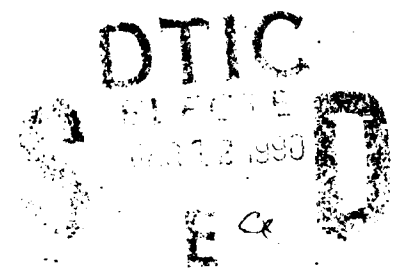


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Isolation of a new herpesvirus from human CD4⁺ T cells

(human herpesvirus 7/virus latency/T-cell activation)

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ABSTRACT A new human herpesvirus has been isolated from CD4⁺ T cells purified from peripheral blood mononuclear cells of a healthy individual (RK), following incubation of the cells under conditions promoting T-cell activation. The virus could not be recovered from nonactivated cells. Cultures of lymphocytes infected with the RK virus exhibited a cytopathic effect, and electron microscopic analyses revealed a characteristic herpesvirus structure. RK virus DNA did not hybridize with large probes derived from herpes simplex virus, Epstein-Barr virus, varicella-zoster virus, and human cytomegalovirus. The genetic relatedness of the RK virus to the recently identified T-lymphotropic human herpesvirus 6 (HHV-6) was investigated by restriction enzyme analyses using 21 different enzymes and by blot hybridization analyses using 11 probes derived from two strains of HHV-6 (Z29 and U1102). Whereas the two HHV-6 strains exhibited only limited restriction enzyme polymorphism, cleavage of the RK virus DNA yielded distinct patterns. Of the 11 HHV-6 DNA probes tested, only 6 cross-hybridized with DNA fragments derived from the RK virus. Taken together, the maximal homology amounted to 31 kilobases of the 75 kilobases tested. We conclude that the RK virus is distinct from previously characterized human herpesviruses. We propose to designate it as the prototype of a new herpesvirus, the seventh human herpesvirus identified to date.

Herpesviruses are large DNA-containing viruses that share architectural features of their virion, including a DNA core, an icosahedral capsid with 162 capsomers, an amorphous tegument, and an envelope (1). Members of the *Herpesviridae* family have been isolated from more than 80 different animal species. Six different herpesvirus of man have thus far been described. The most recent one to be identified (2), human herpesvirus 6 (HHV-6), is the causative agent of roseola infantum, a common childhood disease characterized by high fever and skin rash (3). HHV-6 exhibits predominant T-cell tropism (4-8).

In the course of a study of human immunodeficiency virus 1 (HIV-1) at the Naval Medical Research Institute, it was noted that a culture of uninfected activated CD4⁺ cells obtained from a healthy individual (RK) exhibited spontaneous cytopathic effect (CPE). The cells were negative for HIV-1 as judged by reverse transcriptase activity and absence of p24 antigen by antigen-capture assay. The cultures were transferred to the National Institute of Allergy and Infectious Diseases/Twinbrook, where a herpesvirus with distinct properties was isolated and characterized. Restriction enzyme and blot hybridization analyses of viral DNA indicated that the RK virus differed from HHV-6 and other known herpesviruses. According to the rules of the Herpesvirus Study Group of the International Committee for Taxonomy of viruses (9) this virus has been designated HHV-7.

MATERIALS AND METHODS

Purification of CD4⁺ T Cells and T-Cell Activation. CD4⁺ T cells were isolated from peripheral blood lymphocytes (PBLs) by negative selection using immunoadsorption with goat anti-mouse immunoglobulin-coated magnetic particles, as previously described (10, 11). The cells were >99% CD2⁺ and >96% CD4⁺, as determined by flow cytometry. Monocytes were <0.1% as determined by staining with nonspecific esterase. For T-cell activation the cultures were incubated for 2 days with plastic-immobilized CD3 monoclonal antibody (mAb) G19-4 (11). To maintain cell proliferation, the cells were further cultured with interleukin 2 (IL-2; Calbiochem) at 30 units/ml or with CD28 mAb 9.3 (12). Cultures were restimulated at weekly intervals with plastic-immobilized CD3 mAb. The cells were cryopreserved in 7.5% dimethyl sulfoxide.

Virus Propagation. The Z29 strain of HHV-6 (6) was obtained from C. Lopez (Centers for Disease Control, Atlanta). The U1102 strain (5) was obtained from R. W. Honess (National Institute of Medical Research, London). HHV-6 (Z29) was propagated in PBLs as described (13). Briefly, PBLs were precultured for 3 days in RPMI-10% medium [RPMI 1640 medium plus gentamicin (50 µg/ml) with 10% heat-inactivated fetal bovine serum] containing phytohemagglutinin (PHA; Difco) at 10 µg/ml. Infection was done in RPMI-10% medium with PHA at 5 µg/ml. HHV-6 (U1102) and HHV-7 (RK) were similarly propagated in PHA-pretreated PBLs. However, the infection was done in RPMI-10% medium.

Electron Microscopy. Cell pellets were treated sequentially with glutaraldehyde and osmium tetroxide (1% each for 30 min in 0.1 M cacodylate buffer, pH 7.2), stained with 1% uranyl acetate, and dehydrated in graded ethanol solutions (30% to 100%). After propylene oxide treatment the samples were embedded in PolyBed 812 (Polysciences) and sectioned with an ultramicrotome. Sections of thickness 70-80 nm were examined in a Philips-300 electron microscope.

Preparation of Infected-Cell DNA. Unlabeled infected cells were harvested at the peak of CPE. ³²P-labeling of infected cell DNA was as described (13).

DNA Probes. pHeHE was cloned by A. Marchini and E. Kieff (Harvard University). It contains the EBNA-1 and Ori-P DNA sequences of Epstein-Barr virus (EBV). The cloned *EcoK* fragment of varicella-zoster virus (VZV) DNA and purified intact VZV DNA were obtained from W. T. Ruyechan (Uniformed Services University of the Health Sciences). pON203, obtained from E. S. Mocarski (Stanford

University). contains human cytomegalovirus (HCMV) DNA sequences. HCMV infected-cell DNA was prepared from human foreskin fibroblasts infected with HCMV (Towne). pHD9, pSMD2, and pSAD2a are clones of HHV-6 (U1102) obtained from R. W. Honess (National Institute of Medical Research, London). pNF182 contains the *Bgl* II N fragment of herpes simplex virus 2 (HSV-2) strain 333 DNA (R. R. Spaete and N.F., unpublished results). pNF1021, pNF1022, pNF1019, and pNF1023 contain *Sal* I fragments of HHV-6 (U1102) DNA, whereas pNF1001, pNF1006, pNF1010, and pNF1013 contain *Sal* I or *Hind* III fragments of HHV-6 (Z29) DNA (R.M.D., D. Di Luca, and N.F., unpublished results).

Southern Blot Hybridization. DNA was digested with restriction enzymes and electrophoresed in 0.7% agarose gels. Following ethidium bromide staining, the DNA was blotted onto Nytran (Schleicher & Schuell). The membranes were prehybridized overnight at 67°C in hybridization buffer containing 6 × SSC (1 × is 0.15 M NaCl/0.015 M sodium citrate, pH 7), 2 × Denhardt's solution (14), 0.5% SDS, and 150 µg of denatured salmon sperm DNA per ml. The probe DNA was labeled with [α -³²P]-dCTP (NEN) by using the multiprime DNA labeling system (Amersham). Hybridization was at 67°C in hybridization buffer (50 µl/cm²) containing 300,000 cpm/ml. The blots were washed at 67°C in 6 × SSC, 1 × SSC, and 0.5 × SSC prior to autoradiography.

RESULTS

Initial Isolation of HHV-7 Strain RK. HHV-7 (RK) was isolated following activation of CD4⁺ T cells that were purified from the peripheral blood of a healthy individual, RK. RK, age 26, remains healthy 1 year after virus isolation. He had no recent history of infection with known human herpesviruses, except a VZV infection at age 22. *Mycobacterium tuberculosis* infection was diagnosed at age 23 by conversion to a positive skin reaction with purified protein derivative. He had no significant exposure to animals. He had no blood transfusions and has been seronegative for hepatitis B virus and HIV-1.

To induce T-cell activation, the CD4⁺ cells were preincubated for 2 days with immobilized CD3 mAb. One portion of the treated culture was then incubated with IL-2 at 30 units/ml. A second portion was incubated with CD28 mAb without added IL-2 (10, 12). Aliquots of both cultures were cryopreserved before activation (day 0) and on days 6, 9, and 13 after initiation of culture. CPE was noted in both cultures by day 15. Cocultivation of the cells with fresh PBLs in the presence of PHA at 5 µg/ml resulted in the propagation of an agent exhibiting CPE characterized by ballooning of cells and the appearance of small syncytia. Cells showing such CPE are shown in Fig. 1 *b-d*; mock-infected counterparts are shown in Fig. 1*a*. The new virus will be referred to as HHV-7 (RK) on the basis of further analyses described below.

Repeated Isolation of HHV-7 (RK). To test whether the new virus originated from the purified CD4⁺ cells or from the PBL culture used for cocultivation, we analyzed the cultures, which were frozen as described above, using different PBLs for cocultivation. The test cultures included (i) the nonactivated cells frozen on day 0; (ii) replicate cultures treated with CD3 mAb and then frozen 6, 9, and 13 days after the addition of the CD28 mAb; (iii) cultures treated with CD3 mAb and frozen 9 and 13 days after IL-2 addition; (iv) nonactivated CD4⁺ T cells from RK, which were obtained and frozen 5 months prior to the present experiment (–5-month culture); (v) CD4⁺ T cells purified from a second healthy individual (DS) and frozen prior to activation; and (vi) CD4⁺ T cells from DS frozen after activation with CD3 mAb plus CD28 mAb.

The fraction of cells containing infectious virus was determined by cocultivating 10-fold dilutions of cells from the cryopreserved cultures with excess mitogen-activated fresh



FIG. 1. Virion structure and CPE exhibited by HHV-7 (RK). (*a-d*) Cells viewed in the light microscope after mock infection (*a*) or 7 days after infection with HHV-7 (RK) (*b-d*). In *b-d*, note refractile ballooning cells with distinct binding membrane and cells that appear to have undergone fusion. (×600.) (*e* and *f*) Virions viewed in the electron microscope. (×41,000 and ×118,000, respectively.)

PBLs. Eight cultures were tested for each dilution, starting with 10⁵ test cells per culture. For cultures where no infection was noted, the cells constituting the first cocultivated dilution were blindly cocultivated with fresh PBLs one more time to allow further amplification of any virus.

The results revealed the following. No virus could be isolated from the cells derived from the second individual (DS) or the nonactivated RK cells from day 0 or the –5-month cultures. Similarly, no virus could be propagated by cocultivation of the 6- and 9-day activated cultures. However, virus could be propagated from the CD3 mAb/IL-2-activated culture (isolate 1) and CD3/CD28 mAb-activated culture (isolate 2) 13 days after T-cell activation. It was calculated that the two cultures contained 1 infected cell per 1.6 × 10⁵ and 2 × 10⁵ cells, respectively.

Electron Microscope Analyses of the RK Virus. Analysis of thin sections prepared from cells infected with isolate 2 revealed typical herpesvirus virions (1), 170 nm in diameter and containing an electron-dense cylindrical core, a capsid, a tegument, and an envelope (Fig. 1 *e* and *f*). The virions contained a very distinct tegument layer between the capsid and the envelope, similar to that observed in HHV-6 virions (E.R. and N.F., unpublished data).

Restriction Enzyme Analyses of Viral DNA. Because of the T-cell origin of the RK virus, we suspected that it was a strain of HHV-6. Cells were infected with isolates 1 and 2 and with the HHV-6 strains Z29 (6) and U1102 (5). We have previously shown that host DNA replication is shut off after HHV-6 infection (13). Therefore a significant fraction of [³²P]orthophosphate can be preferentially incorporated into viral DNA. ³²P-labeled HHV-6 and HHV-7 infected-cell DNAs were analyzed using 21 restriction enzymes. Representative patterns are shown in Fig. 2. The results can be summarized as follows. (i) The restriction enzyme patterns of the HHV-6 strains Z29 and U1102 were not identical but were generally similar and many of the fragments comigrated in the gel. It should be noted that variation in a single restriction enzyme site is expected to result in nonidentity of three bands when the two patterns are compared. (ii) In contrast, the cleavage patterns of the RK isolates differed significantly from the Z29 and U1102 patterns. In fact, the majority of the fragments did not comigrate. Some enzymes produced a different distribution of fragments: large fragments from the RK virus DNA compared to relatively small fragments from the Z29 and U1102 DNAs (e.g., *Sal* I, *Pst* I, *Sac* I, and *Mlu* I, Fig. 2) and vice versa (*Hind* III, Fig. 2). (iii) The patterns exhibited by isolates 1 and 2 of the RK virus were very similar and a majority of the fragments comigrated in the gel. However, in

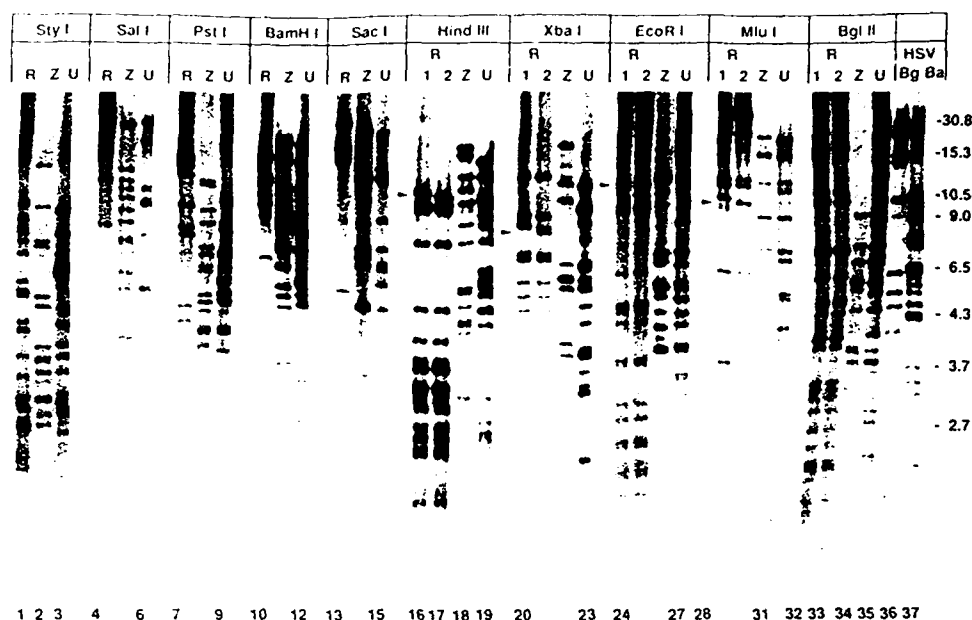


FIG. 2. Restriction enzyme patterns. 32 P-labeled DNA was prepared from PBL cultures infected with HHV-6 strain U1102 (U) or Z29 (Z) or with HHV-7 strain RK (R). Cleavage of DNA from isolates 1 and 2 with *Hind*III, *Xba*I, *Eco*RI, and *Mlu*I produced similar patterns except the variant bands marked with arrowheads. The *Bgl*II patterns exemplify patterns in which no differences can be detected in the migration of fragments of isolates 1 and 2. In the *Sty*I, *Sal*I, *Pst*I, *Bam*HI, and *Sac*I patterns, no differences are noted and hence only isolate 2 is shown. The lack of variations with some enzymes probably reflects variable bands that comigrate with other bands in the gel. Lanes 36 and 37 contain size markers consisting of *Bgl*II (Bg)- or *Bam*HI (Ba)-cleaved 32 P-labeled DNA from Vero cells infected with HSV-1 (Justin). Marker sizes are indicated in kilobases (kb) at right.

some of the patterns some subtle differences were noted (arrowheads, Fig. 2). The explanation for these minor variations awaits the results of further mapping analyses and of studies designed to test the stability of the viruses after clonal purification and serial propagation. The current data are consistent with the isolates representing genetic variants of the RK virus. These variants could have arisen from independent activation of genomes latent in the original CD4⁺ T cells or from deletion and/or other alterations of sequences during virus propagation in culture.

Homology to pHD9 Clone of HHV-6 (U1102). The marked differences between the restriction enzyme patterns of RK virus DNA and those of the two HHV-6 strains raised the question whether these viruses share DNA sequences. DNA from cells infected with the RK virus or with HHV-6 strain Z29 or U1102 was cleaved with restriction enzymes, blotted, and hybridized with a number of probes derived from the two HHV-6 strains. In the first hybridization (Fig. 3, lanes 1–27), DNA from the RK virus and HHV-6 strains Z29 and U1102 was cleaved with 11 enzymes and hybridized with the probe pHD9, which contains a 9-kb *Hind*III insert of U1102 DNA. The probe hybridized strongly to both U1102 and Z29 DNAs, yielding very similar patterns. In contrast, no hybridization was noted in the RK lanes. Three lines of evidence suggest that the lack of hybridization represented lack of homology between RK DNA and the probe sequences rather than the presence of insufficient amounts of RK virus DNA on the blot. (i) Ethidium bromide staining of the gels prior to blotting revealed the presence of large amounts of infected-cell DNA (data not shown). (ii) The same quantities of DNA were loaded onto sister blots which gave positive signals with other probes (see below). (iii) After autoradiographic exposure, the blot shown in Fig. 3 was hybridized with RK virus infected-cell DNA, labeled *in vivo* with 32 P. The specific activity of the *in vitro* labeled pHD9 probe was >1000-fold higher than that of the *in vivo* labeled infected-cell DNA. Nonetheless, the *in vivo* labeled DNA hybridized to the homologous RK DNA on the blot, yielding all of the expected bands in the pattern (Fig.

3, lanes 28–31). No additional hybridization was noted with the heterologous HHV-6 strains Z29 and U1102 and the only visible bands remained the previously hybridized pHD9 bands. This result confirms our conclusion that sufficient RK DNA was available on the blot. Therefore, the lack of pHD9 hybridization reflected the lack of homology.

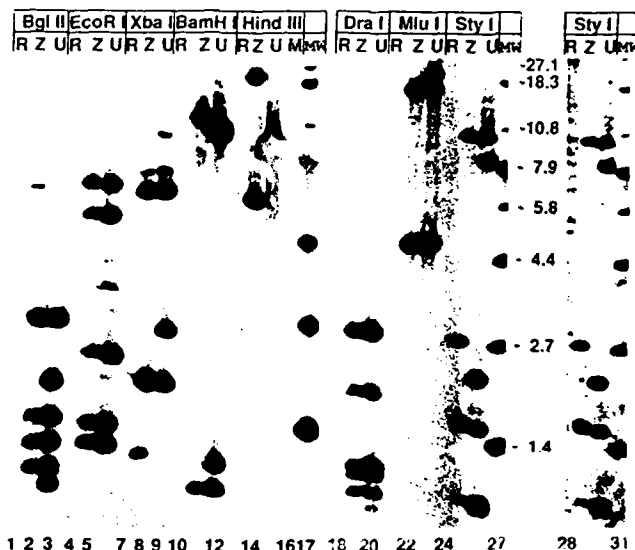


FIG. 3. Hybridization of pHD9 to infected-cell DNAs. Lanes 1–27: hybridization with 32 P-labeled pHD9 probe derived by Honess and coworkers from HHV-6 (U1102). The blot contained restriction enzyme-cleaved DNAs from mock-infected cells (M) or from cells infected with HHV-6 strain Z29 (Z) or U1102 (U) or with RK virus (R). The blot was then hybridized (without stripping the pHD9 probe) with 32 P-labeled RK virus DNA labeled *in vivo*. Lanes 28–31: rehybridized blot portion shown in lanes 24–27. Lanes 17, 27, and 31: molecular weight (mw) markers that hybridize with the plasmid portion of the pHD9 probe; sizes are indicated in kilobases.

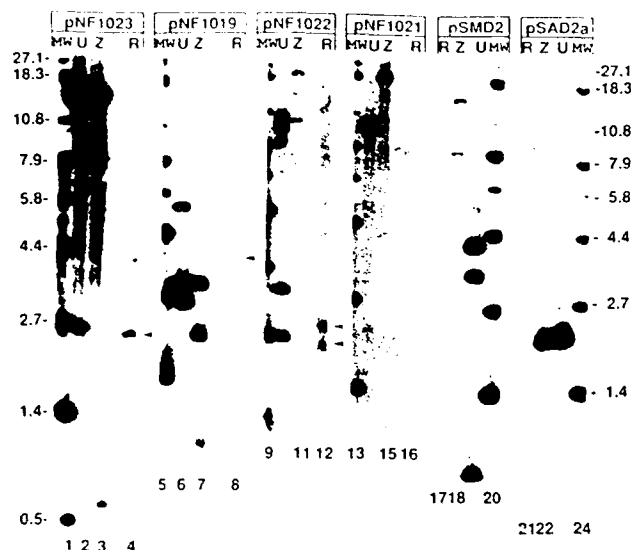


FIG. 4. Hybridization of HHV-6 (U1102) probes to HHV-6 [Z29 (Z) or U1102 (U)] and to RK virus isolate 2 (R) DNAs. *Hind*III-digested DNA was blotted and hybridized with the ³²P-labeled probes shown. MW, size markers that hybridize with the plasmid portions of the probes. Arrowheads point to the hybridizing HHV-7 (RK) bands.

Hybridizations with Other U1102 and Z29 Probes. Additional analyses with six U1102 probes and four Z29 probes are shown in Figs. 4 and 5. The results can be summarized as follows. The U1102 probes pNF1021, pSMD2, and pSAD2a, containing inserts of 6 kb, 2.1 kb, and 2.3 kb, respectively, did not hybridize with RK virus DNA (Fig. 4, lanes 14–24). The remaining three probes, pNF1023, pNF1019, and pNF1022, containing inserts of 11.5 kb, 5.8 kb, and 3.9 kb, respectively, yielded weak hybridization bands (Fig. 4, arrowheads, lanes 1–13). In each case, the aggregate molecular size of the hybridized fragments was lower than the complexity of the U1102 fragment used as probe. For example, the 11.5-kb insert in pNF1023 hybridized only to a 2.3-kb RK virus DNA

fragment (Fig. 4, lane 4). We estimate that a maximum of 11.2 kb hybridized with the 40.5 kb of U1102 test probes.

The four Z29 probes used were *Sal* I or *Hind*III clones containing relatively large inserts of sizes 5, 8, 8, 9, and 12 kb. Of these clones, pNF1013 (5-kb insert) did not hybridize to RK virus DNA (Fig. 5, lane 24). The larger clones revealed weak hybridization apparent only after lengthy autoradiography (Fig. 5, lanes 4, 9, and 20). Once again, the aggregate sum of the molecular sizes of the hybridizing fragments was lower than the size of the Z29 test insert. The probes, totaling 34.8 kb, hybridized to RK DNA fragments totaling 19.6 kb. The lower intensity of hybridizing bands might reflect incomplete sequence homology resulting in less stable hybrids. Thus, these estimates most likely represent maximal values of homology. Once again, the fact that some hybridization was noted serves as a control for the blotting efficiency.

Hybridization Analyses Using Probes from Other Herpesviruses. Additional tests were done using probes derived from other herpesviruses, including EBV, HCMV, VZV, and HSV-2. No hybridization was noted with these probes (Fig. 6). We conclude that RK virus is not identical with these previously characterized human herpesviruses. However, these hybridizations do not exclude the possibility that RK virus DNA contains more highly conserved sequences of herpesvirus genomes.

DISCUSSION

We have isolated a new herpesvirus, HHV-7 strain RK, from activated CD4⁺ T cells. Viral DNA did not cross-hybridize with DNA probes derived from HSV, EBV, VZV, and HCMV. HHV-7 also differed from the previously character-

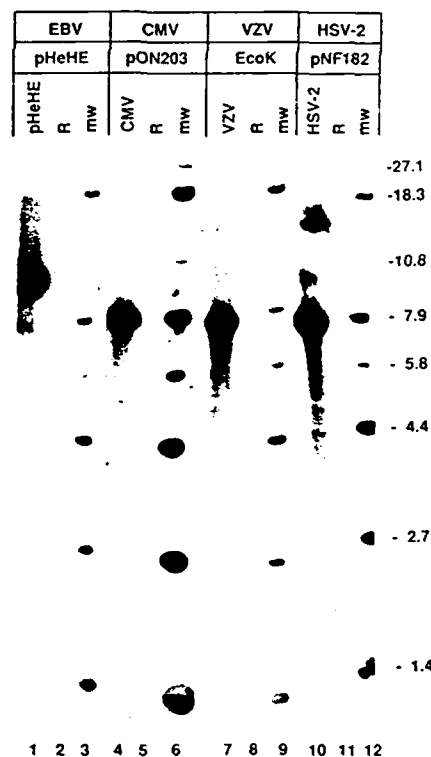


FIG. 6. Hybridization tests using probes derived from other herpesviruses. The blot contained DNA from HHV-7 (RK)-infected cells (lanes 2, 5, 8, and 11), pHeHE DNA (lane 1, *Bam*HI-digested), HCMV (Towne)-infected cell DNA (lane 4, *Bam*HI-digested), VZV DNA (lane 7, *Eco*RI-digested), and DNA from HSV-2-infected cells (lane 10, *Bgl* II-digested). The probes contained 2-kb EBV DNA (pHeHE), 7.6-kb HCMV DNA (pON203), 7.3-kb VZV DNA (*Eco*K), and 7.6-kb HSV-2 DNA (pNF182) fragments. mw, Size markers.

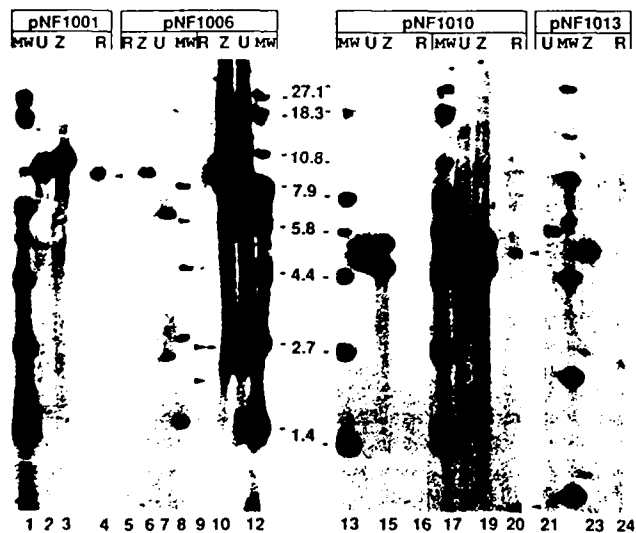


FIG. 5. Hybridizations using HHV-6 (Z29) probes. Blots containing *Hind*III digests of DNAs from cells infected with HHV-6 [Z29 (Z) or U1102 (U)] or with RK isolate 2 (R) were hybridized with the HHV-6 (Z29) probes shown. Lanes 9–12 show long exposure of lanes 5–8, and lanes 17–20 show long exposure of lanes 13–16. The HHV-7 (RK) hybridized bands are marked with arrowheads. MW, size markers.

ized T-lymphotropic HHV-6 by several criteria. (i) While the DNAs of the Z29 and U1102 strains of HHV-6 exhibited limited restriction enzyme polymorphism, the digestion patterns of HHV-7 DNA were very distinct. (ii) Large DNA fragments of HHV-6 DNA did not hybridize to HHV-7 DNA or exhibited only partial homology. By comparison, the genomes of HSV-1 and HSV-2 (which are classified as different herpesviruses) are colinear and exhibit cross-hybridization with all large probes. Preliminary data indicate that HHV-6 possesses homology to limited HCMV DNA sequences (ref. 15; E.C.S. and N.F., unpublished results). HHV-7 also cross-hybridizes with some HCMV sequences (data not shown). Thus, there are sequences that appear to be conserved among the cytomegalovirus-like viruses, including HHV-6 and HHV-7. Eventually, more precise definition of the relatedness of these viruses will come from comparison of their DNA sequences. (iii) Preliminary results (L.S.W. and N.F., unpublished results) have revealed that HHV-7 differs from HHV-6 with respect to antigenic properties of its proteins. Such differentiation will be essential in studies designed to determine the prevalence of HHV-7 infection in humans. In addition, because HHV-6 and HHV-7 exhibit similar CPE, it remains to be determined whether HHV-7, like HHV-6 (3), is involved in roseola infantum and/or other human diseases.

Activation from Latency. Several lines of reasoning support the hypothesis that the HHV-7(RK) isolates were activated by conditions leading to T-cell activation. Specifically, no virus was recovered from the nonactivated CD4⁺ T cells by cocultivation with fresh PBLs, nor could it be recovered from the activated cells 6 or 9 days after activation. Virus was present, however, in the 13-day cultures. The inability to recover virus in the earlier cultures and the recovery of isolates 1 and 2 in the 13-day cultures could reflect a low level of an ongoing asymptomatic infection *in vivo*. Alternatively, it is possible that virus genomes were associated with the cells in a latent state and were independently reactivated at low frequency following the induction of cell proliferation. Finally, the two isolates could have reflected spontaneous deletions/variations arising during virus propagation in culture.

P. Pellet and C. Lopez (personal communication) have characterized DNA variants of HHV-6 (Z29) differing in a small region of their genome. In our own studies, we were able to purify the two HHV-6 (Z29) variants and we have shown that they were genetically stable through at least 15 serial passages. Although the details of events leading to the generation of these variants are not clear, it is tempting to speculate that latent variants present in the same individual were reactivated in the same cell population, resulting in mixed virus stocks.

HHV-7 Is a Newly Recognized Herpesvirus. In the course of studies of HHV-6 we have found that virus replication is enhanced by T-cell activation (N.F., E.C.S., G.K., and C.H.J., unpublished results). These studies led us to postulate that T-cell activation plays a role in the activation of HHV-6 from a putative latent state. The finding of HHV-7, though unexpected, is not entirely surprising. It is yet another example that the identification of new viruses can be facilitated by the ability to grow and propagate their host cells (16)

and by exposing these cells to conditions that affect the state of viruses that these cells are harboring. Herpesviruses typically remain latent in their host throughout the life of their host. It can therefore be expected that new members of this virus family will be identified following the development of procedures for the cultivation of differentiated cells. Further studies will be necessary to determine the prevalence of these new viruses in the human host and to assess their potential role in human disease.

A preliminary report of these studies was presented in a special symposium in honor of Dr. Bernard Roizman at the University of Chicago, May 1989. We thank Ms. Nancy Craighead for excellent technical assistance; Mr. Douglas M. Smoot for flow cytometry; Dr. Donald Smith (Blood Bank Naval Hospital) and Mr. Eldon W. Hayes for leukopheresis; workers at the National Institutes of Health blood bank for buffy coats; Dr. J. A. Ledbetter (Oncogene Corp.) for the mAbs; Dr. Robert W. Honess (National Institute of Medical Research, London) for the pH9, pSAD2a, and pSMD2 clones; Dr. William T. Ruyechan (Uniformed Services University of the Health Sciences) for the VZV DNA and the *EcoK* clone; Dr. Elliott Kieff (Harvard University) for the pHeHE clone of EBV; and Dr. Edward S. Mocarski (Stanford University) for the gift of pON203. We thank Dr. Bernard Roizman for most helpful discussions and Dr. Gerald L. Linette for comments on the manuscript.

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