American Journal of Pathology, Vol. 150, No. 1, January 1997

Detection of the Herpesvirus-Like DNA Sequences in Matched Specimens of Semen and Blood from Patients with AIDS-Related Kaposi's Sarcoma by Polymerase Chain Reaction *in Situ* Hybridization

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The DNA sequences of a novel human γ-herpesvirus type 8 (HHV-8) have recently been detected in Kaposi's sarcoma (KS) lesions obtained from different populations in whom this neoplasm occurs, suggesting that this virus may be implicated in the etiology and/or pathogenesis of KS. To study the distribution and possible means of transmission of the putative viral agent, specimens of KS skin lesions, matched uninvolved skin, peripheral blood mononuclear cells (PBMCs), and semen were collected from 12 HIV-positive homosexual men with acquired immune deficiency syndrome (AIDS)-related KS (AIDS-KS) and 2 human immunodeficiency virus (HIV)-negative homosexual men with KS. HHV-8 virus DNA was detected by polymerase chain reaction (PCR) studies in all 14 of these KS specimens and in 6 of 14 biopsies of normal-appearing skin distant from any KS lesions including 1 uninvolved skin specimen from an HIV-negative homosexual male with KS. In addition, 3 of 12 PBMC samples and 3 of 12 semen samples from the AIDS-KS patients were positive for HHV-8. The DNA sequences of HHV-8 were not detected in the matched semen and PBMC specimens obtained from 2 HIV-negative homosexual men with KS, 4 HIV-positive homosexual patients without KS, 2 HIV-seronegative healthy homosexual men, 5 HIV-positive heterosexual male intravenous drug users, or 5 healthy HIV-negative heterosexual donors. Using PCR in situ, positive signals for HHV-8 were demonstrated in the B lymphocyte subsets of PBMCs and/or in spermatozoa and mononuclear cells in the semen from some of the PCR-positive specimens from the AIDS-KS patients examined. These data show that HHV-8 is present in and could possibly be transmitted via semen and/or blood from some homosexual men with AIDS-KS. (Am J Pathol 1997, 150:147–153)

The DNA sequences of what appears to be a unique, human γ-herpesvirus type 8 (HHV-8) have recently been detected in acquired immune deficiency syndrome (AIDS)-associated Kaposi's sarcoma (AIDS-KS).[1] The increased prevalence of the DNA sequences of this virus have also been found by polymerase chain reaction (PCR) in KS specimens obtained from distinct and unrelated non-human-immunodeficiency-virus (HIV)-infected populations, including the classical indolent type of KS, a rare disease seen primarily on the lower extremities in elderly men of Eastern European or Mediterranean origin; the more aggressive, endemic forms of KS frequently occurring in Central Africa; the iatrogenic KS occurring in immunosuppressed patients after organ transplantation; and in a group of 29 HIV-negative, immunocompetent, homosexual men with a mild form of KS being followed at New York Uni-

Supported in part by the Howard Gilman Foundation and by National Institutes of Health grants CA62782 and HB67021.

Accepted for publication September 5, 1996.

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versity Medical Center.[2–5] These data support the association of HHV-8 with KS among the various populations in which KS occurs and suggests a possible role for HHV-8 in the pathogenesis of this neoplasm.

Various epidemiological studies concerning AIDS-KS predominantly seen in homosexual men in the United States have suggested that this disease may be related to a sexually transmissible agent other than HIV.[6,7] KS is infrequently seen among AIDS patients in the United States who acquired their HIV infection perinatally, by heterosexual transmission, or through blood such as hemophiliacs or intravenous drug users (IVDUs).[8] It seems likely that the putative agent, HHV-8, is prevalent among homosexual males with AIDS-KS and may potentially be more readily transmitted by sexual activity between homosexual men.

Recent *in situ* hybridization studies by Boshoff et al[9] and by us[10] have shown that DNA of HHV-8 is found in the endothelial cells lining the vascular spaces and perivascular spindle-shaped cells histologically characteristic of KS tumor lesions.

In this study, we examine matched specimens of KS tumor lesions, uninvolved skin, PBMCs, and semen from 12 patients with AIDS-KS and 2 HIV-negative young homosexual men with KS for the presence of HHV-8 by PCR and/or PCR *in situ* hybridization (PCR-ISH).

Materials and Methods

Samples and Preparation

Fresh 4-mm punch biopsy samples of KS skin tumors, normal-appearing skin, semen, and blood were obtained from 14 patients with biopsy-proven KS; 12 were HIV-positive homosexual men with AIDS, and 2 were HIV-negative, immunocompetent, homosexual men being followed at New York University Medical Center since 1986. Control specimens included paired blood and semen specimens from 4 homosexual male AIDS patients without KS, 2 asymptomatic HIV-seronegative homosexual men, 5 heterosexual HIV-positive patients who were IVDUs, and 5 healthy HIV-negative heterosexual donors. The subjects who volunteered to participate in these studies gave informed consent.

The PBMCs were separated from the 15 ml of fresh heparinized blood collected from each donor by Ficoll gradient centrifugation. An aliquot of each PBMC sample was incubated with monoclonal antibody to CD21 (Dako Corp., Carpenteria, CA) at 4°C for 30 minutes, followed by the addition of sheep anti-mouse IgG-coated magnetizable particles. The B lymphocytes of these samples were then separated and harvested magnetically (Dynal, New York, NY) as previously described.[11]

Fresh semen samples were collected in sterile plastic screw top jars; the semen specimens were centrifuged at 5000 rpm for 15 minutes at 4°C in plastic centrifuge tubes to separate the cells from the semen supernatant fluids.

The DNA used for the PCR studies was extracted from each of the specimens including the KS skin lesions, uninvolved normal-appearing skin, PBMCs, sera, semen cell pellets, and semen supernatants from each donor by proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation, as previously described.[2]

PCR Analysis

Special precautions were taken to avoid carry-over contamination between the specimens analyzed by PCR in our laboratory as previously described.[2] The handling of the various tissue specimens and extraction of the DNA from the tissues before PCR evaluation was carried out in a specially isolated room, distant from the laboratory where the PCR analyses were performed. Moreover, the primer pair used for the PCR in this study (5′-TCA CAT CTG ACG TTG CCT ATT TCC-3′, 27 to 50 bp, and 5′-GTA CCG CAT AAT GTC TTC CTT GTG-3′, 287 to 310 bp) were derived from open reading frame 25 and are different from primers used in previous publications.[1,2] The addition of uracil-DNA glycosylase and dUTP were also included in the PCR performed in these experiments, which has been shown to eliminate possible carry-over DNA contamination between specimens undergoing concurrent evaluation.[12] The PCR products were then analyzed on a 1.5% agarose gel, transferred to nylon membrane, and hybridized with [γ-^{32}P]ATP-end-labeled internal probe (5′-AGC GTT TGT TCT ACG ACC ATT CAA TAC CAG-3′, 123 to 152 bp).[1]

Southern Blot Analysis

For genomic DNA Southern blot analysis, the DNA preparations were digested with the restriction endonuclease *Hin*dIII (Boehringer Mannheim, Indianapolis, IN), electrophoresed on a 0.8% agarose gel, blotted to Genescreen membranes (DuPont, Wilmington, DE), and then hybridized with [α^{32}P]dCTP-labeled probe specific for the HHV-8 DNA sequences.[2] After sequential washing in 2X standard saline citrate (SSC), 1X SSC, and then 0.5X SSC (1X SSC

contains 0.15 mol/L sodium chloride, 0.015 mol/L sodium citrate) at 65°C, the membranes were then exposed to Kodak x-ray film for 24 to 48 hours.

PCR-ISH

The PCR-ISH[13] studies were performed on fresh B lymphocyte and semen cell pellets that were smeared onto glass slides, air dried, fixed with 10% buffered formalin, and then treated with proteinase K (1 μg/ml) for 15 minutes at 37°C. After washing, PCR was performed on the slides using the hot-start PCR method in 50 μl of a solution containing 3 U of *Taq* polymerase, 4 mmol/L $MgCl_2$, 200 μmol/L dNTP, 0.05% bovine serum albumin, and 20 μmol/L primers mentioned above. The smeared specimens were denatured at 94°C for 5 minutes, followed by 20 cycles of 94°C for 1 minute and 55°C for 1 minute in a Gene Amp *in situ* PCR thermocycler (Perkin-Elmer, Norwalk, CT). The slides were washed and dehydrated with 100% ethanol. The HHV-8 DNA probe used was the same internal sequence as described above, labeled with digoxigenin by oligoprobe end-labeling using Genius Kit (Boehringer Mannheim). Hybridization solution containing the labeled probe was applied to the slides and then denatured at 95°C for 10 minutes. After incubation at 42°C overnight, the slides were washed twice for 10 minutes at room temperature with 2X SSC, twice for 10 minutes at 55°C with 2X SSC, and then twice for 10 minutes at 55°C with 1X SSC.

Immunological studies were performed by applying the anti-digoxigenin antibody conjugated with alkaline phosphatase (Boehringer Mannheim) to the slides. Color staining was observed at the site of the target nucleic acid by the addition of a solution containing a substrate-chromogen with nitroblue tetrazolium and also levamisole (Sigma Chemical Co., St. Louis, MO), which inhibits the endogenous alkaline phosphatase activity.[13] The positive control amplifications and hybridizations were carried out using primer pairs and probe for β-globin.[9] The omission of *Taq* polymerase or the primer pair in the PCR reaction or the use of a probe with digoxigenin-labeled pBR322DNA (Boehringer Mannheim) served as negative controls.

Results

In agreement with previously published reports, the DNA fragment of HHV-8 was detected by PCR in all 12 of the AIDS-KS tumor specimens and the KS tissues from the 2 HIV-negative homosexual men with KS.[1–4] Positive signals were also observed by PCR in 5 of the 12 uninvolved, normal-appearing skin specimens obtained from the AIDS-KS patients and 1 of the 2 uninvolved skin samples examined from the HIV-negative homosexual men with KS.

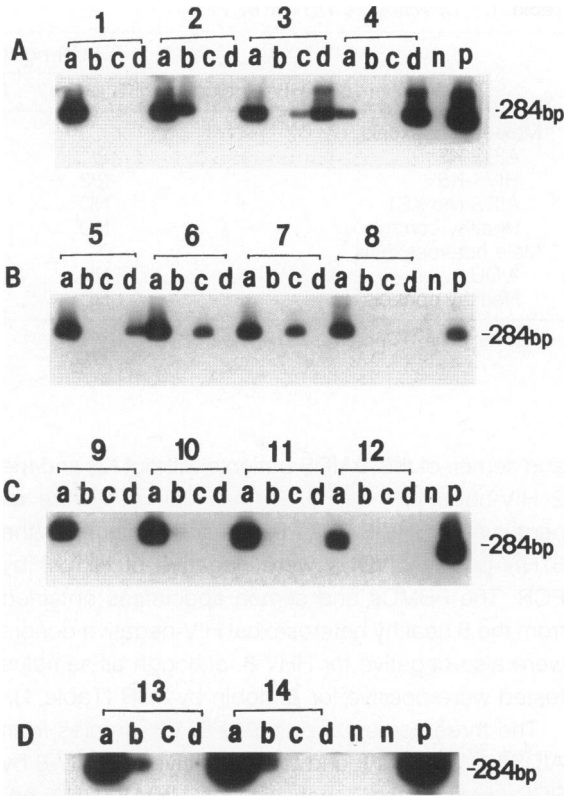

Figure 1. *Detection of herpesvirus-like DNA in 14 matched KS lesions, uninvolved skin, semen, and PBMCs by PCR followed by Southern blot hybridization with a specific HHV-8 probe.* **Lanes 1** *and* **2,** *specimens from an HIV seronegative patient with KS;* **lanes 3** *to* **14,** *samples from AIDS-KS; lanes* **a,** *KS tumor; lanes* **b,** *Uninvolved skin. lanes* **c,** *Semen. lanes* **d,** *PBMCs;* **n,** *negative controls;* **p,** *positive controls. The size of amplified products is shown on the right.*

The HHV-8 DNA was detected by PCR in 3 semen cell pellets and 3 PBMC samples from the 12 patients with AIDS-KS. Of the AIDS-KS patients, 5 were found to be positive for HHV-8 in either their semen and/or their PBMCs; only 1 of these cases was positive for HHV-8 in both his semen and PBMCs (Figure 1). In addition, positive signals for HHV-8 by PCR were observed in 3 samples of the B-lymphocyte-enriched fraction of PBMCs and in 1 sample of semen supernatant fluid from an AIDS-KS patient whose PBMCs and semen cell pellet were also positive for this virus. However, HHV-8 sequences were not detected by PCR in any of the sera samples examined, including those individuals whose PBMCs were positive. No positive signals for HHV-8 were detected in either the PBMCs or semen from the 2 HIV-negative homosexual men with KS. The PBMCs

Table 1. *Detection of HHV-8 by PCR*

Cases	HIV serology	PCR positive (+) for HHV-8/total tested				PCR-ISH	
		KS lesion	Normal skin	Semen	PBMCs	Semen	B lymphocyte
Male homosexuals							
AIDS-KS	+	12/12	5/12	3/12	3/12	3/12	3/12
HIV$^-$-KS	−	2/2	1/2	0/2	0/2	0/2	0/2
AIDS (no KS)	+	ND	ND	0/4	0/4	0/4	0/4
Healthy controls	−	ND	ND	0/2	0/2	0/2	0/2
Male heterosexuals							
IVDU	+	ND	ND	0/5	0/5	0/5	0/5
Healthy controls	−	ND	ND	0/5	0/5	0/5	0/5

ND, not done; +, positive; −, negative.

and semen of the 4 AIDS patients without KS and the 2 HIV-negative healthy homosexual men were all negative for HHV-8. The PBMCs and semen from the 5 HIV-positive IVDUs were negative of HHV-8 by PCR. The PBMCs and semen specimens obtained from the 5 healthy heterosexual HIV-negative donors were also negative for HHV-8, although all samples tested were positive for β-globin by PCR (Table 1).

The three semen and three PBMC samples from AIDS-KS patients found to be positive for HHV-8 by PCR were further analyzed for HHV-8 DNA sequences by genomic DNA Southern blot. No hybridized band was observed in any of these samples; however, positive bands were observed in Southern blots of the DNAs from eight of nine AIDS-KS tumor specimens examined (Figure 2).

Using the PCR-ISH method, positive signals were detected by amplification and hybridization with β-globin primers and probe (Figures 3A and 4A), whereas negative controls, amplification without *Taq* polymerase or using PBR 322 DNA probe, were negative. By using the primer pair and probe specific for HHV-8, some B cells were shown to be positive for HHV-8 DNA sequences in three blood samples from the PCR-positive specimens obtained from the AIDS-KS patients (Figure 3). Furthermore, positive signals by PCR-ISH were also observed in a few mononuclear cells and spermatozoa from three semen samples that had been shown first to be positive for HHV-8 by PCR (Figure 4). The positive stainings were largely confined to nuclei of cells. No positive staining was observed in either the smears of blood or semen cell pellets of two HIV-negative homosexual men with KS or from any of the control individuals without KS examined by PCR-ISH, including five HIV-positive homosexual men without KS, five healthy HIV-negative heterosexual males, or five HIV-positive heterosexual IVDUs.

Discussion

The sudden increased incidence of an aggressive, disseminated form of KS among young homosexual men in New York and California heralded the AIDS epidemic in 1981.[6–8] Homosexual men account for 95% of all AIDS-KS cases seen in the United States.[8] The occurrence of KS among otherwise healthy HIV-

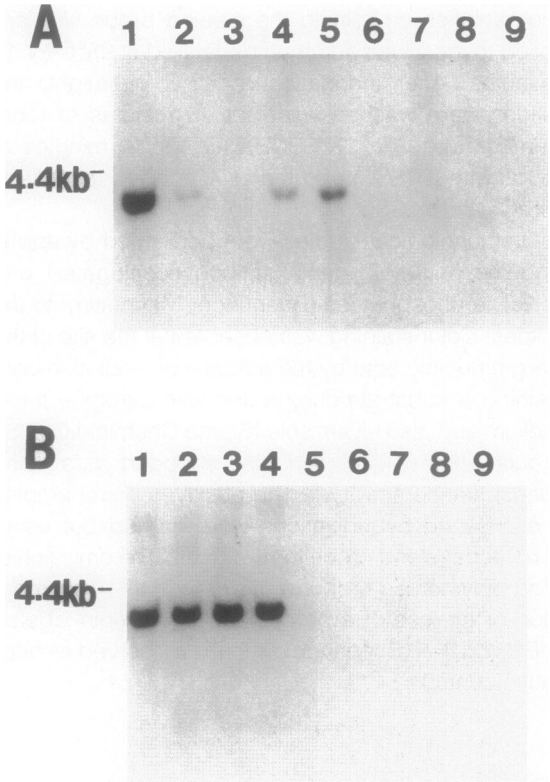

Figure 2. *Genomic DNA Southern blot hybridization for detection of HHV-8 in KS, semen, and PBMCs.* **A: Lanes 1** *to* **5,** *KS lesions;* **lanes 6** *to* **8,** *DNA of semen samples positive for HHV-8 by PCR;* **lane 9,** *control semen sample from healthy HIV-negative donor.* **B: Lanes 1** *to* **4,** *various KS lesions;* **lanes 5** *to* **7,** *DNA of PBMC samples positive for HHV-8 by PCR;* **lanes 8** *and* **9,** *control PBMC samples from healthy individuals.*

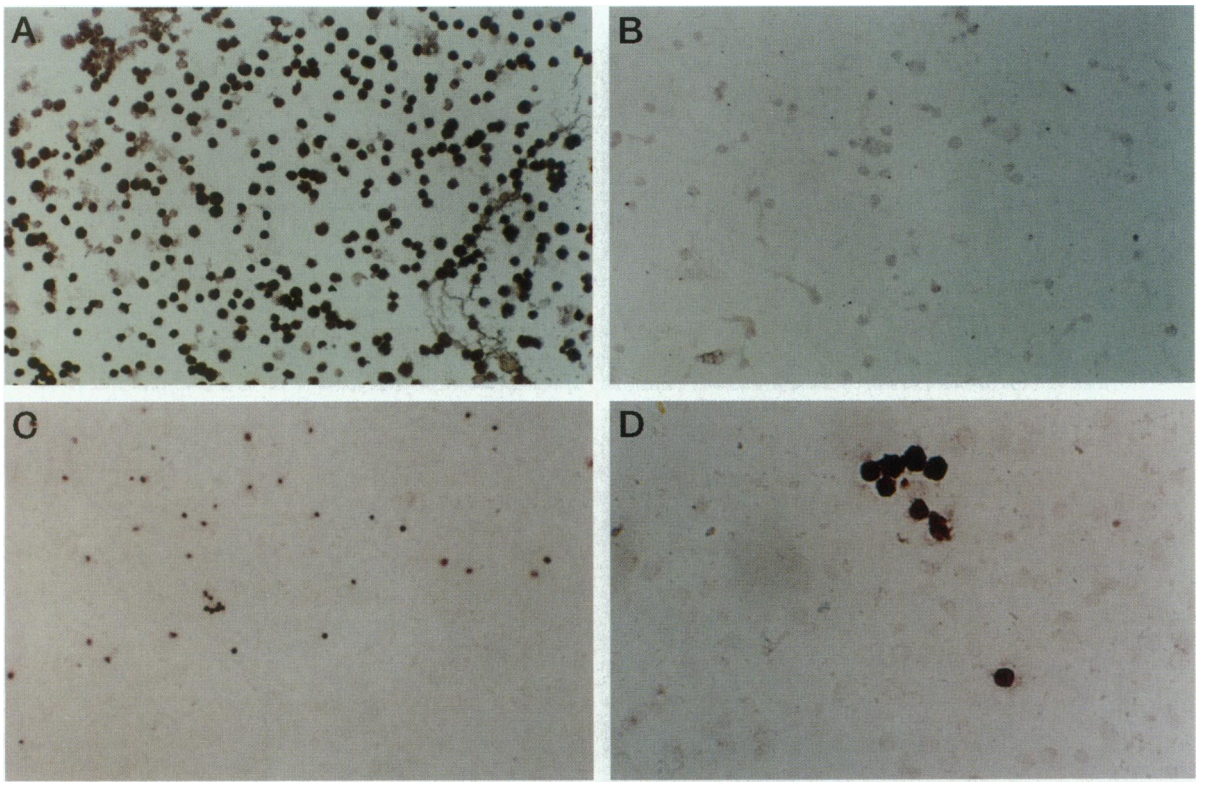

Figure 3. *Detection of HHV-8 DNA sequences in B lymphocytes of AIDS-KS patients by PCR-ISH.* A: *Detection for β-globin as a positive control. Magnification, ×200.* B: *Negative control using primer pair for β-globin and probe for PBR 322. Magnification, ×200.* C: *Positive signals were observed in some B lymphocytes from an AIDS-KS patient. Magnification, ×100.* D: *Higher magnification of* C *showing the positive stainings, whereas cells around are negative. Magnification, ×400.*

negative homosexual men has suggested that the epidemic form of KS predominantly seen among homosexual men with AIDS may be associated with a sexually transmissible agent, other than HIV, that may have been introduced into the homosexual male population at risk for AIDS at or about the same time as HIV.[7,8] The effective safer sex educational campaign that resulted in significant modifications in sexual behavior within the homosexual male community in the United States may have contributed to the remarkable reduction in the incidence of HIV infection in homosexual men and also to the decreased incidence of AIDS-KS from approximately 43% of the homosexual men with AIDS seen in the United States between 1981 and 1983 to less than 15% in 1994.[8] The prevalence of AIDS-KS in all of the other groups at risk for HIV infection has remained extremely low, despite the continuing increased incidence of HIV infection among heterosexual IVDUs, their female sexual partners, and children born to HIV-infected women.

The detection of HHV-8 DNA in semen and/or PBMCs of 3 of 12 of the AIDS-KS patients examined suggests that this virus could potentially be sexually transmitted in the semen of the patients with AIDS-KS. Other human herpesviruses such as Herpes simplex virus have been found in semen and has been infrequently detected in prostatic fluid or biopsied tissue.[14,15] HHV-8 DNA was detected in one sample of semen supernatant from an AIDS-KS patient in this study. The origin of HHV-8 DNA in this semen supernatant is not clear. As both PBMCs and semen pellet from this patient were positive for HHV-8 by PCR, the viral DNA detected in semen supernatant could be released from lysed sperm cells or lymphocytes in the semen, which both harbor HHV-8. However, other possibilities, such as HHV-8 infection of the prostate and the presence of HHV-8 in prostatic fluid in AIDS-KS patients needs to be examined.

This study confirms the high prevalence of HHV-8 in KS skin tumors from homosexual men with AIDS-KS and HIV-negative men with KS and the presence of this virus in normal-appearing skin of some of these patients as well. The detection of HHV-8 DNA sequences in 3 of 12 (25%) of the PBMCs samples from AIDS-KS patients studied by both PCR and PCR-ISH, indicates that the PBMCs can also be shown to be infected with this virus, which is similar to findings recently reported by other investigators.[16,17] In the PCR experiments reported here,

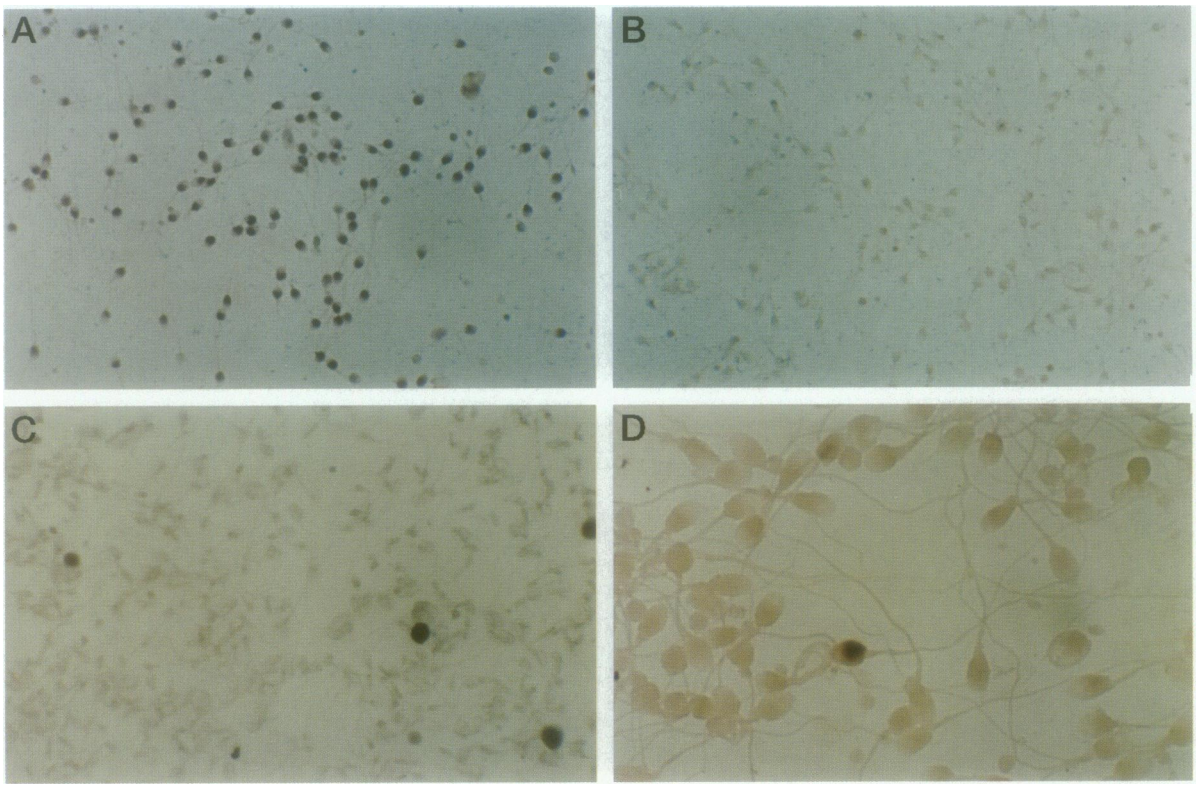

Figure 4. *Detection of DNA sequences by HHV-8 in semen samples by PCR-ISH.* **A:** *Smear of a semen sample shows positive staining using β-globin primers and probe as a positive control. Magnification, ×200.* **B:** *Semen sample shows negative staining when* Taq *polymerase is excluded from amplification step for β-globin. Magnification, ×200.* **C:** *Positive stainings were observed in several mononuclear cells and one sperm cell from an AIDS-KS patient. Magnification, ×200.* **D:** *HHV-8-positive signal in nucleus of a sperm cell from a patient with AIDS-KS. The surrounding sperm cells are negative. Magnification, ×1000.*

we included dUTP and uracil-*N*-glycosylase, which has been shown to prevent carry-over contamination between different specimens being examined simultaneously in our laboratory. A group of British investigators have recently found that 52% of PBMCs from patients with AIDS-KS were positive for HHV-8 using a nested PCR technique.[18] The differences between the rate of positivity in that study and the findings reported here may be due to the different PCR methods employed and/or differences in the prevalence of HHV-8 in the AIDS-KS patient population examined in the United States and Great Britain. Furthermore, it is the subset of B lymphocytes of the PBMCs that have been shown to contain HHV-8 DNA sequences; however, the B cells containing HHV-8 DNA were found to be infrequent. Using PCR-ISH, only 1 to 2% of the B lymphocyte population of the PBMCs from some patients with AIDS-KS examined were found to harbor HHV-8. We detected HHV-8 in 3 of the 14 semen samples examined from AIDS-KS patients; however, in none of the other PBMC or semen samples we examined by PCR was HHV-8 detected. A recent report has shown that HHV-8 was not detected in any of the 5 semen samples examined from AIDS-KS patients.[17] HHV-8 DNA sequences were detected by PCR in another study in which they found 30 of 33 (91%) frozen stored semen samples from HIV-1-positive homosexual men and 7 of 30 (23%) specimens from healthy donors.[17,19] The present study shows that both spermatozoa and mononuclear cells in semen can be shown to be infected by HHV-8, although the HHV-8-positive cells in semen were found to be less than 1% of the total cell population.

The DNA sequences of the HHV-8 agent were detected by PCR and PCR-ISH in some of the semen and/or PBMC specimens from the AIDS-KS patients examined; however, the viral sequences were not detected in these samples by either the conventional ISH method we had initially performed or by genomic DNA Southern blot analysis, suggesting that the copy number of the HHV-8 DNA sequences is much lower in semen and PBMC specimens than in the KS tumors from the same donors. These results are consistent with our PCR-ISH studies that showed that only a few cells in the PBMC B lymphocyte subset and semen samples examined were HHV-8 positive. Nevertheless, these data suggest that semen and

blood could serve as possible means of transmission for HHV-8 in homosexual men with AIDS-KS. The role of HHV-8 in the pathogenesis of KS and the epidemiology of this new human herpesvirus remain to be elucidated.

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