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Immunobiology and Prophylaxis of Human Herpesvirus Infections

IMMUNOBIOLOGY AND PROPHYLAXIS OF HUMAN HERPESVIRUS INFECTIONS

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PLENUM PRESS • NEW YORK AND LONDON

Preface

> No one whose opinion deserves a moment's consideration can doubt that most of the great positive evils of the world are in themselves removable, and will, if human affairs continue to improve, be in the end reduced to narrow limits.
>
> J. S. Mill, *Utilitarianism*, II, 1863

Mill was not writing about herpesviruses, but had he known them as we do, he would have included them among the great positive evils of the world. They cause disease and premature death, and are very costly to our society. There is no loftier aim than to cure or prevent human infections with these viruses.

The objective of much of the current research on herpesviruses is directed toward an understanding of the molecular mechanisms involved in initiation of infection, establishment and termination of latent state, virus multiplication, and the destruction of cells which ultimately is the basis of the diseases caused by these viruses. At no time during the past 80 years, since members of the herpesvirus family were first discovered, has there been so much progress in our understanding of the biology of these viruses as in the past few years. Along with the development of a greater understanding of the molecular biology of the well-known herpesviruses we have witnessed the isolation of new human herpesviruses. This book deals with the new viruses and the molecular basis of diseases caused by viruses that have been known to us for some time, with diagnostic tools, current or promising therapeutic agents, and vaccination. It reflects not merely the results of current research but also the current ideas in the field. Without the efforts of the contributors of this book, there would be no hope of reducing the threat of infection with these agents, and to them we dedicate this book. While the book reflects the scientific advances of the field, its format and organization are the work of Erik Carlson, the true editor of this volume.

The Editors
April, 1990

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Cellular and Growth-Factor Requirements for the Replication of Human Herpesvirus 6 in Primary Lymphocyte Cultures

Niza Frenkel,[1,*] Ehud Roffman,[1] Eric C. Schirmer,[1] George Katsafanas,[1] Linda S. Wyatt,[1] and Carl H. June[2]

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Introduction

Human herpesvirus 6 (HHV-6) was first isolated in 1986 from peripheral blood of AIDS patients and patients with lymphoproliferative disorders (1). Yamanishi and coworkers (2, 3) have further shown that HHV-6 is the causative agent of exanthem subitum (roseola infantum), a common childhood disease characterized by high fever and skin rash. Analyses of HHV-6 cell tropism have yielded complex results. The GS strain isolated by Salahuddin et al. (1) was shown to replicate in a variety of established cell lines including B cells positive for Epstein-Barr virus, continuous T-cell lines, megakaryocytes, and glioblastoma cells (4). Other HHV-6 strains were reported to replicate in continuous T-cell lines (5, 6). In contrast, the Z29 strain does not readily replicate in these cells (7, 8). While more quantitative studies are needed to evaluate the relative efficiencies of virus replication in different cell types, it is commonly accepted that HHV-6 isolates readily replicate in T cells (5–7, 9). Moreover, Takahashi et al. (10) reported that HHV-6 isolates infect predominantly $CD4^+$ T cells in the peripheral-blood lymphocytes (PBL) of exanthem subitum patients.

As part of our studies of HHV-6 replication we began a series of experiments designed to elucidate cellular and molecular requirements for virus replication. Since the T lymphocyte is a quiescent, nondividing cell unless activated by a specific antigen or nonspecific mitogens, we have asked whether T-cell activation was a prerequisite for HHV-6 replication, and whether the efficiency of virus propagation was affected by interleukin 2 (IL-2). The results of these studies are reviewed below.

Results

HHV-6 Replication is Enhanced by T-Cell Activation

Three sets of studies revealed that T-cell activation enhances HHV-6 replication and that efficient virus replication occurs only in host cells capable of responding to cell activation signals.

In the first study (L. S. Wyatt, N. Balachandran, and N. Frenkel, manuscript in prepara-

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tion) virus replication was compared in nonactivated PBL and in PBL exposed to the polyclonal T-cell mitogen phytohemagglutinin (PHA). We employed in this work the HHV-6 strain U1102, isolated by Downing et al. (5), and strain Z29, isolated by Lopez et al. (7). The two strains differed in their ability to propagate in continuous cell lines. Thus, the U1102 strain was shown to replicate readily in T-cell lines (5), whereas the Z29 strain does not replicate in many of these cell lines (8, and L. S. Wyatt and N. Frenkel, unpublished results). Replication efficiency in the activated and nonactivated PBL was assessed by viral cytopathic effects (CPE), the yields of infectious virus, the accumulation of viral DNA, and the presence of viral antigens as determined by immunofluorescence (IF) assays employing an HHV-6 monoclonal antibody (11). These analyses are exemplified in figure 1 with data from the infectious virus yields. The results show that the replication of the U1102 and Z29 strains in fresh peripheral-blood lymphocytes is significantly enhanced by T-cell activation.

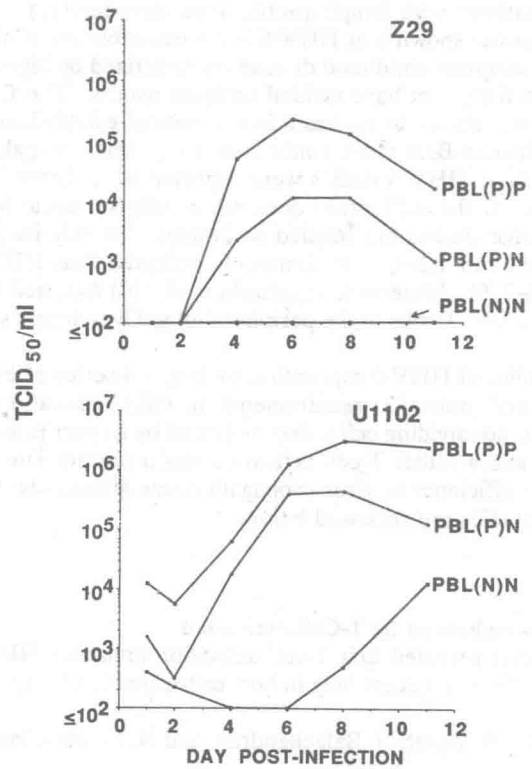

Figure 1. Infectious virus yield of HHV-6 strains in nonactivated and activated PBL cultures. PBL cultures were incubated in the absence or in the presence of PHA prior to infection with HHV-6 strains U1102 and Z29. PBL(N)N = no PHA present prior to or during infection; PBL(P)N = PHA present only prior to infection; PBL(P)P = PHA present prior to and during infection. Data are from L. S. Wyatt, N. Balachandran, and N. Frenkel (manuscript in preparation).

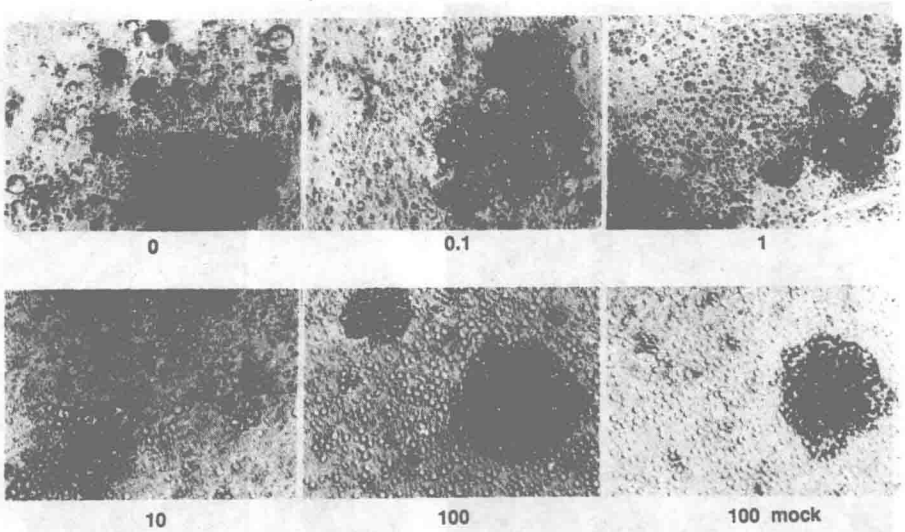

Figure 2. Bright field light microscopy demonstrating the effect of different IL-2 concentrations on HHV-6-infected mature thymocytes. Data are from reference (12).

In a second study (N. Frenkel, E. Schirmer, G. Katsafanas, and C. H. June, manuscript in preparation) we tested the ability of HHV-6 (Z29) to replicate in freshly isolated purified T cells. The results of this study showed that virus replication in the purified T cells required PHA as well as the addition of low concentrations of exogenous IL-2 (see below), suggesting that full T-cell activation mediated by interaction of IL-2 with its receptor was required for virus replication.

Finally, a third line of evidence comes from studies with human thymocytes. This study (12) involved analyses of virus replication in mature and immature thymocytes, separated on the basis of their agglutination with peanut agglutinin. The results of this study showed that immature thymocytes were not responsive to PHA-mediated cell activation and did not support virus replication as judged by CPE and IF assays. In contrast, pronounced virus replication was observed in the mature PHA-activated thymocytes.

IL-2 at High Concentrations Inhibits HHV-6 Replication

T-cell activation results in the accumulation of multiple soluble and cell-associated proteinaceous T-cell products (13). One of these soluble molecules, IL-2, is an obligatory mediator for the completion of the T-cell cycle (14). In the course of the studies concerning T-cell activation we were surprised to note that virus replication was inhibited by high concentrations of IL-2. The inhibitory effect was noted with both natural and recombinant IL-2 preparations. Two sets of studies were done. In the first study, mature thymocyte cultures were treated first with PHA, allowing cell activation. The cells were then infected with HHV-6 (Z29) in the absence of exogenously added IL-2 or in the presence of recombinant IL-2 at concentrations of 0.1, 1, 10, and 100 U/ml. Virus replication was first assessed by CPE and by the presence of antigens detected by IF assays using an HHV-6 specific monoclonal antibody derived by Balachandran et al. (11). Figure 2 shows the CPE observed in these thymocyte cultures by day 7 postinfection. The culture infected in the absence of exogenous IL-2 exhibited considerable CPE as judged by the appearance of multinucleated and giant cells, which are the hallmark of HHV-6 CPE. By day 9 postinfection 53% of these cells contained abundant viral antigens as judged by a bright IF staining. Considerable CPE was visible also in the cultures containing 0.1 and 1 U exogenous

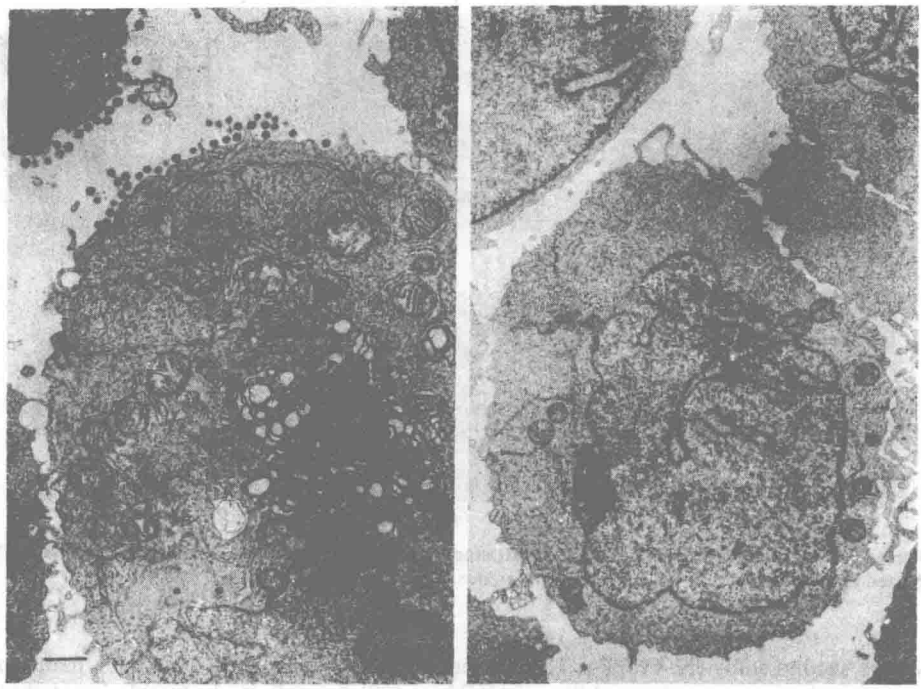

Figure 3. Electron micrographs of mature thymocytes infected with HHV-6 in the presence of (*a*) 1 U and (*b*) 100 U recombinant IL-2/ml. The bar represents 1 μm.

IL-2/ml. In contrast, addition of 10 U IL-2/ml significantly reduced the observed CPE, and no visible infection was noted in the culture incubated with 100 U IL-2/ml. On day 9 postinfection an estimated 20% of the cells incubated with 100 U IL-2/ml appeared to contain viral antigens as determined by the IF assay. However, the abundance of the HHV-6 antigens in the cells was low, inasmuch as only faint fluorescence was observed. We conclude on the basis of these results that IL-2 at high concentrations interferes with virus replication and with the normal progression of virus infection in mature thymocytes.

Additional analyses of the infected thymocyte cultures were done by transmission electron microscopy. Figure 3*a* shows representative cells cultured in the presence of 1 U IL-2/ml. The large cells exhibited both cytoplasmic and extracellular particles. However, in the presence of 100 U IL-2/ml (fig. 3*b*), the fraction of cells containing virus structures was significantly reduced. Moreover, the infected cells contained mainly nuclear nucleocapsids and morphologically resembled the mock infected culture. Quantitative analyses of the electron microscopy data are summarized in table 1. In the presence of 10 U IL-2/ml the fraction of infected cells (evidenced by the presence of viral structures) was reduced from 79% to 23%. The cytoplasmic and extracellular viral structures were diminished, whereas only minor inhibition was noted in the number of nuclear naked nucleocapsids. The data suggest that IL-2 interferes with virion maturation and with the egress of nucleocapsids from the nucleus into the cytoplasm. Moreover, these data also allow a more clear interpretation of the IF data above. Thus, the yield of infectious virus per infected cell is reduced, resulting in a slower rate and lower multiplicity of secondary virus infections in the culture. This less aggressive virus infection explains the lower fraction of cells showing positive IF reaction as well as the lower intensity of IF in the positive cells, reflecting a

Table 1. Electron Microscopic Enumeration of Viral Structures in Mature Thymocytes

IL-2 (units/ml)	Percentage of Cells Infected	Nuclear Nucleocapsids	Cytoplasmic Capsids/Virions	Extracellular Virions
0.1	79	679 (8.6)	176 (2.2)	682 (8.6)
10	23	153 (6.7)	20 (0.9)	46 (2.0)

Note.—Randomly chosen cell sections were screened for nuclear and cytoplasmic viral structures, as well as extracellular virions. The numbers show the total virus structures in 100 cells from these sections. Data in brackets indicate numbers of viral structures per section of one infected cell. Data are from reference (12).

lower abundance of viral antigens synthesized per infected cell.

In a second set of studies (N. Frenkel, E. C. Schirmer, G. Katsafanas, and C. H. June, manuscript in preparation) PBL cultures were infected in the presence of natural or recombinant IL-2. The inhibitory effect of IL-2 on viral replication was assessed by measuring infectious virus yields, by IF assays, and by determination of the accumulation of viral DNA by Southern blot analyses. Examples for two such experiments are shown in figures 4 and 5. The experiment exemplified with IF data in figure 4 involved infection of nonactivated PBL (termed "None" in figure 4) or infection of PHA-activated PBL (i) in the absence of added IL-2, (ii) in the presence of 30 U recombinant IL-2/ml, and (iii) in the presence of both IL-2 and antibody to IL-2. The results of this experiment clearly showed that the addition of IL-2 at 30 U/ml significantly reduced virus replication and spread. Again, low levels of IL-2 induced T-cell activation as judged by ^{3}H thymidine uptake, and enhanced virus replication. The results of the Southern blot analyses supported this conclusion. In a second experiment, PBL were infected in the absence of IL-2 or in the presence of increasing concentrations of natural IL-2. Virus replication was again assessed by titration of infectious virus yields, IF assays, and viral DNA replication. The results have shown 20–50-fold reduction in the titer of infectious virus. Furthermore, as shown in figure 5, natural IL-2 at 2.5 U/ml slightly increased the viral DNA content as indicated by the hybridized counts per minute. This result represents an IL-2 concentration in which it exerts growth promotion activity on both the cell and the virus. However, when the higher IL-2 concentrations are included, viral DNA is dramatically diminished (fig. 5).

Discussion

We have briefly reviewed our studies concerning the effect of T-cell growth and of the T-cell growth factor, IL-2, on HHV-6 replication. The studies showed that T-cell activation was essential for virus replication. However, IL-2 at high concentrations had an inhibitory effect. At present there are two non–mutually exclusive mechanisms which could underlie the requirement for cell activation. First, T-cell activation may be required for the presence of cell receptors which play a role in efficient virus entry into the cell. Second, HHV-6 may adhere to and penetrate into resting or immature T cells, but cell activation is required for the expression or accumulation of factors essential for virus gene expression or viral DNA replication. It is noteworthy in this respect that HIV-1 replication in CD4$^+$ cells was shown to depend on T-cell activation and the synthesis of NF-κB (15–19). In addition, the human cytomegalovirus immediate early promoter is known to contain NF-κB-responsive and cAMP-sensitive elements (20–22). It is thus tempting to speculate that, by analogy with HIV-1 and HCMV, HHV-6 also contains promoter elements driving essential viral genes which are turned on in the process of T-cell activation.

Mitogenic activation and IL-2 addition have been routinely employed by investigators who propagate HHV-6 in fresh lymphocytes. The inhibitory effect of high IL-2 concentrations has not been previously documented. Taken together, the data indicate that viral

Figure 4. IL-2 inhibition of HHV-6 replication in PBL cultures. PBL cultures were infected in the presence of additives as indicated in the figure. rIL-2(30) = recombinant IL-2 at 30 U/ml; anti-IL-2 = polyclonal anti-IL-2 antibody at 15 μg/ml. The cultures were harvested 7 days postinfection and assayed by IF with the monoclonal antibody 9A5D12 (11). Data are from N. Frenkel, E. C. Schrimer, G. Katsafanas, and C. H. June (manuscript in preparation).

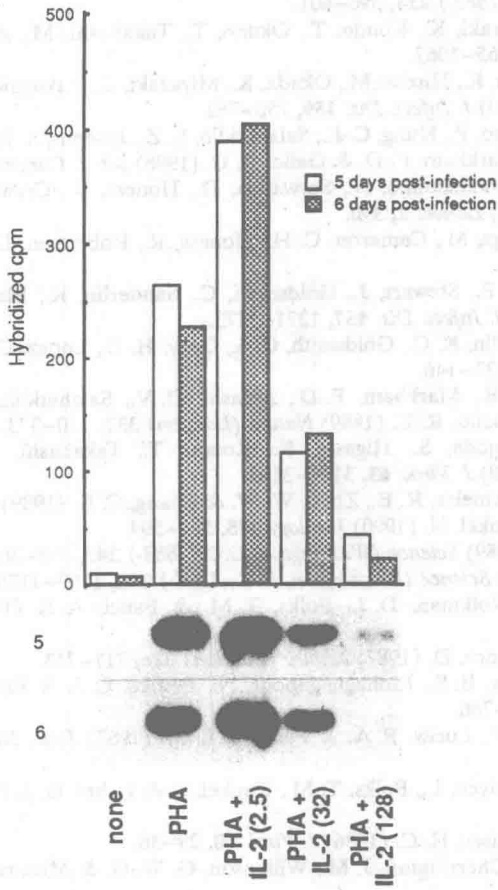

Figure 5. Replication of HHV-6 DNA in PBL cultures infected in the presence of additives as specified. IL-2 denotes natural IL-2 (U/ml indicated). DNA harvested from the infected cells on days 5 and 6 postinfection was analyzed by blot hybridization with a 12.5-kb probe derived from HHV-6 (Z29) DNA. The hybridized bands shown at the bottom of the figure were scanned with a Betascope, and the relative hybridized counts are plotted in the bar graphs.

gene expression and/or viral DNA replication are affected by both positive and negative factors related to T-cell activation and T-cell growth. Although the detailed mechanism underlying these effects is at the moment unclear, we wish to hypothesize that these effects may play a role in HHV-6 in vivo infections. Thus, it is possible that the inhibitory effect of high concentrations of IL-2 reflects cellular factor(s) which play a role in the resolution of HHV-6 infection during the acute phase of in vivo infections (e.g., in exanthem subitum) and/or in the establishment of a latent state. Conversely, T-cell activation by non-relevant antigenic induction (e.g., caused by an inflammatory reaction to bacterial and/or different viral infection) might play a role in the activation of virus replication from a putative latent state.

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Genomic Heterogeneity of Human Herpesvirus 6 Isolates

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The Problem

Human herpesvirus 6 (HHV-6) is the causative agent of the mild childhood disease exanthem subitum (1). Its role in adult disease and in more severe disease of children has not been defined, although preliminary studies have found evidence for changes in HHV-6 serologic status in association with episodes of mild, afebrile illness presenting with symmetrical lymphadenopathy (2). Viral DNA sequences have been found in B-cell lymphoma tissue (3) and cervical lymph nodes of normal individuals (4). HHV-6 has been isolated from people of several continents (1, 5–10). Seroprevalence studies indicate that most individuals have been infected (11–15) and that initial exposure to the virus occurs early in life (1, 11, 13, 14). The route or routes of infection have not been defined. Acquisition via breast milk has not been ruled out, but it is clear that another route of transmission must exist (16). The question of whether an individual can be infected by multiple strains of the virus has not been studied.

To conduct rigorous studies of virus transmission mechanisms, methods for identifying individual virus strains are needed. Such studies with other herpesviruses are routinely and reliably performed by restriction-endonuclease fingerprinting. The technique involves comparing the banding pattern of DNA fragments generated by the digestion of DNA isolated from individual strains. By appropriate choice of the restriction endonuclease, most, if not all, independent isolates may be distinguished from each other. A short list of the uses of this technique includes tracking transmission of individual strains in nosocomial outbreaks of herpes simplex virus type 1 (HSV-1) (17), demonstrating the occurrence of exogenous reinfection with HSV-2 (18), differentiating wild-type varicella-zoster virus from vaccine virus (19), and demonstrating mother-to-child transmission of cytomegalovirus (20). As has been described elsewhere (21), to conduct such studies it is essential both to understand the structure of the viral genome and to measure the stability of the virus genome upon passage in tissue culture so regions of intrastrain variation may be identified.

The genome of HHV-6 has variously been described as having a length of >110 kilobases (kb) (22), approximately 150 kb (6), and approximately 170 kb (23). The paper in the latter case suggested, on the basis of the absence of submolar restriction-endonuclease fragments, that the HHV-6 genome possesses no invertible segments. Our experiments revealed the following properties of the HHV-6 (Z29) genome: (i) an individual HHV-6 strain can vary in length upon passage in tissue culture from approximately 163 kb to 170 kb; (ii) the HHV-6 genome possesses two unique termini; (iii) the length variation maps within a directly repeated sequence which varies from 10 to 13 kb in length and is found in a single copy at each end of the genome; (iv) the only restriction-endonuclease fragments

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Edited by C. Lopez *et al.*, Plenum Press, New York, 1990

of other than unit molarity arise from the terminal repeat structure (G. J. Lindquester and P. E. Pellett, unpublished manuscript). Thus, in agreement with Josephs et al. (23), we found that HHV-6 has no large invertible segments. These findings are summarized in figure 1.

Genomic heterogeneity between strains of HHV-6 has not been studied extensively. Many of the earlier studies consisted of comparisons of hybridization patterns with a cloned fragment of HHV-6 (GS), pZVH14 (6, 7, 9, 10, 23). These studies confirmed the identity of several viral isolates as HHV-6. Interstrain variation in hybridization patterns produced by this probe as well as other cloned fragments has been noted (10, 24). HHV-6 strains GS and Z29 were compared by both hybridization with pZVH14 and their *Eco*RI digestion profiles (23). Although the hybridization patterns produced by the cloned probe were identical, differences in the migration of several *Eco*RI fragments from other regions of the genomes were noted. The nature of this variation was not explored.

The objectives of this work were twofold: (i) to assay the nature and extent of strain-to-strain variation of HHV-6 by comparing the restriction-endonuclease profiles of several isolates of HHV-6 to lay the foundation for future molecular epidemiologic studies of HHV-6 transmission, and (ii) to determine the generality of the model of HHV-6 genomic architecture derived from studies of HHV-6 (Z29).

Experimental Design and Results

Viruses and DNA

Whole-cell DNA was purified from human cord-blood lymphocytes infected with each of eight HHV-6 strains isolated from Japanese exanthem subitum patients (strains C, E, H, K, L, M, P, and R) (1, 16), a strain isolated from an American hemophiliac with acquired immunodeficiency syndrome (AIDS) (strain 5) (8), and a strain isolated from a Zairian AIDS patient (strain Z29) (8). In addition, whole-cell DNA was obtained from a culture of Mo-T (25) cells persistently infected with HHV-6 (Z29) and passaged one or two times per week for 18 mo without adding fresh cells or virus. Comparison of this DNA with that obtained from the parental strain which was passaged in fresh human cord-blood lymphocytes gives some measure of the genomic stability of the virus.

Restriction-Endonuclease Profiles of HHV-6 Strains

Aliquots of DNA from each of the DNA preparations were digested with *Bam*HI, *Cla*I, *Hin*dIII, or *Sal*I, separated in 0.8% agarose gels, blotted to nitrocellulose, and hybridized with nick-translated whole viral HHV-6 DNA. After hybridization, blots were washed at room temperature, then incubated at 65°C for 2 hr in 0.15 M NaCl, 0.015 M sodium citrate, and 0.1 % sodium dodecyl sulfate. Fragment nomenclature is based on that of HHV-6 (Z29) (G. J. Lindquester and P. E. Pellett, unpublished manuscript). Fragments are designated in alphabetical order from the largest to the smallest, with the exceptions that terminal fragments which span the variable portion of the terminal repeat structure are designated TermL and TermR, depending on whether they map to the left or right end of the prototype orientation of the genome, respectively, and internal fragments which span this region are designated Het_i.

***Hin*dIII Profiles.** The pattern of fragments comigrating with or smaller than *Hin*dIII D is nearly identical in all isolates (fig. 2*A*) with the exception of *Hin*dIII P and R. *Hin*dIII P of HHV-6 (C) is slightly shorter than that of the other isolates. The *Hin*dIII R fragment is absent in the exanthem subitum isolates from Japan. More extensive variation is seen in fragments of high molecular weight where no other isolate has a fragment comigrating with *Hin*dIII TermL of HHV-6 (Z29). *Hin*dIII C has slightly greater mobility in the Japanese isolates than in the other isolates. A fragment slightly longer than *Hin*dIII B is seen in several isolates, and other variation is found in the region of the gel containing *Hin*dIII A and TermR.

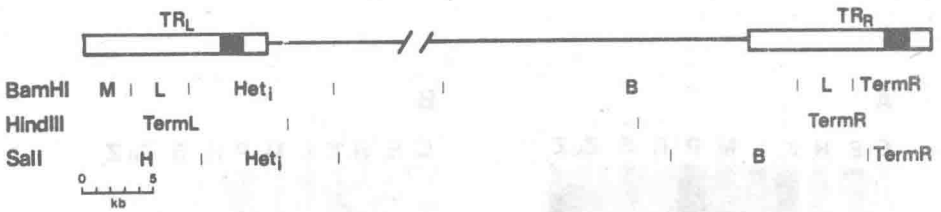

Figure 1. Schematic diagram of the structure of the termini of the HHV-6 genome. The boxed area represents the terminal repeat structure. The filled region within the boxes represents the region which varies in length from strain to strain and within a strain. The precise location and extent of this heterogeneous region within the terminal repeat structure is not known. *Bam*HI, *Hin*dIII, and *Sal*I restriction fragments containing some or all of the terminal repeat structure are indicated.

***Cla*I Profiles.** The most obvious difference between the strains in *Cla*I-generated fragments is in the region of the gel between *Cla*I B and *Cla*I C, where generally diffuse patterns with two major discernable bands appear (fig. 3*A*). Other differences include the lack of a fragment comigrating with *Cla*I E in the Japanese isolates and the presence of additional bands near *Cla*I J,K and N,O in these same isolates.

***Bam*HI Profiles.** The patterns obtained after digestion with *Bam*HI (fig. 4*A*) appear to be more complex than those seen in the digests with *Hin*dIII or *Cla*I. In all the Japanese isolates except one (strain R), *Bam*HI B is longer than that found in HHV-6 (Z29). A band which could correspond to *Bam*HI M is seen in all strains, but it varies from that seen in HHV-6 (Z29) by ±200 base pairs. The relative intensity of *Bam*HI C in comparison to the *Bam*HI D, E, Het$_i$ band varies between strain 5 and strain Z29. The pattern of fragments in the portion of the gel containing fragments I through M is unique in all of the strains except for strain 5 in comparison to strain Z29. Similarly, the portion of the gel between fragments B and F shows marked interstrain variation.

***Sal*I Profiles.** As with the *Bam*HI profiles, the pattern for every isolate is unique (fig. 5*A*).

Hybridizations with Fragments Derived from the Terminal Repeat

Replicas of the blots shown in figures 2*A*, 3*A*, 4*A*, and 5*A* were hybridized with gel-purified *Cla*I Term1, a 10.9-kb fragment derived from one of the genomic termini which contains a complete copy of the terminal repeat and approximately 600 base pairs of the adjacent unique region. Similar blots were also hybridized with cloned *Bam*HI L from HHV-6 (Z29), an internal component of the terminal repeat element found in two copies per genome (G. J. Lindquester and P. E. Pellett, unpublished manuscript).

Panels *B* of figures 2–5 show the patterns of fragments to which *Cla*I Term1 hybridized. In comparison to the patterns of hybridization seen with whole viral DNA (figs. 2–5, panels *A*), it is clear that the regions hybridizing with the *Cla*I Term1 fragment account for most of the interstrain variability. This region of interstrain heterogeneity has also been identified as the location of significant intrastrain restriction-fragment-length heterogeneity (G. J. Lindquester and P. E. Pellett, unpublished manuscript).

***Hin*dIII and *Cla*I Profiles.** The patterns of hybridization with *Cla*I Term1 in the *Hin*dIII digests of the various isolates (fig. 2*B*) range from the example of two predominant bands for strains C and Z29, to the presence of two fragments nearly comigrating with *Hin*dIII Term1 and a single fragment of higher molecular weight than Term2 (HHV-6 [H] and HHV-6 [R]), to the more complex pattern of HHV-6 (M), which has two bands in the vicinity of Term1 and three in the vicinity of Term2. The patterns seen in the *Cla*I digests (fig. 3*B*) mirror those seen in the *Hin*dIII digests in the extent of fragment size