Latent human herpesvirus 6 infection of human monocytes/macrophages

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Human herpesvirus 6 (HHV-6) DNA was detected in peripheral blood from exanthem subitum patients during the acute and convalescent phases of infection using the polymerase chain reaction. Although DNA could be detected in non-adherent and adherent mononuclear cells during the acute phase, it was detected predominantly in adherent cells during the convalescent phase; furthermore, viral DNA was found in adherent cells of healthy adults. When adherent mononuclear cells were cultured in vitro, virus was found to replicate well in differentiated cells cultured for 7 days in vitro before infection. When cells were cultured for more than 1 month, no detectable antigen and no evidence of virus growth was observed, but viral DNA could be detected. These apparently latently infected monocytes were treated with phorbol ester, after which virus could be recovered from the cultures. Therefore, we have developed an in vitro latency system for HHV-6; our results suggest that HHV-6 may latently infect monocytes in vivo and in vitro and that it may be reactivated in cells by some factors.

Introduction

A novel human herpesvirus, now named human herpesvirus 6 (HHV-6), has been isolated independently by several groups from patients with lymphocytic disorders (Salahuddin et al., 1986; Tedder et al., 1987; Downing et al., 1987; Agut et al., 1988; Lopez et al., 1988). This virus was initially named human B lymphotropic virus because it infected B lymphocytes (Salahuddin et al., 1986); however, it was found later to infect and replicate mainly in lymphocytes of the T cell lineage (Ablashi et al., 1987; Lusso et al., 1988, 1989; Takahashi et al., 1989). Preliminary characterization of HHV-6 indicated that it was antigenically and genetically distinct from other human herpesviruses [cytomegalovirus, herpes simplex virus types 1 and 2, varicella-zoster virus and Epstein-Barr virus (EBV) (Josephs et al., 1986; Salahuddin et al., 1986; Lopez et al., 1988)]. However, it has been reported that the HHV-6 genome shows sequence homology to that of human cytomegalovirus (Efthathiou et al., 1988; Lawrence et al., 1990).

Most children have antibodies against HHV-6 by 2 years of age (Okuno et al., 1989), but it was not known whether HHV-6 caused disease in humans. We discovered recently that HHV-6 is the causative agent of exanthem subitum (ES) (Yamanishi et al., 1988) and that it can be isolated from CD4+ CD8− and CD3+ CD4+ mature T lymphocytes, but not from CD4− CD8+, CD3− T cells and other cells in the peripheral blood of ES patients (Takahashi et al., 1989). A novel technique, the polymerase chain reaction (PCR) for the amplification of DNA or RNA in vitro, has been developed recently and can be used for many purposes, such as the analysis of inherited disorders and the detection of somatic diseases (Saiki et al., 1985, 1988; Mullis & Faloona, 1987). One of the most important applications of this method, which gives results in a short time, has been in the detection of infectious agents that are present in small numbers in clinical samples. It has been reported that the PCR can be used to detect HHV-6 DNA in peripheral blood cells of patients with AIDS and various lymphoproliferative disorders, and ES patients (Buchbinder et al., 1988; Kondo et al., 1990). Here we report the use of the PCR for the detection of HHV-6 in peripheral blood mononuclear cells of ES patients during the convalescent phase, and also in healthy adults. We also report that HHV-6 infects monocytes of adults in vitro and replicates in them if the monocytes are cultured for 7 days before infection. Furthermore, viral DNA could be detected in infected monocytes over a long period in the absence of expression of viral antigens.

Methods

Virus and titration. The Hashimoto strain of HHV-6, which was isolated from a patient with ES, was used for infection. The method for titration of HHV-6 has been described previously; results are expressed as f.f.u. (Asada et al., 1989).
Preparation of mononuclear cells from patients and healthy adults.
BLOOD samples (approximately 1 ml) from acute and convalescent phase ES patients and healthy adults (approximately 1 ml), were collected in heparinized tubes. Mononuclear cells were separated, resuspended (0-5 x 10^6 to 2 x 10^6 cells/ml) in RPMI 1640 supplemented with 25% horse serum, and cultured in plastic plates coated with gelatin (Packard et al., 1982; Hassan et al., 1986). Cultures were incubated for 1 h at 37°C in an atmosphere of 5% CO2; non-adherent cells were collected and added again to gelatin-coated dishes to obtain purer non-adherent cells. To obtain adherent cells, dishes were washed several times with RPMI 1640 prewarmed at 37°C and adherent cells were incubated in RPMI 1640 supplemented with 25% horse serum. After 12 h incubation, dishes were washed several times with prewarmed RPMI 1640, and PBS free from Ca and Mg and supplemented with 10 mM EDTA was added to the plates, which were incubated at room temperature for 20 to 30 min to detach adherent cells. Cells were then washed with RPMI 1640 three times by centrifugation and resuspended in RPMI 1640. Cell preparations were tested for non-specific esterase-positive cells. By this method, more than 95% of adherent cells were non-specific esterase-positive, and more than 90% of non-adherent cells were non-specific esterase-negative.

**DNA extraction.** Adherent and non-adherent cells were harvested as described above and incubated for 16 h at 60°C in NET buffer (150 mM-NaCl, 15 mM-Tris-HCl, 1 mM-EDTA) with 0-1% SDS and 1-0 mg/ml proteinase K (Boehringer Mannheim). The mixture was extracted three times with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), treated with chloroform twice and DNA was precipitated by adding ethanol. In the case of samples from ES patients, 5 μg tRNA was used as a carrier; between 5 and 20 μg tRNA did not interfere with the sensitivity of the PCR (data not shown). The DNA was washed three times with 80% ethanol and resuspended in distilled water. The DNA concentration was determined by sequential dot blot hybridization using human placental DNA as a probe. Approximately 100 ng of ES patient DNA and about 1 μg of healthy adult DNA were used in the PCR system.

Oligomer synthesis and PCR. The primers were parts of the SalI fragment (approximately 6 kbp) from HHV-6 strain Hashimoto and had the sequences (5’ to 3’) 5’ GTGTTCCATTGTACT-GAAACCGGT 3’ and 5’ TAAACATCAATGCGTTGCATACAGT 3’. Since the physical map of HHV-6 had not been published, it was not clear which region the DNA fragment used belonged to. All Japanese isolates have been amplified using these oligomers (Kondo et al., 1990 and our unpublished data). These oligomers were synthesized in a DNA synthesizer (Applied Biosystems); the PCR amplification product was 776 bp in length.

DNA was amplified in a total volume of 100 μl of reaction mixture consisting of 50 mM-KCl, 10 mM-Tris-HCl, pH 8.3, 1-5 mM-MgCl2, 0-01% (w/v) gelatin, 200 μM each of dGTP, dATP, dTTP and dCTP, and 5 units of Taq polymerase. The sample was first denatured at 94°C for 10 min and then subjected to 30 amplification cycles of annealing at 62°C for 2 min, extension at 72°C for 5 min and denaturation at 90°C for 1 min; the primers were used at 1.0 μM each and the reaction was carried out in a DNA thermal cycler (Perkin-Elmer/Cetus). In the case of samples of cells from healthy adults, 50 μl of PCR product was mixed with an equal volume of new reaction mixture and subjected to a further 15 amplification cycles as described above. β-Globin primers (Saiki et al., 1985) were used as a positive control in samples in which amplification of viral DNA was not observed; DNA from mononuclear cells of umbilical cord blood was used as a negative control.

Detection of amplified product. The amplified product was detected by Southern blot hybridization using a cloned DNA probe (part of the Safl fragment). The reaction mixture (10 μl) was subjected to electrophoresis on a 1.5% agarose gel and M marker were located by u.v. fluorescence after staining with ethidium bromide. DNA was transferred to a Hybond-N membrane (Amersham) by alkaline blotting. Briefly, we used alkaline transfer buffer (0-4 M-NaOH), capillary blotting for 3 h and rinsing of the membrane in 2 x SSPE (0.36 M-NaCl, 0.02 M-sodium phosphate, 0.002 M-EDTA pH 7.7). The membrane was then hybridized with a homologous 32P-labelled cloned probe (2 x 10^6 c.p.m./ml) using the rapid hybridization system multiprime (Amersham) for 2 h. The filter was washed twice with 2 x SSPE, 0-1% SDS for 10 min each time at room temperature, once with 1 x SSPE, 0-1% SDS for 15 min at 65°C, and then twice in 0-1 x SSPE, 0-1% SDS for 20 min each time at 65°C. Bound probe was detected by autoradiography at -70°C for 16 h with intensifying screens. When the sensitivity of the system was estimated, at least five copies of HHV-6 DNA were detectable after amplification and the amount of DNA could be counted semi-quantitatively (Fig. 1).

Infection of adherent cells with HHV-6. When peripheral blood was collected from healthy adults, HHV-6 DNA was not detectable in 10^6 cells by PCR. After separation of adherent cells as described above, cells (4 x 10^6 to 8 x 10^6 cells/plate) were infected immediately with HHV-6 at a multiplicity of 0.05 f.f.u./cell and incubated for 1 h. Cells were washed twice with RPMI 1640 and cultured in RPMI 1640 supplemented with 25% horse serum. After 7 days of cultivation, the cells had decreased in size and more than 99% were non-specific esterase-positive; no fibroblastic colonies had appeared by 45 days of cultivation. Adherent cells were cultured also for 1 week and infected as above.

Infectious centre assay (ICA), detection of viral antigen and detection of DNA by PCR. For the ICA, cells in dishes were detached by incubation with 10 mM-EDTA in PBS for 20 to 30 min at room temperature. Dispersed cells were suspended in 1 ml RPMI 1640 and serially diluted 10-fold in the same medium. Diluted cells were co-cultured with phytohaemagglutinin (PHA)-stimulated umbilical cord blood cells in 96-well plates. In each well, approximately 2 x 10⁵ cells in 0-3 ml RPMI 1640 with 10% foetal bovine serum were seeded; four wells were used at each dilution. The medium was changed twice a week and, after
HHV-6 infection in monocytes

During the 10 days, cells were stained for detection in an immunofluorescence assay (IFA) using monoclonal antibodies against HHV-6 (Okuno et al., 1990). The number of infectious centres was expressed as the TCID50.

For the detection of viral antigen in infected cells by the IFA, about 1 × 10⁶ cells were centrifuged, pellets were mounted on 10-spot glass slides, fixed in acetone at -20°C for 10 min and stained for immunofluorescence. For the detection of DNA in infected cells, the PCR was used. Serially diluted cells were collected by centrifugation in Eppendorf tubes, solubilized and DNA was purified as described above. Four tubes of each dilution were tested by PCR and the number of HHV-6 DNA-positive cells was estimated.

**Treatment of monocytes with 12-O-tetradecanoylphorbol 13-acetate (TPA).** Adherent cells in plastic dishes were infected with HHV-6 and cultured for 45 to 60 days. The cells were detached from the plates, serially diluted (2 × 10⁴ to two cells/culture) and cultured with non-infected monocytes as a feeder layer in 24-well plates. The feeder layer was used for keeping the small number of cells alive during the experiment. Cells were treated with TPA (20 ng/ml; Sigma) for 5 days and cultured for another 7 days with PHA-stimulated cord blood cells to detect infectious virus.

**Detection of HHV-6 DNA in infected cells by Southern blotting.** Cells (approximately 10⁵), cultured for 45 days after infection as described above, were lysed by treatment with solubilizing buffer and proteinase K and DNA was extracted and purified by ethanol precipitation. DNA (1 µg) from cells or 10⁶ copies of HHV-6 DNA prepared from virions was digested with EcoRI, loaded onto an agarose gel, transferred to a membrane and hybridized using the rapid hybridization system (Amersham) with two cloned EcoRI restriction fragments of approximately 4.0 kb and 5.5 kb as probes. The concentration of the probes was about 2 × 10⁶ c.p.m./ml. Autoradiography was done at -70 °C with two intensifying screens for 2 days.

**Antibody test.** Detection of anti-HHV-6 antibody was done by an IFA as described previously (Kondo et al., 1990).

**Results**

**Detection of HHV-6 DNA in adherent and non-adherent cells from peripheral blood of ES patients**

Blood samples were collected during the acute and convalescent phases from five clinically and serologically diagnosed ES patients. All acute phase samples were collected from febrile patients; convalescent phase samples were obtained from patients 1.5 to 2.5 months after the onset of the illness.

Sera were tested first for anti-HHV-6 antibody by IFA. Antibodies were not detected (<1:10) in acute phase sera, but the antibody titre became 1:640 to 1:2560 during the convalescent phase. We then attempted to detect HHV-6 DNA in adherent cells (monocytes) and non-adherent cells from ES patients using the PCR. Using small amounts of DNA and 30 cycles of PCR amplification, the accumulation of amplification product appeared to be an unlimited process and the product was thought to increase in proportion to the original HHV-6 DNA copy number in the samples (data not shown). HHV-6 DNA was detected in both adherent and non-adherent cells collected during the acute phase, predominantly from non-adherent cells from patients 1 and 2 (Fig. 2). On the other hand, HHV-6 DNA was detected mainly in adherent cells collected during the convalescent phase (DNA was detected only in adherent cells from patients 1, 2 and 3), although the amount of DNA appeared to decrease during this phase (Fig. 2).

**Detection of HHV-6 DNA in adherent and non-adherent cells of peripheral blood collected from healthy adults**

The detection of HHV-6 DNA in cells collected from healthy adults aged 27 to 47 years was attempted. Thirty blood samples were collected from 20 healthy adults, some being bled two or three times at intervals. The HHV-6 antibody titre of the sera was between 1:160 and 1:320, within the normal range. The HHV-6 DNA in both fractions was examined by PCR. When 30 amplification cycles were used, only one sample (sample 8) was positive; when 45 amplification cycles were used, five samples became positive, two (samples 18a and b)
were derived from one person who was bled three times and the other two (samples 2 and 11) were from persons who were bled twice. Furthermore DNA was only detected in samples of adherent cells (Fig. 2).

**HHV-6 infection of monocytes/macrophages in vitro**

The adherent cells derived from the peripheral blood of healthy adults were collected and infected with HHV-6. Three methods were used to detect the existence of virus in cells: the number of cells expressing viral antigens was determined by an IFA, the number of virus-producing cells was estimated by an ICA and the number of cells containing viral DNA was determined by the PCR. Tests were repeated three times using cell cultures derived from three individuals. Although typical c.p.e. caused by HHV-6 infection of cells was not found during cell culture, the number of cells gradually decreased until approximately half of the original number remained on day 45.

When monocytes were infected with virus on day 0 (12 h after collection of mononuclear cells), antigen-positive cells were detected first on day 5 and the number of antigen-positive cells gradually increased (5 to 9%) until 14 days after infection. No antigen-positive cells could be detected after day 35 (Fig. 3a). Infectious centres were not detected until 3 days after infection and the number of infected cells increased from day 5 until day 14; infectious centres could not be detected after day 35. Finally, infected cells were diluted from $10^4$ cells/ml to $10^{-1}$ cells/ml and the number of cells containing HHV-6 DNA was estimated by PCR. Cells containing HHV-6 DNA were found from the first day (four to eight positive cells/1000 cells) and their number increased gradually until 14 days after infection (100 to 180 positive cells/1000 cells). The number of DNA-positive cells stayed at this level until day 45.

On the other hand, when cells were cultured for 7 days in vitro and infected with virus, antigen-positive cells were first detected 3 days after infection and the proportion of antigen-positive cells increased to 15 to 35% after infection. They then decreased and no antigen-positive cells could be detected 21 days after infection. Infected cells were detected from 1 day, reaching a maximum on day 7. They then decreased gradually and after 3 weeks no infectious centres could be detected. Finally, the number of cells containing viral DNA was estimated using PCR; HHV-6 DNA-containing cells were found from the first day (three to nine positive cells/1000 cells), the number increasing until 7 days after infection (300 to 550 positive cells/1000 cells), and remaining at almost the same level until 45 days after infection (Fig. 3b). Antigen-positive cells 7 days after infection are shown in Fig. 4; viral antigens can be seen in the cytoplasm and nucleus, whereas no specific antigen was detected in non-infected cultures.

Adherent cells which had been cultured for 7 days after separation in dishes and infected with HHV-6 were harvested 45 days after infection and DNA from cells and virions was extracted for Southern blot analysis after digestion with EcoRI as described in Methods. A specific band was observed in lanes loaded with $10^6$ DNA copies (Fig. 5, lanes 1 and 5) but not those to which $10^5$ DNA copies were added (lanes 3 and 7). HHV-6-specific DNA was detected by Southern blot analysis (Fig. 5, lanes 2 and 6), but the density of the band was less than those in lanes 1 and 3. This result suggested that $10^5$ cells contained between $10^5$ and $10^6$ copies of HHV-6 DNA so the number of copies in DNA-positive cells was estimated using the PCR on diluted DNA samples. As shown in Fig. 6, positive bands were observed in undiluted and 10-fold diluted samples but no amplification was observed in 100-fold diluted samples. From this
result, it can be deduced that at least 10 DNA copies are found in a single HHV-6 DNA-positive cell and, from the result of the ICA shown in Fig. 3, viral DNA is maintained in adherent cells with a relatively high copy number (10 to 100 copies/cell) for a long time.

These data showed that HHV-6 might infect undifferentiated or differentiated monocytes/macrophages and replicate in differentiated monocytes/macrophages. Furthermore, viral DNA may persist in these cells for a long time in a form which may be considered latent.

Fig. 4. Immunofluorescence micrograph. (a) Adherent cells, which were cultured for 7 days and infected with HHV-6, were stained with monoclonal antibodies. (b) Uninfected adherent cells as a control.

Fig. 5. Southern blot analysis of HHV-6 DNA in adherent cells infected with HHV-6. Lanes 1 and 5, 10⁶ DNA copies; lanes 2 and 6, DNA from 10⁵ infected cells; lanes 3 and 7, 10⁴ DNA copies; lanes 4 and 8, mock-infected cells. DNA was digested with EcoRI, loaded onto an agarose gel, run, transferred to a membrane and hybridized with EcoRI restriction fragments as probes (lanes 1 to 4, 4.0 kb fragment; lanes 5 to 8, 5.5 kb fragment).

Fig. 6. Detection by PCR of HHV-6 DNA in a latently infected cell. Diluted samples of latently infected cell DNA were amplified by PCR. Lane 1, equivalent to one cell; lane 2, equivalent to 10⁻¹ cells; lane 3, equivalent to 10⁻² cells.
Table 1. Reactivation of HHV-6 in monocytes by treatment with TPA

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<th>Expt.</th>
<th>TPA treatment</th>
<th>Numbers of adherent cells/plate</th>
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<td>2 x 10⁴</td>
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* −, No HHV-6 reactivation; +, HHV-6 was reactivated.

Induction of HHV-6 in infected adherent cells

Cells which were infected with HHV-6 and cultured for 45 days were treated with TPA and virus isolation was attempted. As shown in Table 1, when cells were not treated with TPA no antigen-positive cells were observed in two of three cases, even in undiluted samples (2 x 10⁴ cells/plate). On the other hand, when cells were treated with TPA, c.p.e. could be observed in cultures, and cells expressing viral antigen were observed even in samples diluted 1000-fold. This result suggested that HHV-6 could be reactivated in infected cells by TPA treatment.

Discussion

HHV-6 shows a tropism for CD4+ lymphocytes (Lusso et al., 1988) and also some established cell lines, including HL-60 which was established from monocytic leukaemia (Downing et al., 1987). We have shown previously that HHV-6 can be isolated from non-adherent cells of peripheral blood from ES patients (Takahashi et al., 1989) and that HHV-6 DNA could be detected by PCR in peripheral blood mononuclear cells collected during both the acute and convalescent phases of ES (Kondo et al., 1990). In this communication, we have analysed in detail the tropism of this virus by the PCR after separation of mononuclear cells into the adherent and non-adherent fractions. Viral DNA could be detected not only in non-adherent cells but also in adherent cells collected during the acute and convalescent phases (Fig. 2), and predominantly from the adherent cell fraction of mononuclear cells collected during the convalescent phase. Since it was not possible to isolate virus from the peripheral blood of ES patients during the convalescent phase (unpublished data), HHV-6 might persist in the latent state or as an immune complex with antibody in monocytes. Viral DNA could be detected only in the monocyte fraction of the peripheral blood of healthy adults, although the detection rate was not so high. Therefore, it is unlikely that virus persists as an immune complex in the cells of adults. These results suggest that HHV-6 may stay in monocytes/macrophages in a latent state, although it is not clear whether this cell type is the main site of latency in vivo.

As shown in Fig. 3, HHV-6 replicated in cells cultured for 7 days in vitro faster than it did in cells infected immediately after separation. Morphological and functional studies have shown that when human monocytes are cultured in vitro for several days, they differentiate into macrophages (Hammerstrom, 1979; Zuckerman et al., 1979). Herpes simplex virus (Daniels et al., 1978; Domke-Opitz et al., 1986) and varicella-zoster virus (Arbeit et al., 1982) cannot replicate in human monocytes, but this cell acquires the ability to allow virus replication during maturation in vitro. Our study showed similar results, indicating that undifferentiated monocytes may not be permissive for the replication of HHV-6, but become permissive during maturation in vitro. However, a small amount of infectious virus could be produced in cells infected immediately after cell separation. This leakiness of virus production might be caused by the fact that the differentiation of cells during cultivation is not simultaneous.

The number of antigen-positive cells and infectious centres decreased 10 days after infection in cells which were infected after differentiation (Fig. 3b). Neither viral antigen nor infectious virus could be detected in adherent cells during the late stage of infection, but viral DNA could be found in between 5 and 20% of cells. Furthermore, HHV-6 DNA in adherent cells was maintained at a relatively high copy number, as determined by Southern blot analysis (Fig. 5) and PCR (Fig. 6), during the late stage of infection. These results suggest that virus can latently infect macrophages in vivo. Although viral antigen was not detected in infected adherent cells which had been cultured for more than 1 month, virus could be recovered from these cells following treatment with TPA (Table 1). Numerous activation signals have been shown to prevent the latency of various viruses. Since TPA has pleiotropic effects on cells, such as the induction of cell differentiation and oncogenic viruses, and the enhancement of the replication of human immunodeficiency virus (HIV) (Harada et al., 1986), this agent has been commonly used to reactivate virus from latently infected cells. Phorbol esters have been shown to activate EBV replication in lymphoblast cell lines that are non-producers (zur Hausen et al., 1978; Yamamoto & zur Hausen, 1979). Therefore, latent HHV-6 in monocytes/macrophages might also be reactivated by TPA treatment.

It has been reported recently that HHV-6 may be a cofactor in AIDS because this virus trans-activates the
HHV-6 infection in monocytes

1407


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