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

C hang 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, 1994) and with many cases of multicentric Castleman’s disease (Soulie et al, 1998). 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 (D’Alberti 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, 1999; Lebbe et al, 1997a, b; MacKenzie et al, 1997; Marcelin et al, 1997; Masood et al, 1997; Parravicini et al, 1997b; Whithby 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

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

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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, not Hodgkin’s 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 autopsy.

Patients (by seroassay) (by nested PCR) hybridization)

<table>
<thead>
<tr>
<th>Patients</th>
<th>HHV8 antibodies (by seroassay)</th>
<th>HHV8 DNA (by nested PCR)</th>
<th>HHV8 mRNA (by <em>in situ</em> hybridization)</th>
</tr>
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<tbody>
<tr>
<td>PNP</td>
<td>0/24</td>
<td>–</td>
<td>–</td>
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<tr>
<td>PV</td>
<td>0/19</td>
<td>0/5</td>
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<tr>
<td>PF</td>
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<td>0/5</td>
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<tr>
<td>KS</td>
<td>25/26</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Healthy volunteers</td>
<td>0/20</td>
<td>–</td>
<td>–</td>
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</table>

*Not tested.

*Tested on numerous occasions (>20) and always positive.

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 (Qagen, 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<sup>+</sup> 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 HHV8 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).

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<sup>+</sup> 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).

Figure 2. No evidence of HHV8 DNA in lesional skin of patients with PF or PV by *in situ* hybridization. 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).

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<sup>+</sup> 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).
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-indolyl 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 BCC-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., 1997b), PNP patients were studied for evidence of HHV8 infection. Sera from 1997a) and PNP (Anhalt, 1997; Jansen et al., 1997b) were used as positive controls.

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**REFERENCES**


