HLA-DR14 and HLA-DQ1 with pemphigus foliaceus (PF),[8,9] whereas HLA-DR17 contributed to resistance against PV and PF.[6,10] Moreover, HLA antigen alleles susceptible to pemphigus were different among ethnic groups.[2-15] Environmental factors, including thiol drugs, burn, UV and radiographs, virus infection, neoplasm, hormones, pregnancy, and nutritional and psychologic conditions, were also reported to trigger pemphigus.[16,17]

In 1974, Krain[18] first reported a pemphigus case accompanied with herpes simplex virus infection.

Abbreviations used:

ELISA:	enzyme-linked immunosorbent assay
HHV:	human herpesvirus
KS:	Kaposi's sarcoma
ORF:	open reading frame
PBMC:	peripheral blood mononuclear cell
PCR:	polymerase chain reaction
PF:	pemphigus foliaceus
PV:	pemphigus vulgaris

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Supported by the Biological Research Fund of China Medical University.
Conflicts of interest: None identified.
Accepted for publication October 15, 2004.

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0190-9622/$30.00

doi:10.1016/j.jaad.2004.10.882

Thereafter, several reports described pemphigus cases that were associated with infection of other herpesviruses, such as varicella-zoster virus, Epstein-Barr virus, cytomegalovirus, and human herpesvirus (HHV)-6.[19-28] Some of these associations were confirmed by detection of viral DNA sequence in lesional skin biopsy specimens and/or in peripheral blood mononuclear cell (PBMC).[26-28] However, because herpesvirus infection was demonstrated only in a subset of patients with pemphigus, further studies should be performed to clarify herpesvirus involvement in the pathogenesis of pemphigus.

Historically, a high incidence of Kaposi's sarcoma (KS) and Castleman's disease was reported for patients with pemphigus.[29,30] In 1994, Chang et al[31] discovered a new member of HHV, HHV-8, in a patient with AIDS-associated KS. Subsequent studies demonstrated HHV-8 infection in patients with AIDS-associated, classic, endemic, and iatrogenic KS but rarely in healthy individuals, which proved the causal association of HHV-8 with KS. HHV-8 has also been detected in patients without KS such as those with primary effusion lymphoma and multicentric Castleman's disease.[32-34] Some researchers reported detection of HHV-8 DNA sequence in lesional skin from patients with PV and PF,[35-37] especially in perilesional vascular endothelial cells and basal keratinocytes.[36] They proposed a causal relationship of HHV-8 with pemphigus. However, it is still unclear whether HHV-8 plays any role in the pathogenesis of pemphigus, because other groups reported inconsistent results.[28,38-44]

To further investigate the possible association of pemphigus with HHV-8, we enrolled a larger number of patients than that of the earlier studies by others. We determined the prevalence of HHV-8 DNA in their cryopreserved lesional skin biopsy specimens and PBMCs and that of HHV-8-specific IgG antibodies in their sera.

METHODS

Specimens from patients with pemphigus

A total of 78 specimens, including 36 cryopreserved lesional skin biopsy specimens, 13 PBMCs, and 29 sera, were obtained from 58 patients with pemphigus (29 men and 29 women, age 21-78 years, mean age 50.1 years) with their informed consent (Table I). These cases comprised 37 PV, 1 pemphigus vegetans, 4 PF, and 16 pemphigus erythematous types. All patients were given a diagnosis by clinical and histopathologic findings and by direct immunofluorescence testing for intercellular deposits in lesional or nonlesional skin. Some patients were simultaneously tested for serum antibody against intercellular substance by indirect immunofluorescence on substrate of guinea pig esophagus or tongue. Among these patients, only 9 received oral prednisone (<80 mg/d) for less than 1 week before the specimens were taken, whereas the others did not receive any systemic immunosuppressive therapy.

Specimens from control subjects

In all, 18 normal skin specimens were obtained from excised skin tissues for plastic surgery (11 men and 7 women, age 13-40 years, mean age 38.1 years). In all, 230 samples of PBMCs and 109 sera were obtained from 230 healthy blood donors in our hospital (171 men and 59 women, age 19-54 years, mean age 34.6 years).

Nested polymerase chain reaction

Total genomic DNAs were extracted from skin biopsy specimens and PBMCs with a standard phenol-chloroform method. Concentration and purity of the extracted DNA were determined by a spectrophotometry (Spectronic, Rochester, NY).

Primer set KS1/KS2 and nested polymerase chain reaction (PCR) primer set NS1/NS2 were used to amplify a 233-base pair (bp) HHV-8 KS330Bam fragment and a 160-bp fragment, respectively, as previously described.[31,40] A 25 μL reaction system included 10 mmol/L Tris-HCl, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 100 μg/mL bovine serum albumin, 0.2 mmol/L deoxyl nucleoside triphosphate, 0.625 U γ-Taq DNA polymerase (TaKaRa Biotechnology Dalian Co, Dalian, China), 0.1~0.25 μg template DNA, and 0.8 μmol/L of each primer. PCR amplification was performed in a T-Gradient thermalcycler (Biometra, Gottingen, Germany). Thermocycling conditions for the first round of PCR with KS1/KS2 primers were initial denaturation at 94°C for 2 minutes, 35 cycles of 94°C for 1 minute, 62°C for 1 minute, 72°C for 1 minute, and a final extension at 72°C for 5 minutes. One tenth (2.5 μL) of the PCR products were used as template in the second round of PCR with NS1/NS2 primers. Thermocycling conditions for the second round of PCR were the same as the first round. All amplified products were analyzed on 2% agarose gels. DNA extracted from a primary effusion lymphoma cell line (BC-1) that was HHV-8- and Epstein-Barr virus-positive[45] was used as a positive control. Primer set PC03/PC04 was used to amplify a 110-bp fragment of human β-globin for all samples to confirm the integrity of DNA samples and for lack of inhibitors in the amplification reactions.[46]

Restriction endonuclease analysis of PCR products

All 233-bp and 160-bp products were retrieved with DNA fragment recovery kit (TaKaRa

Table I. Patients with pemphigus

No./sex/age, y	Intercellular deposits on DIF	Anti-intercelluar substances on IIF	Type	HHV-8 DNA		Serum IgG	ISP therapy
				Lesional skin	PBMCs		
1/M/78	IgG	/	PV	–	/	/	×
2/M/37	IgG, IgA	/	PV	–	/	/	×
3/M/41	IgG	/	PV	+	/	/	×
4/M/52	IgG, C3	/	PF	–	/	/	×
5/M52	IgG, C3	/	PV	+	/	/	×
6/M/43	IgG, IgA, C3	/	PE	–	/	/	×
7/M/43	IgG, C3	/	PE	–	/	–	×
8/F/56	IgG, C3	1:64	PE	+	/	+	×
9/M/76	IgG, C3	1:64	PF	+	/	+	√
10/M/76	IgG, C3	–	PV	–	/	–	×
11/M/50	IgG	1:16	PV	–	/	+	√
12/F/60	IgG	/	PVe	+	/	/	×
13/F/53	IgG, C3	/	PV	–	/	/	×
14/F/40	IgG, C3	/	PV	–	/	/	×
15/F/55	IgG, C3	/	PE	–	/	/	×
16/F/40	IgG, C3	1:128	PE	–	/	/	×
17/M/44	IgG, C3	1:128	PV	+	/	+	×
18/F/67	IgG, C3	/	PV	+	/	/	×
19/F/67	IgG, C3	/	PV	–	/	/	×
20/M/62	IgG	/	PV	–	/	/	×
21/F/75	IgG, IgA, C3	/	PV	+	/	/	×
22/F/41	IgG	/	PE	+	/	+	×
23/F/58	IgG	/	PV	–	/	+	√
24/F/34	IgG	/	PF	–	/	/	×
25/M/73	IgG, C3	/	PE	+	/	/	×
26/F/45	IgG, C3	/	PV	–	–	/	×
27M/28	IgG	/	PV	–	–	–	×
28/M/56	IgG	/	PV	–	–	/	×
29/M/?	IgG, C3	/	PV	–	–	/	×
30/M/73	IgG, C3	/	PV	+	+	/	√
31/F/28	IgG	/	PV	–	–	/	√
32/M/25	IgG, C3	/	PF	–	–	–	×
33/M/58	IgG, IgA, C3	/	PV	+	/	/	×
34/F/36	IgG	/	PV	–	–	/	×
35/F/36	IgG	/	PE	+	+	/	×
36/M/37	IgG, C3	1:64	PV	–	–	/	×
37/F/46	IgG	/	PV	/	+	/	√
38/M/47	IgG	/	PV	/	–	/	×
39/M/60	IgG	/	PV	/	+	/	√
40/F/64	/	1:64	PE	/	/	–	×
41/F/30	/	1:1024	PF	/	/	+	×
42/F/56	/	1:256	PE	/	/	–	×
43/M/21	IgG	/	PV	/	/	–	×
44/F/?	/	1:256	PE	/	/	–	×
45/M/40	/	1:256	PV	/	/	–	×
46/M/59	IgG	1:64	PV	/	/	–	×
47/F/29	/	1:128	PE	/	/	–	×
48/F/57	–	1:80	PV	/	/	–	×
49/F/44	/	1:16	PV	/	/	–	×
50/F/53	/	1:128	PE	/	/	–	×
51/M/64	/	1:32	PV	/	/	+	√
52/M/60	/	1:32	PE	/	/	–	√
53/F/40	/	1:128	PE	/	/	–	×
54/F/49	/	1:32	PV	/	/	+	×

Table I. Cont'd

No./sex/age, y	Intercellular deposits on DIF	Anti-intercelluar substances on IIF	Type	HHV-8 DNA		Serum IgG	ISP therapy
				Lesional skin	PBMCs		
55/M/47	/	1:1024	PV	/	/	+	×
56/M/36	/	1:64	PV	/	/	−	×
57/F/54	/	1:32	PV	/	/	−	×
58/F/55	IgG, IgA, C3	/	PV	/	/	−	×

C3, Complement 3; *DIF*, direct immunofluorescence; *F*, female; *HHV*, human herpesvirus; *IIF*, indirect immunofluorescence; *ISP*, immunosuppressive; *M*, male; *PBMCs*, peripheral blood mononuclear cells; *PE*, pemphigus erythematous; *PF*, pemphigus foliaceus; *PV*, pemphigus vulgaris; *PVe*, pemphigus vegetans; /, not available; +, positive; −, negative; √, received; ×, not received.

Biotechnology Dalian Co) as recommended by the manufacturer. Retrieved products (5 μL) were digested with 10 U of restriction endonuclease PstI (Gibco-BRL, Gaithersburg, Md) at 37°C for 3 hours. The digests were analyzed on 12% polyacrylamide gels.

ELISA

Enzyme-linked immunosorbent assay (ELISA) using HHV-8 open reading frame (ORF) 65 and K8.1 oligopeptides as antigens were undertaken as described previously.[47] A serum dilution of 1:100 was used. HHV-8-specific IgG antibody positive sera that were obtained from patients with HIV-positive KS and distilled water were used as positive and negative controls, respectively. ELISA without coating the oligopeptides was performed to monitor nonspecific reactions. The cut-off value was defined as the mean plus 5 SD of optical density values obtained from control subjects whose PBMCs were negative for HHV-8 DNA in the nested PCR. When 1 of the 3 types of the oligopeptide antigens (ie, ORF65, K8.1, and their mixture) provided positive results in ELISAs, tested serum specimens were considered HHV-8-specific antibody positive.

Statistical analysis

The differences in prevalence of HHV-8 DNA or specific IgG antibodies between the pemphigus cases and control subjects were analyzed by chi-square test.

RESULTS

HHV-8 DNA sequence in lesional skin and PBMCs of patients with pemphigus

After first round PCR, HHV-8 DNA was detected in 2 pemphigus lesional skin biopsy specimens, 1 pemphigus PBMC, and 5 blood donors' PBMCs. After nested PCR, HHV-8 DNA was detected in 13 pemphigus lesional skin biopsy specimens, 4 pemphigus PBMCs, 1 normal skin biopsy specimen, and 18 blood donors' PBMCs (Table II and Fig 1). In all positive cases, PstI digestion generated 138-bp and 95-bp fragments from the 233-bp fragment, and 99-bp and 61-bp fragments from the 160-bp fragment, confirming the PCR products were HHV-8 specific (Fig 2). In addition, the presence of HHV-8 DNA in subsets of the specimens was further examined by using a nested PCR assay with a different primer set, and concordant results were obtained in this additional assay (data not shown). Prevalence of HHV-8 DNA detected in lesional skin biopsy specimens and in PBMCs from patients with pemphigus were both statistically higher than those of control subjects (chi-square = 4.35, $P < .05$; chi-square = 5.32, $P < .05$). There was perfect concordance of the PCR results between lesional skin biopsy specimens and PBMCs from the patients with pemphigus (2 positive and 8 negative cases).

Table II. Human herpesvirus-8 DNA and specific IgG antibodies in pemphigus and control subjects

	Pemphigus	Control subjects
DNA in lesional skin	13/36 (36.1%)	1/18 (5.6%)
DNA in PBMCs	4/13 (30.8%)	18/230 (7.8%)
Serum IgG antibodies	10/29 (34.5%)	8/109 (7.3%)

PBMCs, Peripheral blood mononuclear cells.

HHV-8-specific IgG antibodies in sera of patients with pemphigus

Of 29, 10 (34.5%) sera from patients with pemphigus and 8 of 109 (7.3%) blood donors' sera were positive for HHV-8-specific IgG antibodies (Table II). Positive rate of serum IgG antibodies for patients with pemphigus was statistically higher than that of blood donors (chi-square = 12.58, $P < .005$). There was good concordance among ELISAs using the individual ORF65 and K8.1 oligopeptides and their mixture. In addition, the mean and SD of the ELISA optical density values from patients with seropositive pemphigus were 1.36 and 0.38, respectively, whereas those from seropositive control subjects were 0.81 and 0.20, respectively, in the ELISA using both oligopeptides as antigens. In 10 patients tested

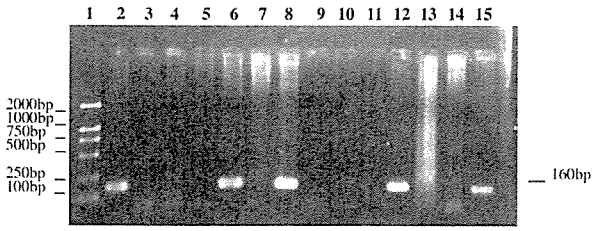

Fig 1. Amplified 160-bp products by nested polymerase chain reaction. *Lane 1*, DNA marker; *lane 2*, BC-1 DNA (345 ng of total DNA); *lane 3*, negative control; *lanes 4 to 15*, results in some patients.

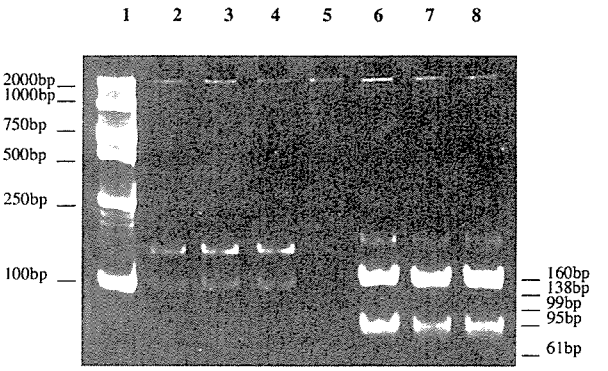

Fig 2. PstI-digests of 233-bp and 160-bp products. *Lane 1*, DNA marker; *lanes 2 to 4*, digests of 233-bp products; *lane 5*, negative control; *lanes 6 to 8*, digests of 160-bp products.

for both HHV-8 DNA and specific antibodies, all 4 patients who were positive for HHV-8 DNA were also HHV-8 seropositive.

Insignificance of immunosuppressive therapy and pemphigus types

In the 15 patients who were HHV-8 DNA positive, only 4 received immunosuppressive therapy before their specimens were taken. In addition, in the 6 patients who were positive for both HHV-8 DNA and antibodies, only 3 received an immunosuppressive therapy.

Based on positive detection of either HHV-8 DNA or IgG antibody, HHV-8 infection was found in 14 of 37 cases of PV, 1 of 1 case of pemphigus vegetans, 2 of 4 cases of PF, and 4 of 16 cases of pemphigus erythematous. A statistical analysis of these results did not find any significant difference in the positive rates between PV/pemphigus vegetans and PF/pemphigus erythematous (chi-square = 0.509, $P > .05$).

DISCUSSION

HHV-8 DNA sequence has been detected in lesional skin biopsy specimens from patients with PV who had neither HIV infection nor KS. Detection of viral DNA in perilesional vascular endothelium and basal keratinocytes, but not in nonlesional skin, of the patients or normal skin of the control subjects suggested an important role of HHV-8 in the onset, development, or both of pemphigus.[35-37] In this study, HHV-8 DNA sequence was detected in 36.1% of the lesional skin biopsy specimens and 30.8% of PBMCs; HHV-8-specific IgG antibodies were detected in 34.5% of sera from patients with pemphigus. A statistical analysis of the results obtained in the two different assays revealed a higher prevalence of HHV-8 infection in patients with pemphigus than in control subjects. In addition, the average IgG titers against HHV-8 of the patients who were seropositive were also higher than those of the control subjects. Of 58, 21 (36.2%) patients were positive for HHV-8 DNA sequence and/or specific IgG antibodies. These results are partly in agreement with the previous reports that described the association of pemphigus with HHV-8, although the HHV-8 prevalence in the patient group of our study was lower than those studies.

Because of the high sensitivity of PCR, there is always an argument whether positive detection is real or a result of cross-contamination in a laboratory. The previous studies demonstrated the specificity of their PCR results by comparing HHV-8 DNA sequences of the PCR products.[35,36] In this study, we took precautions to avoid cross-contamination: extraction of DNA from specimens was undertaken before DNA amplification; each procedures for DNA extraction and for mixing PCR reagents was undertaken in a separate room in which neither virus preparation nor amplification of plasmids encoding the PCR region has been operated previously; positive-displacing pipettes and filtered pipette tips were used; and positive, negative, and internal controls were included in the nested PCR to monitor cross-contamination. Importantly, a nested PCR assay with a different primer set provided consistent results. There was also perfect concordance of the PCR results between lesional skin biopsy specimens and PBMCs in the 10 patients from whom both specimens were obtained. Thus, we do not think that our results represent laboratory cross-contamination. Moreover, we conducted a serologic assay for HHV-8 and confirmed the higher prevalence of HHV-8 infection in the pemphigus group. A high concordance of the results between HHV-8 DNA and specific IgG antibodies in the patients whose DNA and sera were available also argues against the potential PCR contamination.

Several groups reported results inconsistent with ours.[28,38-44] To interpret the discrepancy between the positive and negative findings of HHV-8 association

with pemphigus, some issues should be considered. First, our study demonstrated a higher prevalence of HHV-8 for patients with pemphigus, but the viral components were found only in one third of the patients, indicating that HHV-8 is a contributing rather than a sole or decisive factor. The onset and progression of pemphigus may depend on a combination of genetic and environmental factors, including HHV-8 infection. In other words, HHV-8 prevalence and presence of a risk factor or factors accompanied with the infection may be different among the studied populations. HHV-8 prevalence varies by the risk factors for transmission and by geographic locations.[48] Among healthy individuals worldwide, HHV-8 is more prevalent in Africa than in the United States and northern Europe. In the United States and Europe, HHV-8 transmits predominantly by a sexual route, and some types of behavioral and sexual practices are considered risk factors. In contrast, in Africa, it is likely that most transmission occurs postnatally from mothers to children or between siblings. Although KS is rare in China, the HHV-8 prevalence and the main transmission route in the whole regions of China is not defined yet. Compared with the publications by others, the positive rate of HHV-8 DNA in control subjects of our study was surprisingly high. Interestingly, the HHV-8 seroprevalence in the northwestern area of China was reported very high.[49] Further studies might be useful to examine environmental or behavioral risk factors for activation of HHV-8 lytic infection in the northern parts of China, because a high incidence of nasopharyngeal carcinoma in the southern China exemplified the importance of an environmental factor accompanied with viral infection. Practical issues that may cause the discrepancy include enrollment size, source of materials, and assay methods. Former studies enrolled a relatively small number of patients, and their specimens examined were mainly formalin-fixed and paraffin-embedded.[36,41,44] In our study, a relatively larger number of freshly excised or cryopreserved lesional skin biopsy specimens, PBMCs, and sera were used, which may increase the sensitivity and reliability. Some studies reporting the negative association used latent antigen-based serologic assays.[38,39,44] Although the latent antigen-based assays were useful for KS cases, they were less sensitive than the lytic antigen-based assays for detection of HHV-8 infection in non-KS populations.[50] In our study, we used ELISAs based on the ORF65 and K8.1 oligopeptides, of which performance was good and well-defined.[50]

What is the implication of the higher HHV-8 prevalence in patients with pemphigus? It is possible that immunosuppressive therapy for autoimmune diseases allows an opportunistic infection or reactivation of HHV-8 as observed in bullous pemphigoid.[51-53] However, HHV-8 DNA sequence was detected in lesional skin from patients with PF and PV who had not received any immnuosuppressive therapy.[36] In our study, 14 of 21 patients who were positive for HHV-8 DNA sequence and/or specific IgG antibodies had not received any systemic immnuosuppressive therapy before the collection of the specimens, suggesting that HHV-8 infection induced by the therapy is unlikely. In addition, HHV-8 DNA was detected in the lesional biopsy specimens of only 1 of the 3 seropositive cases with the therapy (Nos. 9, 11, and 23), whereas it was detected in all of the 3 seropositive cases without the therapy (Nos. 8, 17, and 22), suggesting that the therapy has little effect on reactivation of latently infected HHV-8 or on proliferation of cells latently infected with HHV-8. It is also possible that pemphigus and HHV-8 infection share common risk factors and that HHV-8 infection is just a bystander of pemphigus. However, an alternative and attractive working hypothesis is that HHV-8 infection contributes to triggering and/or progression of pemphigus. For example, HHV-8 infection could potentially mediate the tissue damage by expressing its cytokine homologs, or by upregulating cellular factors that contribute to the development of pemphigus. There is some similarity in the involvement of IL-6 in the lesions between HHV-8-associated neoplasms and pemphigus. IL-6 that can act as an autocrine or paracrine growth factor is overexpressed in HHV-8-associated neoplasms.[54-58] Higher IL-6 in sera was reported not only in multicentric Castleman's disease but also for patients with pemphigus,[59,60] and IL-6 and tumor necrosis factor alpha were expressed around the pemphigus blister.[61] HHV-8 encodes viral IL-6 that induced cell proliferation and immune evasion of interferon activity in lymphoma cells[62]; HHV-8 gene products also induce cellular IL-6 synthesis.[63,64] Alternatively, in genetically predisposed patients, HHV-8 infection might enhance the presentation of autoantigen. Cytokines such as interferon gamma produced by virus-activated T lymphocytes might induce the expression of HLA antigen type II antigens on membranes of kerationcytes, and then form immnuoactivated structural sites of pemphigus antigen. If it is accompanied with virus infection or reactivation, cytokines may shift from T helper 1 cell type to T helper 2 cell type, with more IL-4 and IL-10 being produced, which further stimulates the antibody response. In this manner, autoantigen chain can be activated, more autoantibodies can be produced, and then pemphigus may be induced

through some cytotoxic effectors and pathogenic autoantibodies.[25,43] To elucidate the role of HHV-8 in the pathogenesis of pemphigus, further studies are necessary. These may include monitoring viral load and immune responses, immunohistochemistry, in situ hybridization and electron microscopic analyses of lesional and nonlesional skin biopsy specimens, and epidemiologic studies to screen risk factors.

In conclusion, higher prevalence of HHV-8 infection in pemphigus was demonstrated in our study, which warrants further studies on the HHV-8 association with the pathogenesis of pemphigus.

We thank Y. Chang for BC-1 cells, T. Spira for HIV positive KS patient sera, C-P. Pong for the HHV-8 oligopeptides, and F. R. Stamey for English-language editing.

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