Evidence for putative immediate early antigens in human herpesvirus 6-infected cells

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Human herpesvirus 6 (HHV-6) induced nuclear antigens in cells as early as 3 h after infection. These nuclear antigens were induced by all three strains of HHV-6 tested, and their de novo synthesis required the function(s) of the intact viral genome. Their appearance was not affected by 2,2'-anhydro(1-β-D-arabinofuranosyl)cytosine, but was completely inhibited by cycloheximide. However, the nuclear antigens did appear if cycloheximide was replaced with actinomycin D. Thus, the nuclear antigens seem to be equivalent to the immediate early antigens of other herpesviruses.

A novel human herpesvirus (Salahuddin et al., 1986), now referred to as human herpesvirus 6 (HHV-6), is T cell lymphotropic in vitro (Lusso et al., 1988) and in vivo (Takahashi et al., 1989), and is the causative agent of exanthem subitum (Yamanishi et al., 1988). The DNA of HHV-6 is unique (Josephs et al., 1986; Lopez et al., 1988), but has some homology with that of human cytomegalovirus (HCMV) (Estathiou et al., 1988). Indeed, the sequence and arrangement of the predicted open reading frames of HHV-6 closely resemble those of the corresponding regions of HCMV (Lawrence et al., 1990).

The proteins of the members of the Herpesviridae, e.g. herpes simplex virus, are synthesized under cascade regulation (Honess & Roizman, 1974, 1975). Viral immediate early antigens (IEAs) are those expressed by host cell transcription and translation systems without prior viral protein synthesis, and are key antigens for subsequent synthesis of early and late antigens. HHV-6-specific polypeptides have been analysed by several investigators (Balachandran et al., 1989, 1991; Shiraki et al., 1989; Okuno et al., 1990), but no evidence for IEAs has been reported. In this paper, we described evidence for IEAs in HHV-6-infected cells treated with inhibitors of DNA, RNA or protein synthesis.

Cord blood mononuclear cells (CBMCs) were separated on a Ficoll-Conray gradient and grown in RPMI 1640 medium supplemented with antibiotics [100 units (U)/ml penicillin and 100 μg/ml streptomycin] and 10% foetal bovine serum (FBS) in the presence of 5 μg/ml phytohaemagglutinin P (Honen Oil Co.) and 1 U/ml recombinant human interleukin 2 (rIL-2) (Takeda Chemical Industries). MT-4 cells (Miyoshi et al., 1982) were also grown in RPMI 1640 medium supplemented with antibiotics and 10% FBS (growth medium).

Three strains of HHV-6 (Hashimoto, HG and KA) were used. The Hashimoto strain was kindly provided by Dr K. Yamanishi, Osaka University, Osaka, Japan and the other two strains were isolated in our laboratory. The HG and KA strains were grown in CBMCs. Infected CBMCs were sonicated at 60 W for 30 s in a Sonifier (Heat Systems-Ultrasonics) and, after centrifugation at 3000 r.p.m. for 15 min, the supernatant was stored at −80 °C as a cell-free virus stock. Cell-free Hashimoto strain virus was prepared from persistently infected MT-4 cells (Y. Eizuru et al., unpublished data) in the same manner. The infectivity of virus was titrated in MT-4 cells by the endpoint dilution method using quadruplicate wells (2 × 10⁵ MT-4 cells/well) in a 96-well microtitre plate (Flow Laboratories). Virus was inactivated either by u.v.-irradiation with a 15 W germicidal lamp at a distance of 30 cm or by heating at 56°C.

HHV-6 antigens in infected cells were examined by anti-complement immunofluorescence (ACIF) as follows. Infected cells were spotted on heavy Teflon-coated slide glass (Bokusui-Brown), fixed in acetone at −20 °C for 5 min and dried. The fixed cells were reacted sequentially with heat-inactivated (56°C, 30 min) human serum, guinea-pig serum (1:10 dilution) as complement, and anti-guinea-pig C3 goat serum labelled with fluorescein isothiocyanate (1:80 dilution) (Cappel Laboratories) at room temperature for 45 min. The cells were washed three times in PBS pH 7.4 at the end of each reaction. After staining, the cells were mounted in glycerol and examined under an immunofluorescence microscope (Olympus).
Table 1. Antibody titres of sera used in this study

<table>
<thead>
<tr>
<th>Serum*</th>
<th>Putative IEA†</th>
<th>HHV-6 antigens‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 1 Unadsorbed</td>
<td>&lt;10</td>
<td>2560</td>
</tr>
<tr>
<td>No. 1 Adsorbed</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>No. 7 Unadsorbed</td>
<td>80</td>
<td>160</td>
</tr>
<tr>
<td>No. 7 Adsorbed</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>No. 8 Unadsorbed</td>
<td>40</td>
<td>160</td>
</tr>
<tr>
<td>No. 8 Adsorbed</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>No. 25 Unadsorbed</td>
<td>10</td>
<td>320</td>
</tr>
<tr>
<td>No. 25 Adsorbed</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

* Both sera no. 1 and no. 7 were collected from patients with ATL, serum no. 8 was from a patient with mycosis fungoides, and serum no. 25 was from a patient with exanthem subitum. Each serum was adsorbed with recombinant Protein G-agarose at room temperature for 15 min or left unadsorbed, and then used for ACIF.
† HHV-6-infected CBMCs at 5 h.
‡ HHV-6-infected CBMCs at 7 days.

The human sera used in this study were collected from patients with various diseases, but mostly adult T cell leukaemia (ATL), and from eight healthy adults. Anti-HHV-6 antibodies in the sera were examined by ACIF using CBMCs which had been infected for 7 days as antigen.

The chemicals used for the inhibition of DNA, RNA and protein synthesis were 2,2'-anhydro(1-β-D-arabinofuranosyl)cytosine (Cyclo-C; Kojin), actinomycin D (Act D; Sigma) and cycloheximide (CH; Nakarai Chemicals), respectively. These were dissolved in distilled water and sterilized by filtration through a filter of pore size 0.2 µm. Working solutions were made with growth medium just before experiments were performed.

An attempt was made to find a novel antigen–antibody system in cells infected with HHV-6. As most of the sera were collected from patients with ATL, CBMCs infected with the HG strain, an isolate from a patient with exanthem subitum, were used for this purpose. CBMCs were inoculated with HHV-6 infected at a multiplicity of approximately 0.01 TCID₅₀/cell. After adsorption for 2 h at room temperature with gentle agitation, the cells were washed once with growth medium. The cells were resuspended in growth medium and incubated at 37 °C for 5 h in an atmosphere of 5% CO₂. Using acetone-fixed infected CBMCs as antigen, 67 human sera positive for anti-HHV-6 antibody were screened for a novel antigen–antibody system. Of those sera, two (nos. 7 and 8) gave nuclear fluorescence in cells within 5 h after infection. However, these nuclear antigens were not detected by other sera positive for anti-HHV-6 antibody, even that with high titre (no. 1).

Nuclear antigens were also induced in cells infected with either the Hashimoto strain or the KA strain. The antinuclear antigen antibody titres of sera nos. 7 and 8 were 80 and 40, respectively (Table 1). When the sera were adsorbed with recombinant Protein G-agarose (Life Technologies), no fluorescence was detected in infected CBMCs. Thus, the antibodies against the nuclear antigens seemed to be IgG.

Owing to the inefficiency of infection in the CBMC/HG strain system, further analysis of the nuclear antigens was carried out using MT-4 cells and the Hashimoto strain of HHV-6. To examine whether a function(s) of the viral genome is required for the appearance of the nuclear antigens, MT-4 cells were inoculated with HHV-6 irradiated with u.v. light of various doses and nuclear antigen-positive cells were counted 5 h after infection. The number of positive cells reduced exponentially as a function of u.v. dose, whereas the reduction in viral infectivity was more rapid (Fig. 1). In addition, heat-inactivated virus did not induce nuclear antigens in MT-4 cells.

MT-4 cells were inoculated with HHV-6 at a multiplicity of approximately 1.0 TCID₅₀/cell. After adsorption for 2 h at room temperature with or without inhibitors, the cells were washed as described above. Then cells were resuspended in growth medium with or without inhibitors and incubated at 37 °C in 5% CO₂ for various periods. MT-4 cells infected with HHV-6 were stained with either serum positive for antibody against nuclear antigens (no. 8) or convalescent serum from the patient with exanthem subitum (no. 25). Neither serum gave fluorescence in uninfected MT-4 cells (Fig. 2a, b). Serum no. 8 gave nuclear fluorescence in infected MT-4 cells as early as 3 h after infection, and approximately
65% of cells became positive at 8 h after infection (Fig. 2c, e); infected cells stained with serum no. 25 showed no fluorescence (Fig. 2d, f). In the presence of CH or Act D from the initiation of infection, the appearance of the nuclear antigens was inhibited in a dose-dependent fashion (Table 2). The lowest doses of CH and Act D to inhibit completely the appearance of nuclear antigens were 2.5 μg/ml and 0.25 μg/ml, respectively (Fig. 2g, h). Cyclo-C (25 μg/ml) showed no inhibitory effect on the appearance of nuclear antigens (Fig. 2i, j). The replacement of CH (2.5 μg/ml) with Act D (0.25 μg/ml) at 5 h after infection allowed nuclear antigens to appear in infected MT-4 cells (Fig. 2k, l). This suggested that the HHV-6 nuclear antigens are IEAs.
As HHV-6 antigens for immunofluorescence tests, many investigators use cells which have been infected for approximately 7 to 10 days. Even in the most rapid system reported, HHV-6 antigens were detected 25 h after infection (Asada et al., 1989). In comparison, the nuclear antigens described here appear extremely rapidly after infection. Analysis of the appearance of the nuclear antigens in cells treated with inhibitors of RNA and protein synthesis indicated that the nuclear antigens are not antigens from virus inoculated into the nucleus, but are synthesized de novo after infection. In addition, the transcription of HHV-6-specific RNA is limited in the presence of CH (Dr K. Yamanishi, personal communication). These lines of evidence strongly suggest that the nuclear antigens in HHV-6-infected cells are equivalent to the IEAs of other human herpesviruses (Beth et al., 1976; Geder, 1976; Michelson-Fiske et al., 1977; Tanaka et al., 1979; Shiraki & Hyman, 1987). It was also shown that the de novo synthesis of nuclear antigens requires the function(s) of the intact viral genome, suggesting that they are virus-encoded. However, whether the nuclear antigens are really HHV-6 IEAs must await mapping of their genes on the viral genome because the induction of host cell genes, e.g. CD4 in the T cell (Lusso et al., 1991) cannot be excluded.

We attempted to analyse putative HHV-6 immediate early polypeptides by PAGE. However, it was difficult to determine their number and size, probably because the titre of antibodies against the putative IEAs was too low. Therefore, we are preparing monoclonal antibodies against the putative IEAs.

The function(s) of the putative IEAs of HHV-6 is not clear, but they may be involved in the control of expression of other HHV-6 gene(s), shutoff of host cell DNA replication (Di Luca et al., 1990) and/or transactivation of other viral regulatory elements. Recently, Martin et al. (1991) have identified a trans-activating function for other viral regulatory elements in the putative IE region of the HHV-6 genome. Although the function and arrangement of the putative IE region of HHV-6 is very similar to that of the major IE region of HCMV, the gene product(s) has not yet been identified and we do not know whether our IEAs are encoded in this region of HHV-6.

Antibodies against the putative IEAs of HHV-6 were detectable in sera positive for HHV-6 antibody only rarely. This was true for sera from patients with ATL and other disorders with high anti-HHV-6 antibody titres; only two sera were positive for anti-putative IEAs. Even extremely high titre serum contained no antibody against the putative IEAs of HHV-6 (Asada et al., 1989). Yoshida et al. (1990) also failed to find positive serum from patients with exanthem subitum. Two patients with anti-putative IEA antibodies had no common clinical background, which would have indicated a relationship between these antibodies and the stage of disease. Therefore, the clinical significance of anti-putative HHV-6 IEA antibodies remains to be elucidated.

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References


Short communication


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