Immunosuppressive Effect of Human Herpesvirus 6 on T-Cell Functions: Suppression of Interleukin-2 Synthesis and Cell Proliferation

By Louis Flamand, Jean Gosselin, Irina Stefanescu, Dharam Ablashi, and José Menezes

Human herpesvirus 6 (HHV-6), the etiologic agent of roseola, is ubiquitous, establishes latency in the host, and can infect a variety of immunocompetent cells, with CD4+ T lymphocytes being the targets in which it replicates most efficiently. The present study was undertaken to learn more about specific immunobiologic effects of HHV-6 infection on T-lymphocyte functions. Our data demonstrate that infection of peripheral blood mononuclear cells (PBMC) by HHV-6 results in suppression of T-lymphocyte functions, as evidenced by reduced interleukin-2 (IL-2) synthesis and cellular proliferation. In fact, HHV-6-infected PBMC secreted 50% less IL-2 than mock-infected cells after mitogenic stimulation with OKT3 antibody or phytohemagglutinin (PHA). The inhibition of IL-2 by HHV-6 was also observed in enriched T-cell cultures, suggesting a direct effect of this virus on this cell type. Messenger RNA (mRNA) analysis by reverse-transcriptase polymerase chain reaction (PCR) indicated that HHV-6 diminishes IL-2 mRNA levels in mitogen-stimulated peripheral blood T cells. These results were also confirmed by Northern blot using the leukemic T-cell line Jurkat. This inhibitory effect of HHV-6 did not require infectious virus, as the use of UV-irradiated HHV-6 produced similar results. Moreover, HHV-6-infected PBMC showed up to an 85% reduction in their mitogen-driven proliferative response, as compared with sham-infected cells. Proliferation of both CD4+ and CD8+ T cells was affected by HHV-6. Taken together, our data show that infection of T cells by HHV-6 results in immune suppression characterized by a downregulation of IL-2 mRNA and protein synthesis accompanied by diminished cellular proliferation.

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The human herpesvirus 6 (HHV-6) is the second most recently identified member of the herpesvirus family. HHV-6 was initially isolated in 1986 from patients with acquired immunodeficiency syndrome (AIDS) and other lymphoproliferative disorders. Immunologic, morphologic, and molecular analyses have clearly distinguished HHV-6 from other known herpesviruses. Restriction fragment length polymorphism analysis of genomes from different virus isolates showed the existence of two HHV-6 types: type A HHV-6 is represented by phenotype strains GS or U1102, whereas type B HHV-6 is associated with prototype strain Z29. Transmission of HHV-6 is believed to be mediated through saliva, from where infectious virus can be recovered. Infection by HHV-6, which occurs generally within the first 3 years of life, often causes a febrile illness followed by a cutaneous rash known as Exanthem subitum (ES), commonly called roseola, or can result in more serious pathologic abnormalities such as infectious mononucleosis, hepatitis, and meningencephalitis. Transmission of HHV-6 by transplant of infected organs and subsequent reactivation in immunocompromised patients has been reported.

It has been observed that HHV-6 can infect various cell types, most of which are constituents of the immune system. T and B lymphocytes, monocytes/macrophages, and megakaryocytes are all susceptible to infection by HHV-6, although CD4+ T lymphocytes represent the cell population in which HHV-6 most efficiently replicates. Previous studies have demonstrated that HHV-6 has immunomodulatory activities by inducing the secretion of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interferon-α (IFN-α) in leukocyte cultures in vitro. Furthermore, T cells infected by HHV-6 have reduced surface expression of CD3 molecules, and this virus also impairs the proliferative response of peripheral blood mononuclear cells (PBMC). CD4+ T lymphocytes play a pivotal role in immunity by regulating the immune response of other cell types via cell-to-cell contact and by the secretion of soluble mediators called cytokines. Although CD4+ T lymphocytes can secrete various cytokines, the principal and by far the most studied are IL-2 and IFN-γ. The main functions of IL-2 are to stimulate the proliferation of activated T and B cells, to enhance the generation of cytotoxic T lymphocytes, and to induce natural killer cell activity through induction of IFN-γ gene transcription and translation. Viruses can affect immunologic function in a variety of ways. For example, it has been reported that cytomegalovirus can diminish IL-1 and IL-2 secretions by PBMC, as well as impair the proliferative response of these cells to mitogen. Also, two paramyxoviruses, the respiratory syncytial virus and the measles virus, have been shown to inhibit the proliferative response of T cells. In an effort to further our understanding of the effects of an infection by a predominantly T-lymphotropic human herpesvirus, we studied various immunologic parameters after infection by HHV-6 of PBMC, enriched primary human T cells, and the T-cell line Jurkat. Our results indicate that HHV-6 suppresses IL-2 mRNA and protein synthesis in peripheral blood T-lymphocyte cultures. Furthermore, mitogen-driven proliferative response of both CD4+ and CD8+ T lymphocytes was inhibited by HHV-6. These results indicate...
cate that HHV-6 can alter normal immune functions and, therefore, may contribute to immunopathologic processes associated with HHV-6 infection.

MATERIALS AND METHODS

Cells and virus. PBMC and cord blood mononuclear cells (CBMC) were obtained from healthy donors after centrifugation of heparinized venous blood over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradients and cultured as previously described. The HHV-6 serologic (antibody) status of each donor was determined by testing the sera by indirect immunofluorescence assay on HHV-6-infected HSB-2 cells. Individuals were considered seronegative when the antibody titer against HHV-6 was ≤5. The cell lines HSB-2 and Jurkat E6.1 were purchased from the American Type Culture Collection (ATCC; Rockville, MD) and cultured in RPMI-1640 supplemented with 10% fetal bovine serum and antibiotics. These cell lines were tested for the presence of mycoplasma and were found to be negative. The HHV-6 used in this study (GS strain [1]) was propagated on HSB-2 cells as previously described. Virus was concentrated from culture supernatant by high-speed centrifugation. The viral preparation used in these studies had a titer of >10^6 50% tissue culture infective dose (TCID50) per milliliter. Viral preparation and culture medium, tested for the presence of endotoxin by the limulus amebocyte assay (Sigma, St Louis, MO), were found to contain less than 20 pg/mL.

Isolation of T lymphocytes. Monocytes were removed from PBMC by adherence to plastic. The nonadherent cells were passed through nylon wool column to remove B lymphocytes. A portion of the eluate was analyzed by flow cytometry for T-lymphocyte purity using specific monoclonal antibodies (MoAbs). The cell population was CD3+ (95%), CD19- (1%), and CD14+ (less than 1%). Further purification of T-cell subpopulations was obtained by panning as described. CD4 and CD8 subpopulations used were always greater than 90% pure as estimated by flow cytometry using anti-CD4 and anti-CD8 MoAbs (Becton Dickinson, San Jose, CA).

Cell treatments. PBMC, enriched T cells, and Jurkat E6.1 cells (10⁶ cells per milliliter) were treated with either mock-infected culture supernatant, HHV-6 (10⁶ TCID50), phytohemagglutinin-P (PHA; 10 μg/mL), DIFCO, Detroit, MI), OKT3 MoAb (25 ng/mL; Ortho Diagnostics, Rantian, NJ), and tetracanadecanol-phorbol-13-acetate (PMA; 20 ng/mL), or infected with HHV-6 for 2 hours before mitogenic stimulation. In some experiments, PBMC were preincubated for 1 hour with phosphonoacetic acid, 200 μg/mL (PAA, an inhibitor of viral DNA polymerase activity; Sigma), before infection or stimulation. HHV-6 was inactivated by heat (56°C, 1 hour) or by UV light irradiation (2 x 10⁶ μL). Effectiveness of inactivation treatments was tested by determining the infectivity of viral particles by immunofluorescence assays on HSB-2 cells using MoAb specific for HHV-6 gp110. Consistently less than 1% of cells were found to be positive for HHV-6 gp110 4 days postinfection. As specificity control, HHV-6 neutralized with a reference serum was used. The HHV-6 antibody-positive serum used was from a patient with acute lymphoproliferative leukemia from whom the GS isolate was obtained. GS serum had IgG titer to HHV-6 of 320. As measured by immunofluorescence assay (IFA), and could neutralize 1,000 TCID50 of HHV-6 at a 1:20 dilution. The serum with gold labeling binds to the surface of HHV-6. In control experiments we found that serum samples adsorbed with Epstein-Barr virus, human cytomegalovirus, and herpes simplex virus type 1 lost their neutralizing activities to these viruses but still retained activities to HHV-6. For IL-2 determinations, cell-free supernatants were collected at different times and kept frozen until used. For cell proliferation assays, cultures were incubated at 37°C with 5% CO2 for 3 days before being pulsed with 1 μCi of [3H]TdR (DuPont, Mississauga, Ontario, Canada) for 4 hours. Cells were harvested onto glass fiber filter. Radioactivity was determined by liquid scintillation counting (LSC).

mRNA analyses by reverse-transcriptase polymerase chain reaction (RT-PCR). Levels of mRNA were measured using RT-PCR procedure, essentially as previously described. Peripheral blood T lymphocytes were stimulated either with culture medium, HHV-6, PHA (2 μg/mL) plus PMA (10 ng/mL) or first with HHV-6 followed by PHA plus PMA. Cells were incubated with HHV-6 for 2 hours before ratiogenitic stimulation (time, 0 hour). Polyadenylated (poly-A) RNA was isolated at 6 and 12 hours postmitogenic stimulation. Reverse transcription was performed by incubating poly-A RNA with 3 μmol/L random hexamer (Pharmacia), 5 mMol/mL MgCl2, 50 mMol/L KCl, 10 mMol/L Tris-HCl pH 8.3, 1 mMol/L of the four deoxyribonucleoside triphosphates (dNTPs), 20 U RNA Guard (Pharmacia), and 200 U Mo-MuLV RT (Gibco Life Technologies, Mississauga, Canada) in a total volume of 30 μL for 1 hour at 42°C, followed by heating at 80°C for 10 minutes. The PCR was performed by amplifying 3 μL of the RT reaction. PCR buffer contained 20 mMol/L Tris-HCl pH 8.4, 50 mMol/L KCl, 2.5 mMol/L MgCl2, 0.1% Triton X-100, 0.01% gelatin, 200 μmol/L of the four dNTPs, 1 μmol/L primer A (5' end), 1 μmol/L primer B (3' end), and 2 U of Taq DNA polymerase (Pharmacia). Primer sequences for IL-2 were as follows: primer A, 5'-ATGATCAGGATCGAATTCC- TG-3', primer B, 5'-TCAAGTGATGTTAGA-TGATCTTTTG-ACAAATTTG-3' (462-bp amplified fragment); actin primer A, 5'-CCCTCCTGGCAGTGGACTCCT-3', and primer B 5'-GGAGAAGAATGTACCTTACCTTTGG-T-3' (202-bp amplified fragment). Thirty cycles were performed, each consisting of denaturation at 92°C for 1 minute, annealing at 56°C for 2 minutes, and extension at 72°C for 1 minute using a DNA mini cycler (AECL, Chalk River, Ontario, Canada). Ten percent of the PCR products was resolved on a 1.5% agarose gel band transfer onto a nylon membrane. Blots were hybridized with 5' end-labeled specific oligonucleotide overnight at 55°C, washed twice with 2X standard sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) at room temperature and once at 55°C. Membranes were exposed overnight at -70°C onto XAR films (Eastman-Kodak, Rochester, NY). Intensity of bands was determined by scanning the film with a laser densitometer (Pharmacia).

Northern blot. Jurkat E6.1 cells (10⁶ cells per sample) were either treated with mock-infected culture medium and mitogens (OKT3, 100 ng/mL + PMA, 20 ng/mL) or infected with HHV-6 (10⁶ TCID50) for varying time periods before mitogen stimulation. Total RNA was extracted and electrophoresed through formaldehyde-containing agarose gel, transferred onto nylon membranes, prehybridized [0.75 mol/L NaCl, 0.05 mol/L NaH2PO4, 5 mMol/L (EDTA) X 5, 0.5% SDS, 5X Denhardt’s solution, 100 μmol/mL salmon sperm DNA, 50% formamide] at 42°C for 4 hours, and hybridized overnight at 42°C in prehybridization buffer supplemented with 5% dextran sulfate. IL-2 mRNA expression was detected using a 32P-labeled 462 bp PCR amplified probe (primers described above). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was detected using a 32P-labeled cDNA clone (ATCC). Hybridization signals were quantitated by scanning densitometry.

II-2 determination. IL-2 in the culture supernatant was quantitated using a commercial enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN). The sensitivity of the test was 5 pg/mL.

Surface antigen expression. PBMC were cultured for 48 hours with culture medium, HHV-6, and PHA, or HHV-6 plus PHA. Cells were then washed twice and incubated for 1 hour with either phycoerythrin (PE)-labeled anti-Tac (p55, a chain) MoAb, fluorescein isothiocyanate (FITC)-labeled anti-CD8 MoAb, FITC-labeled anti-CD4 MoAb (Becton Dickinson), or incubated first with anti-p75 (IL-2R β chain; Endogen, Cambridge, MA) MoAb, followed by...
goat anti-mouse IgG FITC-labeled antibodies (Ortho Diagnostics). FITC-labeled IgG2a, and PE-labeled IgG, irrelevant MoAbs (antikeyhole lympet hemocyanin) were used as controls. After three washes, $10^5$ cells were analyzed for surface molecule expression by flow cytometry.

Statistical analyses. Statistical analysis of data was performed using a standard Student's $t$-test. Differences were considered significant when $P < .05$.

RESULTS

Inhibition of IL-2 synthesis by HHV-6. CD4$^+$ T lymphocytes, the main cellular targets for HHV-6 replication, are known to secrete various cytokines, including IL-2 and IFN-$y$, and to proliferate upon activation. The consequences of an HHV-6 infection on the function of these T cells are not well known. The following experiments were designed to evaluate T cell-associated functions after infection with HHV-6. We first evaluated the potential of PBMC and T cells to secrete IL-2 in response to HHV-6. Consistently, levels of IL-2 below the detection limit were observed after infection of PBMC or CBMC with $10^5$ TCID$_{50}$ of HHV-6, despite the fact that IL-1 was present in the same culture supernatants (data not shown). This was the case whether PBMC were derived from HHV-6-seropositive or -seronegative donors. Supernatants were collected and tested for the presence of IL-2 up to 7 days postinfection (PI), and never was there presence of any detectable amount of IL-2 in these cultures (data not shown).

The undetectable levels of IL-2 in culture supernatants from HHV-6–infected PBMC lead us to study the potential inhibitory action of HHV-6 on IL-2 secretion. PBMC and clone E6.1 of the Jurkat cell line, the latter being a CD4$^+$ T cell line that secretes IL-2 upon stimulation, were mitogen-stimulated in the presence or absence of HHV-6. Results indicate (Fig 1A) that high levels of IL-2 were detected when PBMC were stimulated with OKT3. However, when these cells were preincubated with HHV-6 before OKT3 stimulation, a greater than 50% reduction in IL-2 synthesis by PBMC was observed, despite no differences in cell viability. Similar results were obtained using UV-irradiated HHV-6 (data not shown). This reduction in IL-2 synthesis was also observed when PHA was used as mitogen. The specificity of the IL-2 inhibition by HHV-6 was confirmed by neutralizing the virus with specific antibody before OKT3 stimulation. Indeed, neutralized HHV-6 did not suppress IL-2 synthesis (Fig 1A). Cells treated with serum alone secreted similar amounts of IL-2 as did mock-infected cells (data not shown).

Cells were isolated, enriched, treated, and cultured (10$^6$/mL) as described in Materials and Methods. After 24 hours of culture, supernatants were collected and assayed for IL-2 using an ELISA kit. Values (mean ± SD) were calculated from triplicate cultures. Numbers in parentheses indicate the percentage of IL-2 inhibition.

<table>
<thead>
<tr>
<th>Table 1. Effects on HHV-6 on IL-2 Synthesis by Enriched T-Cell Populations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-2 (pg/mL)</strong></td>
</tr>
<tr>
<td>PBMC</td>
</tr>
<tr>
<td>Mock-infected</td>
</tr>
<tr>
<td>HHV-6</td>
</tr>
<tr>
<td>PHA</td>
</tr>
<tr>
<td>HHV-6 + PHA</td>
</tr>
<tr>
<td>CD3$^+$ T cells</td>
</tr>
<tr>
<td>Mock-infected</td>
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<tr>
<td>HHV-6</td>
</tr>
<tr>
<td>PHA + PMA</td>
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<tr>
<td>HHV-6 + PHA + PMA</td>
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<tr>
<td>CD8$^+$ T cells</td>
</tr>
<tr>
<td>Mock-infected</td>
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<tr>
<td>HHV-6</td>
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<tr>
<td>PHA + PMA</td>
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<td>HHV-6 + PHA + PMA</td>
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</tbody>
</table>

Cells were isolated, enriched, treated, and cultured (10$^6$/mL) as described in Materials and Methods. After 24 hours of culture, supernatants were collected and assayed for IL-2 using an ELISA kit. Values (mean ± SD) were calculated from triplicate cultures. Numbers in parentheses indicate the percentage of IL-2 inhibition.

* $P < .05$.

Fig 1. Effect of HHV-6 on IL-2 secretion by mitogen stimulated cells. (A) PBMC were isolated as described in Materials and Methods. PBMC (10$^6$ cells/mL) were either treated with mock-infected culture supernatant, HHV-6 (10$^5$ TCID$_{50}$/mL), OKT3 (25 ng/mL), HHV-6 for 2 hours followed by OKT3, HHV-6 preincubated with an HHV-6 antibody-positive serum followed by OKT3, PHA (10 mg/mL), or HHV-6 for 2 hours before the addition of PHA. After 24 hours, cell-free supernatants were collected and stored at −70°C until used. Their IL-2 content was measured using a commercial ELISA kit. The data shown are from triplicate determinations with PBMC from one donor; when the experiments were repeated with PBMC from three other donors, similar results were obtained ("$P < .05$). (B) Jurkat cells were either mock-infected, infected with HHV-6, and stimulated with mitogens (PHA + PMA) or exposed to HHV-6 for 2 hours before stimulation with mitogens. Supernatants of 24-hour cultures were collected and stored frozen until IL-2 determination. These results represent means of three separate experiments ± SD ("$P < .05$)
shown). PBMC from both HHV-6-seropositive and -seronegative individuals were found to have impaired IL-2 secretion in the presence of HHV-6. Similar results were observed with the cloned CD4^+ T-cell line Jurkat (Fig 1B); ie, infection of Jurkat cells with HHV-6 resulted in reduced IL-2 secretion by these cells after mitogen stimulation.

We next examined if the inhibition of IL-2 synthesis by HHV-6 can be observed using enriched cell populations. As shown in Table 1, a level of IL-2 suppression similar to that observed in PBMC cultures was obtained when enriched CD3^+ T cells were used. PMA was added to the cultures to provide the accessory signal required for T-cell activation in the absence of monocytes. As shown, HHV-6–treated T cells secreted about four times less IL-2 than control cultures. Similarly, HHV-6 reduced mitogen-induced IL-2 secretion in enriched CD4^+ T cells by 75%. Cultures of CD8^+ T cells produced a minimal amount of IL-2 after stimulation.

**Infection of T cells by HHV-6 reduces IL-2 gene transcription.** Knowing that HHV-6 can reduce IL-2 protein secretion by T cells by as much as 75%, we studied the effect of HHV-6 infection on IL-2 gene transcription using RT-PCR. Figure 2A shows the autoradiographs of the amplified RNA using primers specific for IL-2 and actin. Amplification of actin mRNA served as an internal control. Figure 2B represents the calculated levels of IL-2 mRNA after densitometry and normalized against the IL-2 mRNA levels in sham-treated cells. As shown in Fig 2A, the same amounts of mRNA were used for amplification (actin control). Analyses of the IL-2 transcripts in enriched T cells indicate that very little IL-2 mRNA was present 6 hours posttreatment in mock-
treated and infected T cells (lanes 1 and 2). Mitogen stimulation resulted in a 270-fold induction in IL-2 mRNA (lane 3). Infection by HHV-6 before mitogen stimulation resulted in a 35% decrease in IL-2 message (lane 4). At 12 hours posttreatment (lanes 5 through 8), similar results were obtained, although the fold-increase in IL-2 mRNA after mitogen stimulation was much lower (25-fold) when compared with mock-treated cells. Moreover, HHV-6 prevented the spontaneous induction of IL-2 (lane 6) observed in mock-infected cells (lane 5). Knowing that precise quantitation of mRNA by RT-PCR is sometimes difficult to achieve, we decided to confirm our results obtained from peripheral blood T cells using the Jurkat T cell line and Northern blot technique. As shown in Fig 3, kinetics of IL-2 mRNA reduction after infection suggest that nearly total suppression (85%) is observed by 4 hours PI. This effect is specific for IL-2 and does not affect GAPDH mRNA expression. The upper panel represents the calculated IL-2 mRNA levels at different time points after infection, while the lower panel represents the actual autoradiographs.

**HHV-6 abolishes the proliferative response of T cells.** The ultimate step of T-cell activation is proliferation. To continue our study concerning the impact of HHV-6 on T-cell functions, we next observed [3H]TdR incorporation as a means of assessing cellular proliferation in PBMC after HHV-6 infection and mitogenic stimulation. Results (Table 2) indicate that HHV-6 did not induce significant cell proliferation at 3 days PI. Furthermore, when PBMC were first infected with HHV-6 for 2 hours before mitogen stimulation, an 86% to 92% reduction in cell growth was observed, despite the fact that no significant differences in cell viability were observed, as determined by the trypan blue dye exclusion test. When PHA was used as mitogen, similar results were obtained (data not shown). The inhibitory effect of HHV-6 on cell growth was prevented by neutralizing the virus with human serum containing antibodies to HHV-6, as described in Materials and Methods. Neutralizing serum had no stimulatory effect on cell proliferation (data not shown). Heat-inactivated HHV-6 had no inhibitory effect, whereas UV-irradiated virus diminished mitogen-driven cell proliferation by 49% to 75% (Table 2). Furthermore, addition of PAA to cultures, to prevent viral replication, had no effect on the inhibition of cell proliferation (data not shown). The addition of recombinant IL-2 (rIL-2; 100 U/mL) to cultures could not restore the cell proliferative response to normal levels. Infected cells were not able to use IL-2, as witnessed by a 68% to 81% reduction in [3H]TdR uptake. Similarly, infected cells stimulated with OKT3 and IL-2 did not proliferate (Table 2).

**HHV-6 inhibits both CD4+ and CD8+ T-lymphocyte proliferation.** We next determined the effect of depletion of selected cell populations on HHV-6 inhibitory potential. As shown in Table 3, depletion of either CD14+, CD4+ or CD8+ cells from PBMC had no effect on HHV-6-induced inhibition. When monocytes (CD14+ cells) were removed, HHV-6 was able to inhibit [3H]TdR uptake by 43%. Similarly, depletion of CD8+ or CD4+ had no effect on the ability of HHV-6 to affect cell growth. Incubation of PHA-stimulated PBMC with supernatant (10% final volume) from HHV-6-infected PBMC, CD14+, or CD3+ cell cultures did not inhibit the cell proliferation (data not shown).

T-cell activation is accompanied by the induction of various cell surface molecules. Some of these molecules include the IL-2 receptor (IL-2R; α, β, and γ chains) and the class II major histocompatibility complex (MHC) antigens. To assess whether the observed diminished cellular proliferation induced by HHV-6 is attributable to the failure of these cells...
to express such molecules, we analyzed the expression of the IL-2R and HLA-DR on PBMC at 48 hours PI with HHV-6. As shown in Table 3, HHV-6–infected PBMC showed a 10% increased expression of both IL-2R α and β chains when compared with mock-infected cells. HHV-6–infected cells stimulated with PHA had an equal percentage and intensity of expression of both IL-2R α and β chains as did PHA-treated cells. Expression of HLA-DR (not shown) on lymphocytes was similar in infected cells to that of mock-infected cells. HHV-6–infected cells stimulated with PHA showed only marginal reduction of HLA-DR expression by preventing IL-2 synthesis and T-cell proliferation. Furthermore, early in the infection the CD3 molecules expressed on T cells is known to activate the CD3 molecules involved in cell adhesion, was expressed similarly on mock-treated and infected cells (data not shown).

Finally, inhibition of cell proliferation was found to be HHV-6 dose-dependent. As shown in Fig 4, infection of PBMC from two different donors with serial 10-fold dilutions of HHV-6 led to a virus dose-dependent inhibition of [3H]Tdr incorporation in PHA-stimulated PBMC, with the highest HHV-6 concentration causing the greatest inhibition. A dose of 10^6 TCID50 of HHV-6 reduced cell proliferation by 80%, whereas the 50% inhibitory concentration was 10^4 TCID50 of HHV-6. A TCID50 corresponding to 10^2 did not cause a significant reduction in cell proliferation.

### DISCUSSION

The mounting of an effective immune response against a pathogen is a complex event that requires antigen (Ag) processing, Ag presentation and recognition, cell activation and proliferation, and cytokine secretion. Each step of the response involves numerous finely regulated events. Activation of T-helper lymphocytes by Ag requires a minimum of two signals, the first being delivered through the T-cell receptor (TCR) and the second triggered by the presence of costimulatory molecules on the activated protein C (APC). After these signals, IL-2 is produced, IL-2R (α, β, and γ chains) is expressed, and clonal expansion follows. Disturbance of one or more of these mechanisms can greatly alter the overall immune response to a given Ag. This study demonstrates that HHV-6, mainly a T-lymphotropic virus, interrupts the normal flow of events of the immune response by preventing IL-2 synthesis and T-cell proliferation.

In a previous report, we showed that HHV-6 strain GS induces high levels of IL-1β and TNF-α after infection of PBMC in vitro. It was expected that PBMC from seropositive donors for HHV-6 would, as one of the next events of the immune response, secrete IL-2 after in vitro infection by this virus. We could not demonstrate such IL-2 secretion after infection of PBMC by HHV-6. Therefore, we investigated the potential inhibitory effect of this virus on IL-2 secretion from mitogen-stimulated cells. Incubation of cells with HHV-6 for as little as 2 hours greatly affected the cells' ability to secrete IL-2 after mitogenic stimulation with either PHA or OKT3. Repeatedly, infected cells were found to secrete twofold less IL-2 than the uninfected, control cells, despite a lack of effect on cell viability. This phenomenon was observed regardless of the HHV-6–related serologic status of the PBMC donor. Mitogen stimulation of HHV-6–infected enriched CD3+ or CD4+ T lymphocytes also yielded reduced IL-2 secretion, suggesting that the virus acts directly on the T cell and does not require other cell populations to inhibit IL-2 synthesis. Furthermore, early in the infection (ie, 30 minutes), cells showed reduced ability to transcribe the IL-2 gene. Although the precise mechanism by which HHV-6 represses IL-2 transcription is not known, it can be hypothesized that this virus may carry a structural protein (resembling the VP16 tegument protein of herpes simplex virus type 1) that could modulate transcription of cellular genes as well as its own genes.

Interaction of specific antibodies with the TCR or with the CD3 molecules expressed on T cells is known to activate these cells. CD3 molecules are known to transmit the activation signals to the cytoplasm mainly through the activation of a phosphatidylinositol-specific phospholipase C and a tyrosine kinase. HHV-6 has been found to transcriptionally

### Table 2. Effect of HHV-6 on Mitogen-Induced Cellular Proliferation

<table>
<thead>
<tr>
<th></th>
<th>PBMC1</th>
<th>PBMC2</th>
<th>PBMC3</th>
</tr>
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<tbody>
<tr>
<td>Mock-Infected</td>
<td>257 ± 73</td>
<td>349 ± 88</td>
<td>327 ± 69</td>
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<tr>
<td>HHV-6 infected</td>
<td>297 ± 43</td>
<td>512 ± 291</td>
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<tr>
<td>OKT3</td>
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<td>10,485 ± 863 (86)*</td>
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<td>26,849 ± 1,766</td>
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<td>IL-2</td>
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<td>751 ± 50 (68)*</td>
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<td>2,507 ± 294 (85)*</td>
<td>5,364 ± 934 (72)*</td>
<td>ND</td>
</tr>
</tbody>
</table>

Abbreviations: HI, heat inactivated; ND, not determined.

* P < .05.
downregulate the expression of the CD3 molecule while upregulating the CD4 antigen. This can lead to mature T cells having an immature CD3⁻, CD4⁺, CD8⁻ phenotype. It is tempting to speculate that the inhibitory activity of HHV-6 on IL-2 secretion results from such downregulation of CD3 molecules. By reducing the expression of CD3, the virus may prevent the interaction of the CD3 with the mitogen. However, our data suggest that this may not be the mechanism by which HHV-6 inhibits IL-2 synthesis: (1) the blockade of IL-2 synthesis by HHV-6 occurs within 2 hours, a time at which surface expression of CD3 is not altered; (2) downregulation of CD3 molecules by HHV-6 requires infectious virus, whereas IL-2 synthesis is inhibited by UV-irradiated HHV-6; and (3) PAA has no effect on IL-2 inhibition induced by HHV-6. Interestingly, phosphonoformic acid, a drug analog to PAA, had been found by other investigators to prevent CD3 downregulation by the virus. Our results suggest that HHV-6, by interacting with T lymphocytes, triggers an inhibitory signal in the cell. This signal can be induced by the binding of the virus to the cell or it can be a factor brought inside the cell by the virus itself. This signal would then lead to a decrease in IL-2 mRNA levels. It has been well documented that stimulation of cells with OKT3 in the absence of APC can lead to a state of anergy characterized by the inability of these cells to secrete IL-2 and to proliferate. Recent evidence suggests that the reduction in IL-2 synthesis results from the failure to induce transcription factors such as NF-AT or one of the two NF-kB necessary for efficient IL-2 gene transcription. Therefore, it can be hypothesized that HHV-6-induced IL-2 inhibition may arise from a defect in the transcriptional elements controlling the IL-2 gene, an increase in the turnover rate of IL-2 mRNA, or by a deficient signal transduction pathway.

Experiments designed to answer these questions are currently being performed.

In addition to the reduced capacity of HHV-6–infected T cells to secrete IL-2 after OKT3 stimulation is the severely diminished proliferative response of these cells. HHV-6–treated cells showed a greater than 80% reduction in [³H]-TdR uptake when compared with sham-treated cells. Our results are in agreement with those of Horvat et al., who have made similar observations using PBMC. Preliminary results of DNA analysis of HHV-6–infected cells suggest that, after mitogenic stimulation, transition from G1 to S phase does not occur efficiently, and cells accumulate in the G1 phase. The reduced levels of IL-2 in supernatants of HHV-6–treated PBMC seem not to be responsible for the observed effect, as exogenously added IL-2 did not correct this defect. Furthermore, by cell depletion analyses, it was found that HHV-6 affected the proliferation of both CD4⁺ and CD8⁺ T-lymphocyte subpopulations. IL-2R (α and β chains) expression on the surface of infected cells did not differ from that of mock-infected cells, suggesting that the reduced cell growth observed was not a consequence of a

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**Table 3. Proliferative Response and IL-2R Expression of HHV-6–Treated Cells**

<table>
<thead>
<tr>
<th>PBMC or CD8 T cells</th>
<th>% of Cells Expressing IL-2R (mean fluorescence intensity)</th>
<th>α Chain</th>
<th>β Chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock-infected</td>
<td>76 ± 49</td>
<td>22 [59]</td>
<td></td>
</tr>
<tr>
<td>HHV-6</td>
<td>55 ± 126</td>
<td>22 [56]</td>
<td></td>
</tr>
<tr>
<td>PHA</td>
<td>47 ± 113</td>
<td>21 [59]</td>
<td></td>
</tr>
<tr>
<td>HHV-6 + PHA</td>
<td>57 ± 154</td>
<td>47 [106</td>
<td>78 [112]</td>
</tr>
</tbody>
</table>

PBMC were isolated and treated as described in Materials and Methods. Selected cell populations were depleted by panning. After 48 hours of culture, cells were harvested for 4 hours before being pulsed with [³H]-TdR for 4 hours. Cells were harvested onto glass fiber discs, and incorporated radioactivity was determined by LSC. The numbers in parentheses indicate the percentage of inhibition of [³H]-TdR incorporation.

* P < .05.

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**Fig 4. HHV-6 dose-dependent inhibition of cell proliferation.** PBMC from two donors were treated with 10-fold serial dilution of HHV-6 before PHA stimulation. After 3 days, cells were pulsed with [³H]-TdR for 4 hours before being harvested onto filters. Radioactivity was determined by LSC. Results are expressed as percentage of cell growth inhibition relative to PHA-treated cells.
defect in IL-2R expression, although we cannot rule out the possibility of a defect in the expression of the γ chain, essential for transduction of signal activation.64 Interestingly, Proust et al65 have shown that induction of IL-2R (α and β) in the absence of IL-2 can affect the ability of the cell to proliferate after appropriate stimulation. Therefore, the presence of IL-2 during the early phase of T-cell activation seems to be a prerequisite for the expression of a functional IL-2.66 By inhibiting IL-2, HHV-6 may induce an operational defect in the IL-2R signaling pathway leading to the inability of these cells to use IL-2.

In conclusion, the induction of an anergic T-cell state by HHV-6 may seriously impair normal immune functions. For instance, during HHV-6 viremia, where relatively high levels of free virions are present in the blood, a general state of unresponsiveness could be induced, thereby favoring the spread of pathogenic agents, particularly other viruses that may be reactivated in the host during HHV-6 active infection. Furthermore, in immunocompromised patients in which the reactivation of HHV-6 is a common event, the virus may contribute to further diminishing the overall immune functions. Whatever the mechanism by which HHV-6 acts, serious pathologic outcomes may result. Further studies are needed to clarify the immunopathogenesis of these outcomes. Such efforts will help to gain a better understanding of the immune dysregulation triggered by HHV-6 infection.

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