No Evidence of Human Herpesvirus 8 Infection in Patients with Paraneoplastic Pemphigus, Pemphigus Vulgaris, or Pemphigus Foliaceus

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Paraneoplastic pemphigus has been associated with both malignancies and multicentric Castleman's disease; the latter is a rare angiolymphoproliferative disorder that has also been linked with human herpesvirus 8 (HHV8) infection. Other diseases definitively associated with HHV8 include Kaposi's sarcoma and primary effusion lymphoma. In a search for additional HHV8-associated diseases, patients with paraneoplastic pemphigus, as well as patients with pemphigus vulgaris and pemphigus foliaceus, were studied. Using an immunofluorescence assay able to specifically detect antibodies directed against lytically induced HHV8 antigens, HHV8 antibodies were not detected in sera from 24 patients with paraneoplastic pemphigus (including 10 with concomitant Castleman's disease) nor from 19 patients with pemphigus vulgaris. Sera from patients with Kaposi's sarcoma and from healthy U.S. blood donors were positive (25 of 26) and negative (none of 20), respectively. In addition, HHV8 DNA was not found in frozen lesional skin of five patients with pemphigus vulgaris and five patients with pemphigus foliaceus by nested polymerase chain reaction (lower limit of detection = 10 copies viral DNA per μg total cellular DNA). Finally, tissue sections of lesional skin from 10 patients with pemphigus vulgaris were negative for HHV8 by *in situ* hybridization, using probes able to detect both latently and lytically expressed HHV8 genes in Kaposi's sarcoma tissue. In summary, no evidence of HHV8 infection was found in all types of pemphigus using a variety of methods. These findings do not support a general role for HHV8 in skin diseases associated with immunosuppression. ***Key words:*** **in situ *hybridization/ Kaposi's sarcoma/polymerase chain reaction/serology*. *J Invest Dermatol 111:781–783, 1998***

Chang and Moore first described human herpesvirus 8 (HHV8), also known as Kaposi's sarcoma (KS)-associated herpesvirus, in 1994 (Chang *et al*, 1994). Since that discovery, numerous DNA and serologic studies have documented HHV8 in all clinical types of KS (i.e., classic, epidemic, endemic, iatrogenic) and in all tissues with histologic evidence of KS (for recent review, see Blauvelt, 1998). In addition, HHV8 has been associated with all cases of primary effusion lymphoma (Cesarman *et al*, 1995) and with many cases of multicentric Castleman's disease (Soulier *et al*, 1995). Castleman's disease is a rare angiolymphoproliferative disease that can occur in patients infected with human immunodeficiency virus (HIV) (Herrada *et al*, 1998). In HIV-infected individuals, Castleman's disease is nearly always associated with HHV8 infection (>90% of cases), whereas Castleman's disease in HIV-seronegative individuals is associated with HHV8 in ≈40% of cases (Soulier *et al*, 1995; Grandadam *et al*, 1997; Parravicini *et al*, 1997a). Interestingly, Castleman's disease can also occur in patients with paraneoplastic pemphigus (PNP) (Anhalt, 1997; Jansen *et al*, 1995; Lemon *et al*, 1997), a rare autoimmune mucocutaneous blistering disease (Anhalt *et al*, 1990; Anhalt, 1997). Because of this association, patients with PNP (many of whom had concomitant Castleman's disease) were examined for evidence of HHV8 infection in this study.

In addition to KS, primary effusion lymphoma, and Castleman's disease, several other conditions have been reported to be associated with HHV8. Diseases in this group include angiosarcoma (McDonagh *et al*, 1996), angiolymphoid hyperplasia with eosinophilia (Gyulai *et al*, 1996), nonmelanoma skin cancers in immunosuppressed individuals (Rady *et al*, 1995), sarcoidosis (DiAlberti *et al*, 1997), multiple myeloma (Rettig *et al*, 1997), pemphigus vulgaris (PV), and pemphigus foliaceus (PF) (Memar *et al*, 1997a, b). Most of these associations, however, have either not been confirmed or have been disproved by subsequent more carefully performed studies (Chang *et al*, 1994; Adams *et al*, 1995; Boshoff *et al*, 1995, 1996; Dictor *et al*, 1996; Jin *et al*, 1996; Lin *et al*, 1996; Uthman *et al*, 1996; Cathomas *et al*, 1997; Cottoni and Uccini, 1997; Dupin *et al*, 1997; Lebbe *et al*, 1997a, b; MacKenzie *et al*, 1997; Marcelin *et al*, 1997; Masood *et al*, 1997; Parravicini *et al*, 1997b; Whitby *et al*, 1997; Moore, 1998; Regamey *et al*, 1998; Tarte *et al*, 1998). For patients with PV and PF, there have been no subsequent reports confirming or refuting possible links with HHV8 as reported initially by Memar *et al* (1997a, b). Thus, in addition to PNP patients, patients with PV and PF were also examined for evidence of HHV8 infection in this study. Importantly, a combination of serologic assays, *in situ* hybridization, and sensitive polymerase chain reaction (PCR) assays were employed for this investigation.

MATERIALS AND METHODS

Patients PNP sera and frozen pemphigus tissue for PCR assays were obtained from the Dermatology Department at Johns Hopkins University. PNP patients

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Abbreviations: HHV8, human herpesvirus 8; KS, Kaposi's sarcoma; PF, pemphigus foliaceus; PNP, paraneoplastic pemphigus; PV, pemphigus vulgaris.

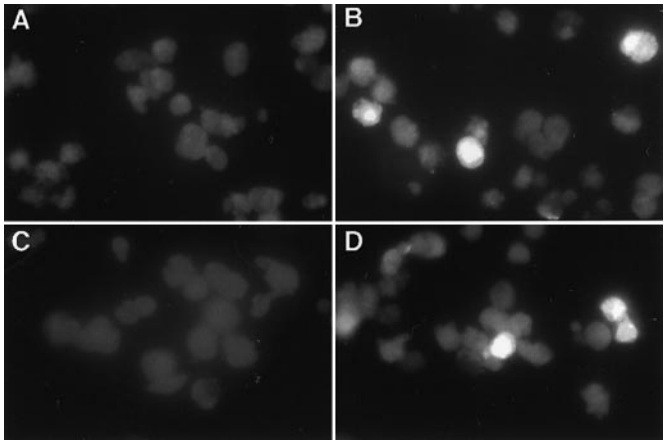

Figure 1. No evidence of HHV8-specific antibodies in sera from patients with PNP. Sera from patients with PNP (*A*) or KS (*B*) were incubated at a dilution of 1:20 with phorbol ester-stimulated HHV8$^+$ primary effusion lymphoma cells. Negative (*C*) and positive (*D*) controls included in the immunofluorescence kits were run each day that patient sera were tested. Primary antibody labeling was detected using fluorescein isothiocyanate-conjugated anti-human antibody and immunofluorescence microscopy. Gray cells indicate no HHV8 antibody binding, whereas white cells (≈30% in positive controls and KS sera) indicate HHV8 antibody binding. All sera were evaluated by an investigator blinded with respect to the source of the sera. The data shown are representative of numerous experiments (summarized in **Table I**).

Table I. Summary of results showing no evidence of HHV8 infection in all types of pemphigus

Patients	HHV8 antibodies (by seroassay)	HHV8 DNA (by nested PCR)	HHV8 mRNA (by *in situ* hybridization)
PNP	0/24	–[a]	–[a]
PV	0/19	0/5	0/10
PF	–[a]	0/5	–[a]
KS	25/26	–[b]	–[b]
Healthy volunteers	0/20	–[a]	–[a]

[a]Not tested.
[b]Tested on numerous occasions (>20) and always positive.

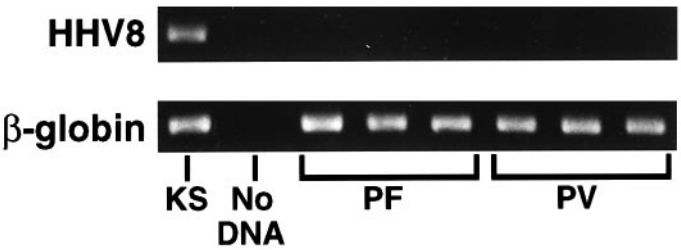

Figure 2. No evidence of HHV8 DNA in lesional skin of patients with PF or PV by nested PCR. DNA was extracted from frozen lesional skin of patients with KS, PF, or PV and examined for HHV8-specific DNA using a sensitive nested PCR assay (see *Materials and Methods*). β-globin-specific DNA was readily detected by non-nested and nested PCR in all samples tested (as a test of the quality of DNA and PCR reagents), whereas HHV8-specific DNA was only observed in KS lesions and BCBL-1 cells. The data shown are representative of numerous experiments (summarized in **Table I**).

had severe mucocutaneous blistering disease and all had characteristic circulating autoantibodies directed against 250, 230, 210, and 190 kDa keratinocyte proteins by western blot (Anhalt *et al*, 1990). Ten PNP patients had associated Castleman's disease. Other disease associations included sarcoma (specifically not KS, four patients), non-Hodgkin's lymphoma (specifically not primary effusion lymphoma, four patients), chronic lymphocytic leukemia (four patients), and myofibroblastic tumor (one patient); one PNP patient had no documented concomitant disease but the patient died with generalized lymphadenopathy and hepatosplenomegaly. Diffuse Castleman's was suspected but not proven at time of death. Sera from healthy US blood donors and from well characterized

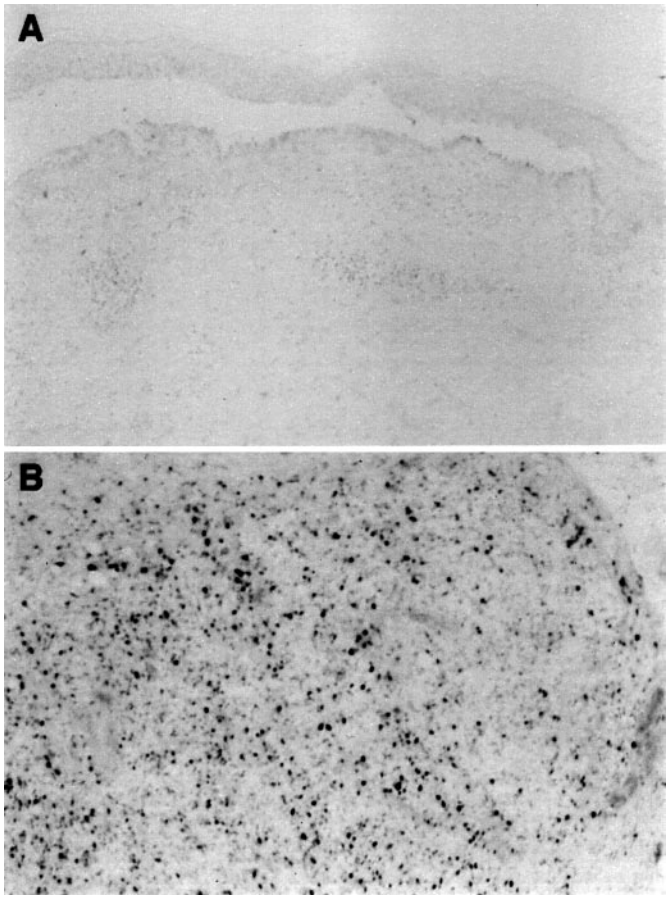

Figure 3. No evidence of HHV8-specific mRNA expression in lesional skin from patients with PV by *in situ* hybridization. Sections of unstained lesional skin from patients with PV (*A*) or KS (*B*) were incubated with a riboprobe (T0.7) able to detect latently infected HHV8$^+$ cells (see *Materials and Methods*). No HHV8-specific signals were observed in PV lesions, whereas the majority of tumor spindle cells in KS lesions were labeled with this probe (black dots). The data shown are representative of numerous experiments (summarized in **Table I**).

patients with PV and PF were obtained from Kim Yancey (Dermatology Branch, National Cancer Institute) and John Stanley (Department of Dermatology, University of Pennsylvania). Tissues used for *in situ* hybridization were obtained from archival specimens in the Pathology Department at University of California, San Francisco. Sera and lesional biopsies were obtained from KS patients (following informed consent) enrolled in studies approved by the Institutional Review Board at the National Cancer Institute.

Serologic assay A commercially available immunofluorescence assay kit (Advanced Biotechnologies, Columbia, MD) was used for all HHV8 serologic studies according to manufacturer's instructions (Masood *et al*, 1997). As a source of viral antigens, this kit uses phorbol ester-stimulated HHV8-infected cells derived from a patient with primary effusion lymphoma (Said *et al*, 1996), i.e., it detects antibodies directed against lytically expressed HHV8 antigens. Sera were tested at either 1:20 or 1:40 final dilution. All assays were performed by one investigator (S.C.), using samples from healthy individuals and patients with pemphigus or KS in random order. Using an immunofluorescence microscope, coded slides were then examined and scored by a second investigator (A.B.), without knowledge of the serum source.

Nested PCR Nested PCR was performed as previously described in detail (Blauvelt *et al*, 1997). Briefly, DNA was extracted from frozen lesional skin obtained from five patients with PV and five patients with PF using a commercially available kit (Qiagen, Valencia, CA), according to manufacturer's instructions. DNA was quantitated by spectrophotometry and 1 μg of total DNA was used as a template for outer PCR reactions (35 cycles), whereas 2% of outer PCR reaction products were used as templates for inner PCR reactions (35 cycles). DNA isolated from KS tissue and from the HHV8$^+$ cell line BCBL-1 were used as positive PCR controls. Negative control reactions contained all PCR reagents without DNA template. This assay is able to detect

as little as 10 copies of HHV8 DNA per μg of total cellular DNA (Blauvelt *et al*, 1997).

***In situ* hybridization** *In situ* hybridization was performed as previously described (Orenstein *et al*, 1997). Briefly, deparaffinized tissue sections from 10 formalin-fixed specimens of lesional PV skin were incubated with either a digoxigenin-labeled T0.7 riboprobe (able to detect latently expressed HHV8 ORF K12 mRNA) or a digoxigenin-labeled T1.1 riboprobe (able to detect lytically expressed HHV8 ORF K7 mRNA). Visualization of probe was carried out with alkaline phosphatase-labeled anti-digoxigenin antibody and nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indoyl phosphate substrate (Boehringer, Mannheim, Germany). Separate PV sections were stained for ALU repetitive DNA sequences to evaluate nucleic acid integrity. KS tissue and cytospins of BCBL-1 cells ($HHV8^+$ primary effusion lymphoma cells, NIH AIDS Research & Reagent Program, Rockville, MD) were used as positive controls.

RESULTS AND DISCUSSION

Given that Castleman's disease can be associated with both HHV8 infection (Soulier *et al*, 1995; Grandadam *et al*, 1997; Parravicini *et al*, 1997a) and PNP (Anhalt, 1997; Jansen *et al*, 1995; Lemon *et al*, 1997), PNP patients were studied for evidence of HHV8 infection. Sera from 24 PNP patients, including 10 with concomitant Castleman's disease, did not demonstrate HHV8-specific antibodies (**Fig 1**, **Table I**). By contrast, antibodies directed against HHV8 lytic antigens could readily and reliably be detected in sera obtained from KS patients (25 of 26) (**Fig 1**, **Table I**). Sera from 20 healthy US blood donors were HHV8-nonreactive using this assay (**Table I**). This study is the first to examine patients with PNP for HHV8 infection, and the results suggest that HHV8 plays no role in the pathogenesis of this paraneoplastic disease.

Furthermore, no evidence of HHV8 infection in patients with other forms of pemphigus was found. Specifically, PV patients demonstrated no HHV8-specific antibodies in sera (**Table I**), no HHV8 DNA was detected in lesional skin of PV or PF patients by nested PCR (**Fig 2**, **Table I**), and HHV8 mRNA expressed by either latently or lytically infected cells was not detected in lesional skin of PV patients by *in situ* hybridization (**Fig 3**, **Table I**). Positive and negative controls gave expected results for each of these assays (**Figs 1–3**, **Table I**). Also, PV tissue used for *in situ* hybridization demonstrated clear ALU repetitive DNA signals, indicating that nucleic acid integrity was intact in the tested specimens (not shown). In short, the results of earlier studies reporting HHV8 within skin of PV and PF patients could not be confirmed (Memar *et al*, 1997a, b). These earlier studies may have demonstrated false positive results because of PCR contamination, as has occurred with other purported HHV8-associated diseases (Moore, 1998). To avoid potential contamination problems that are possible with PCR, future investigations of HHV8 disease associations should ideally include additional assays to detect virus, such as *in situ* hybridization, serologic assays, or electron microscopy.

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