Pathogenic Effects of Human Herpesvirus 6 in Human Lymphoid Tissue Ex Vivo

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Human herpesvirus 6 (HHV-6) is a double-stranded DNA virus originally isolated from immunocompromised patients with lymphoproliferative disorders (24). Shortly thereafter, a primary tropism for CD4+ T lymphocytes was documented both in vitro (21) and in vivo (28). Two major viral subgroups, designated A and B, have been defined, which form two segregated clusters with unique genetic, biologic, and immunologic characteristics. While HHV-6B is highly prevalent in the human population in all geographic areas, the epidemiology of HHV-6A is still largely undefined. Primary infection with HHV-6B, which occurs almost universally during early childhood, has been linked to the etiology of exanthema subitum, a benign febrile disease (30). Conversely, little is known about the time and pathological consequences of primary HHV-6A infection. In adults, HHV-6 infection and/or reactivation has been associated with a wide variety of diseases, although most of these associations have not been substantiated by rigorous epidemiological and virological studies (17). Considerable interest was raised by recent data on a possible link between HHV-6 and multiple sclerosis (2), but the evidence hitherto accumulated is controversial (22, 27).

In immunocompromised people, HHV-6 causes life-threatening infections of the respiratory tract and the central nervous system, as well as bone marrow and organ graft failure (17). Besides this role as an opportunistic agent, however, several lines of clinical and experimental evidence support the concept that HHV-6 may also act as an immunosuppressive agent in its own right. Both HHV-6 subgroups were shown to induce severe thymocyte depletion in heterochimeric SCID-hu thym/liv mice, with a predominant tropism and cytopathicity for immature thymic precursor cells (8). Disseminated coinfection with HHV-6A and -6B was linked to progressive and ultimately fatal immunodeficiency in a human immunodeficiency virus (HIV)-negative child (13). Moreover, active HHV-6 infection, as documented by the presence of plasma viremia, was associated with lymphocytopenia and defective lymphocyte proliferation to recall antigens in patients who had received allogeneic stem cell transplantation (29). Consistent with these observations, it has been suggested that HHV-6, particularly subgroup A, may act as a cofactor in the progression of HIV disease, contributing, either directly or indirectly, to the depletion of CD4+ T cells and other immune dysfunctions associated with full-blown AIDS (19).

In spite of the evidence thus far accumulated indicating that HHV-6 infection can have immunosuppressive effects, progress in this area of investigation has been hampered by the lack of physiologically relevant study models. The systems most com-
monly used to grow HHV-6 in vitro, such as cord blood- and peripheral blood-derived mononuclear cells cultured in suspension (17), require cells to be maximally stimulated with polyvalent activators, such as plant lectins or mitogenic anti-CD3 antibodies, in order to sustain HHV-6 replication. However, such stimulation is unlikely to occur in vivo. For this reason, we decided to investigate the cellular tropism and the pathogenic effects of HHV-6 in the ex vivo lymphoid tissue system. This physiologically relevant model was previously used to study HIV infection (5, 6, 10, 11), as well as the interactions between HIV type 1 (HIV-1) and HHV-6 (9). A series of specific issues were addressed in the present work: (i) whether the lymphoid tissue system can efficiently support the growth of both HHV-6 subgroups (A and B) in the absence of exogenous stimuli; (ii) which cell subsets are primarily targeted by HHV-6A or B; (iii) whether naive and/or memory T cells show differential susceptibility; (iv) whether the infection is cytopathic for tissue-residing lymphocytes and whether it alters the expression of functionally important cell surface molecules such as the viral receptor, CD46 (25), CD3 and CD4; and (v) whether HHV-6 modulates the expression of selected cytokines and chemokines. Our results provide the first evidence, in a physiologically relevant study model, that HHV-6 can profoundly perturb the physiology of human lymphoid tissue.

MATERIALS AND METHODS

Tissue culture and infection. Human tonsils surgically removed during routine tonsillectomy and not required for diagnostic purposes were received within 5 h of excision. Tonsils were dissected into 3-mm blocks and cultured as described earlier (5, 6). For HHV-6 infection, the tissue blocks were either inoculated overnight with 10 μl of viral stock directly pipetted onto the external surface of each fragment. Alternatively we soaked tissue blocks in the undiluted viral stock for 4 h, and then washed with complete culture medium. Both methods of infection resulted in a similar HHV-6 production, and for the purpose of this work we used the former protocol of infection. The viral strains used were HHV-6 GS, a subgroup A isolate originally derived from a patient with a hematological malignancy (24), and HHV-6 PL1, a subgroup B isolate obtained from HHV-6 GS, a subgroup A isolate originally derived from a patient with a hematological malignancy (24), and HHV-6 PL1, a subgroup B isolate obtained from HHV-6 GS, a subgroup A isolate originally derived from a patient with a hematological malignancy (24), and HHV-6 PL1, a subgroup B isolate obtained from a patient with a hematological malignancy (24). The viral strains used were HHV-6 GS, a subgroup A isolate originally derived from a patient with a hematological malignancy (24), and HHV-6 PL1, a subgroup B isolate obtained from a healthy adult subject (8). The viral stocks contained approximately 10^6 cell culture infectious doses per ml, as determined by titration of infectivity on primary human peripheral blood mononuclear cells (PBMC). The viral stocks were produced as described (21) by infecting activated total (for GS) or CD8-depleted (for PL1) PBMC and by collecting cell-free culture supernatants at the time of peak virus-induced cytomorphological transformation (around days 6 to 8 postinfection). The culture medium was changed every three days.

Quantification of HHV-6 DNA by calibrated real-time PCR. DNA extraction from cell-free culture supernatants or from pelleted cells was performed using the phenol-chloroform method. The quantitation of HHV-6 DNA was performed using the TaqMan technology on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, Calif.); a quantitative β-actin detection system (Applied Biosystems) was used to quantify the content of human genomic DNA. At least 1 μg of genomic DNA recovered from each cellular pellet was subjected to real-time PCR analysis. Details about the real-time PCR technique for HHV-6 have been reported elsewhere (9, 16). The primers and the probe were derived from open reading frame U67, which is highly conserved in both viral subgroups (A and B). The primers were 5'-CAA AGCACAATTATCCAGAGGCG-3' and 5'-GGCAGTGGTGGAGATGTCG A-3'; the probe was 5'-CACCAGAGTCACAACCGAGAAG-3'. The probe was covalently linked at the 5' end to the reporter dye 6-carboxy-fluorescein and at the 3' end to the quencher dye 6-carboxy-tetramethylrhodamine. The amplified product was 133 bp long. The reaction was performed in a total volume of 25 μl with dATP, dCTP, and dGTP at 100 μM each; dUTP at 200 μM; TaqMan buffer A, 1×; 5.5 mM magnesium chloride; primers at 300 nM each; 50 μM probe; 0.625 U of AmpliTaq Gold; 0.25 U of uracil-N-glycosylase; and 40% (vol/vol) sample DNA. PCR was performed first by activation of the uracil-N-glycosylase (95°C for 15 min) and by 40 cycles of amplification (denaturation step, 15 s at 95°C; annealing-extension step, 1 min at 60°C). Data analysis was performed using sequence detection software (Applied Biosystems). As a reference for HHV-6 DNA quantitation, the HHV-6 fragment amplified by the above primers was cloned into the pCRRII plasmid (Invitrogen). A standard curve was obtained by serially diluting the plasmid stock previously quantified by spectrophotometry. Serial dilutions of genomic DNA isolated from PBMC of normal donors were used to generate a reference curve for β-actin quantitation. The results were normalized using a synthetic DNA calibrator molecule (10^6 copies per reaction), added to the samples before the extraction step, which allows to control for intersample extraction efficiency and to monitor PCR artifacts; the calibrator molecule was then coamplified with the HHV-6 target sequence. Primers and calibrator probe were covalently linked at the 5'-CACCAGACGTCACACCCGAAGGAAT-3' and 5'-CACCAGAGTCACAACCGAGAAG-3' sequences. For tissues from two donors infected ex vivo with HHV-6A, we compared the amounts of cell-associated and cell-free virus. Comparison of these data obtained from samples collected on days 6 and 9 postinfection revealed that the amount of virus in the culture medium correlates with that associated with cells. For the purpose of this work we based our analysis of HHV-6 replication on quantification of viral DNA in samples of culture medium.

Flow cytometry. At day 12 or 13 postinfection, single-cell suspensions were prepared from tissue blocks by mechanical dissociation. Tissue blocks were placed into a petri dish with complete medium and gently ground with a pestle. As shown previously, such a procedure releases lymphocytes from stromal elements (6). Similarly, cells emigrated into the collagen sponge were mechanically squeezed out and collected by centrifugation. To determine whether the infection resulted in a physiologically relevant study model, that HHV-6 can profoundly perturb the physiology of human lymphoid tissue.

Statistical analysis. Data are presented as means ± standard errors of the mean (SEM). The significance of the differences between various data sets was tested using Student paired t test. The correlation between chemokine production and accumulation of HHV-6 genome equivalents was assessed by linear regression and log transform regression analysis in which the chemokine level was correlated with the log ([HHV-6 genome equivalents] + 1) in order to
RESULTS

HHV-6A and -6B efficiently replicate in the absence of exogenous stimulation in human tonsillar tissue ex vivo. To establish a model system for the study of HHV-6 infection, we tested the ability of different HHV-6 strains to replicate in human lymphoid tissue in the absence of exogenous stimulation. The tissues were dissected into blocks and exposed to HHV-6. The viral strains used were HHV-6 GS, a subgroup A isolate originally derived from a patient with a hematological malignancy (24), and HHV-6 PL1, a subgroup B isolate obtained from a healthy adult subject (8). Viral replication was documented by measuring the kinetics of accumulation of viral genomes in culture medium using a quantitative-calibrated real-time PCR assay. Viral antigen expression was assessed by flow cytometry using MAb directed against a nonstructural nuclear phosphoprotein (pp41/38) and a structural envelope glycoprotein (gH), both of which are expressed late in the viral life cycle and associated with productive infection.

Analysis by real-time quantitative PCR showed a progressive accumulation of HHV-6 genome equivalents over time in the culture supernatants of infected tissues (Fig. 1A), with peak levels between $6.4 \times 10^7$ and $4.1 \times 10^9$ (median, $10^9$) genome equivalents per ml of culture supernatant at day 13 postinfection in tissues infected with HHV-6A ($n = 10$) and between $1.0 \times 10^8$ and $3.0 \times 10^9$ (median, $3.2 \times 10^8$) in tissues infected with HHV-6B ($n = 5$).
with HHV-6B \( (n = 5) \). Increasing levels of HHV-6 over time were also detected by measuring cell-associated DNA extracted from the same tissues (data not shown). The wide interassay variability in virus yield from different ex vivo-infected tissues was not surprising, as similar fluctuations were previously documented with HIV-1 grown in primary lymphoid tissues \( (5, 6, 9–11) \). As expected considering the ubiquitous distribution of latent HHV-6 infection \( (17) \), a low level of endogenous HHV-6 infection was detected in almost all control tissues not exposed to exogenous HHV-6 ex vivo. However, the level of HHV-6 DNA in control tissues was small \( (0.03\% \pm 0.02\% \); \( n = 10 \), and even in the tissues with the highest endogenous infection it did not exceed 1.2\% of the levels measured in matched tissues exposed to exogenous HHV-6.

Productive HHV-6 infection was confirmed by the intracellular expression of the viral pp41/38 antigen, as detected by flow cytometry at day 13 postinfection \( (Fig. 1B) \). The mean proportion of viral antigen-positive lymphocytes was 28\% \( \pm \) 2\% in HHV-6A-infected tissues \( (n = 9) \) and 11\% \( \pm \) 2\% in HHV-6B-infected tissues \( (n = 9) \). In control tissues 1.6\% \( \pm \) 0.2\% \( (n = 9) \) of lymphocytes were HHV-6-antigen positive. Altogether, these data demonstrated that both HHV-6 subgroups productively infect human lymphoid tissue ex vivo in the absence of exogenous stimuli.

**T lymphocytes are the primary target cells for both HHV-6A and -6B in human lymphoid tissue.** The lineage of cells productively infected by HHV-6 in lymphoid tissue was investigated by multicolor flow cytometry using MAb directed against several leukocyte membrane antigens. The vast majority of viral antigen-positive cells were CD2\(^+\), with very limited productive infection in other cell types \( (Fig. 1B) \). Figure 2A shows the proportion of cells expressing viral antigens among CD4\(^+\) and CD8\(^-\) T lymphocytes. At day 13 postinfection with HHV-6A, the mean proportions of productively infected cells among CD4\(^+\) T cells and CD8\(^-\) T cells were similar \( (19\% \pm 3\% \text{ and } 18\% \pm 4\% \text{, respectively}; \ n = 11) \); nevertheless, since CD4\(^+\) T cells represent the predominant T-cell subpopulation in human lymphoid tissue, the majority of the infected cells expressed a CD4\(^+\) phenotype. A small proportion of infected T cells coexpressing CD4 and CD8 was consistently detected \( (2.1\% \pm 0.6\% \text{ and } 1.1\% \pm 0.2\% \text{ in tissues infected with HHV-6A and -6B, respectively}; \ n = 5) \).

In tissues infected with HHV-6B \( (n = 9) \), productive infection was seen in 21\% \( \pm \) 4\% and 5\% \( \pm \) 2\% CD4\(^+\) and CD8\(^-\) T cells, respectively. Thus, infection of CD4\(^+\) T cells occurred with a similar efficiency with the two viral subgroups, whereas CD8\(^-\) T cells were efficiently infected only by HHV-6A. The observation that in control tissues the proportion of endogenously infected CD8\(^-\) T cells was low \( (Table 1 \text{ and data not shown}) \) indirectly confirms the concept that latent HHV-6 infection in healthy subjects is sustained by strains belonging to subgroup B \( (17) \).

**HHV-6-infected T cells in human lymphoid tissue predominantly express a nonnaive phenotype.** Analysis of the cellular phenotype in control tissues showed a prevalence of nonnaive T cells of either CD45RA\(^-\)CD62L\(^+\), CD45RA\(^-\)CD62L\(^-\), or CD45RA\(^+\)CD62L\(^-\) phenotype, with a mean ratio of nonnaive to naive \((\text{CD45RA}\(^+\)\text{CD62L}\(^-\))\) T cells of 2.5 \( \pm \) 0.3 \( (n = 5) \). Statistical analysis of the relative level of HHV-6 infection in the two cell subsets revealed a preferential infection of nonnaive cells by both HHV-6A and -6B variants \( (Table 1 \text{ even after normalization of the results for their relative abundance. In the nonnaive T-cell subset, the proportions of cells expressing viral antigens at day 13 postinfection were 34\% \( \pm \) 7\% and 15\% \( \pm \) 2\% \( P < 0.001 \) \( (n = 5) \) for HHV-6A- and HHV-6B-infected tissues, respectively; among naive T cells, these proportions were 21\% \( \pm \) 9\% and 5\% \( \pm \) 2\% \( P = 0.12 \) \( (n = 3) \) \( (Fig. 2B) \). Thus, the probability for a nonnaive T cell to be infected by HHV-6A was 0.26 \pm 0.03, whereas for naive cells it was 0.10 \pm 0.03. In HHV-6B-infected tissues, these probabilities were 0.14 \pm 0.03 and 0.04 \pm 0.03, respectively.**
TABLE 1. Membrane phenotype of HHV-6-infected cells in ex vivo-cultured lymphoid tissue infected with HHV-6 A or B or naturally infected in vivo (control)

<table>
<thead>
<tr>
<th>Cell subset</th>
<th>Proportion of cells (mean ± SEM)</th>
<th>HHV-6 A</th>
<th>HHV-6 B</th>
<th>Control (endogenous)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>27.7 ± 2.0</td>
<td>10.5 ± 1.6</td>
<td>1.6 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>CD4+</td>
<td>19.2 ± 3.4</td>
<td>20.5 ± 3.6</td>
<td>3.3 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>CD8+</td>
<td>17.9 ± 4.0</td>
<td>5.2 ± 2.3</td>
<td>1.5 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>CD4+ CD8-</td>
<td>19.0 ± 4.1</td>
<td>22.2 ± 3.9</td>
<td>3.9 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>Naive+</td>
<td>14.6 ± 2.4</td>
<td>4.5 ± 2.6</td>
<td>2.8 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Nonnaive*</td>
<td>34.1 ± 7.1</td>
<td>21.4 ± 9.5</td>
<td>3.5 ± 1.9</td>
<td></td>
</tr>
</tbody>
</table>

*CD45RA+ 62L-.

Other phenotypes.

Cytopathic effect of HHV-6 in lymphoid tissue. To evaluate the cytopathic effects of HHV-6 in human lymphoid tissue ex vivo, we measured the absolute numbers of cells recovered from pooled tissue fragments using calibrator beads as a volume reference (10). A significant cell depletion (41% ± 11% of CD4+ and 37% ± 13% of CD8+ T cells with HHV-6A; 49% ± 11% of CD4 and 18% ± 14% of CD8+ T cells with HHV-6B; mean ± SD) was observed at day 12 to 13 postinfection (Fig. 3). Consistent with the phenotype of viral antigen-expressing cells, both HHV-6A- and -6B predominantly depleted CD4+ T cells; a significant loss of CD8+ T cells was observed in tissues infected with HHV-6A, but not in tissues infected with HHV-6B. Thus, HHV-6A depleted CD4+ and CD8+ T cells with similar efficiency (P = 0.26; n = 5), while HHV-6B depleted CD4+ T cells with significantly greater efficiency (P < 0.001; n = 5). These data indicate that in the context of human lymphoid tissue HHV-6 is cytopathic, and that cell depletion involves primarily the cells in which HHV-6 is replicating, without significantly depleting uninfected bystander cells.

HHV-6 induces a dramatic and generalized downmodulation of the CD46 receptor in lymphoid tissue. The CD46 glycoprotein, a member of the regulator of complement activity (RCA) family, was recently identified as a cellular receptor for HHV-6A and -6B (25). Thus, we investigated whether receptor downmodulation, which is a common sequela of viral infections, occurs in the context of human lymphoid tissue upon infection with HHV-6A and -6B. Cells from infected and matched uninfected tissues were stained for HHV-6 pp41/38, CD2 and CD46 expression. As illustrated in Fig. 4A, a marked downmodulation of CD46 expression was observed in CD2+ T cells obtained from tissues infected with HHV-6A. Interestingly, the decrease in fluorescence intensity was seen both in cells expressing viral antigens (mean reduction, 91% ± 4%) and in pp41/38 antigen-negative cells (“bystanders”) T cells (mean reduction, 82% ± 8%; n = 5). In contrast, CD46 downmodulation was less pronounced in tissues infected with HHV-6B, with a lower downmodulation in bystander versus infected T cells (mean reduction, 17% ± 8% versus 54% ± 9%; n = 3). This finding seems to reflect the overall lower efficiency of infection by HHV-6B in lymphoid tissue. Indeed, a significant correlation was seen between CD46 downmodulation in bystander T cells and viral-antigen expression (r = 0.8; P = 0.002). Downmodulation of CD46 in bystander cells was further confirmed by analysis of non-T (CD2+) lymphoid cells. In HHV-6A-infected tissues, the mean decrease in CD46 expression in bystander non-T cells was 80% ± 8%, whereas no significant effect was observed in HHV-6B-infected tissue (mean decrease, 0.3% ± 2.6%). These data demonstrate that HHV-6, particularly subgroup A, induces a marked loss of CD46 in human lymphoid tissue, which may result in an increased susceptibility to tissue damage secondary to spontaneous activation of the complement cascade (26).

HHV-6 alters the expression of T-cell surface antigens of functional relevance. We evaluated by flow cytometry the effects of HHV-6 on the expression of different T-cell surface markers of functional relevance. Similar to CD46, the CD3 antigen was found to be dramatically downmodulated in tissues infected with either HHV-6A or HHV-6B (Fig. 4B). In five HHV-6A-infected tissues studied, the expression of CD3 was virtually abrogated in HHV-6-expressing cells (mean reduction, 99.3% ± 0.1% relative to uninfected controls). However, there was only a limited downmodulation of CD3 in pp41/38 antigen-negative cells (mean reduction, 25.0% ± 2%). Similar results were obtained in three HHV-6B-infected tissues tested, with a marked downmodulation of CD3 in pp41/38 antigen-positive cells (mean reduction, 99.3% ± 0.2%), but a low degree of reduction (13% ± 8%) in uninfected bystander cells. At variance with CD46, no correlation was found in bystander cells between the viral load and CD3 downmodulation (r = 0.68; P = 0.1).

Unlike CD3, the CD4 antigen was upregulated on the surface of cells isolated from tissues infected with HHV-6 (Fig. 4C). In tissues infected by HHV-6A, the mean level of CD4 expression in HHV-6 pp41 antigen-positive cells was 179% ± 10% relative to that of CD4+ T cells in matched uninfected tissue; in tissues infected with HHV-6B, it was 155% ± 5%. By contrast, uninfected bystander CD4+ T cells showed a slight
FIG. 4. Downmodulation of cell surface antigens by HHV-6 in human lymphoid tissue ex vivo. At day 12 or 13 postinfection, the cells were stained with anti-CD46 PE, CD2 TriColor, CD3 APC and anti-HHV-6 nuclear protein pp41/38 antibody coupled with Alexa 488. (A) The left panel shows downmodulation of CD46, expressed as the percent median fluorescence intensity (MFI) in control tissue. The right panel shows typical contour plots at 2% probability representing CD46 versus HHV-6 pp41/38 for a HHV-6A infected tissue gated on CD2⁺ lymphocytes. (B) The left panel shows downmodulation of CD3 expressed as percent MFI in control tissue. The right panel shows typical contour plots at 2% probability representing CD3 versus HHV-6 pp41/38 for an HHV-6A-infected tissue gated on CD2⁺ lymphocytes. (C) Upregulation of CD4 is expressed as a percent MFI in control tissue. The right panel shows typical contour plots at 2% probability representing CD3 versus HHV-6 pp41/38 for a HHV-6A infected tissue gated on CD2⁺ lymphocytes. In all panels, the results represent the mean ± SEM (error bars) from five experiments with HHV-6A and three with HHV-6B, each performed with 54 tissue blocks derived from an individual tonsil donor.
reduction in CD4 expression compared to matched uninfected control tissues (mean levels, 62% ± 3% for HHV-6A and 77% ± 4% for HHV-6B relative to CD4 T cells from uninfected tissues). The difference between infected and uninfected cells was statistically significant (P < 0.05 for both HHV-6A and -6B). In tissues infected with HHV-6A and -6B, CD4 expression in infected cells was (2.9 ± 0.1)-fold and (2.1 ± 0.2)-fold higher, respectively, than in uninfected cells. Two-parameter contour plot analysis (Fig. 4C) documented a discrete population of infected cells exhibiting a very low expression of CD4 (CD4low), which is consistent with the phenotype observed in CD8 T cells and other commonly CD4–cell types in which HHV-6 induced de novo expression of CD4 (18, 20). Altogether, these data demonstrate that HHV-6 has the potential to severely affect the physiology of T cells in secondary lymphoid tissues by altering the expression of critical functional surface molecules, such as CD3 and CD4.

HHV-6 infection upregulates the production of RANTES, but not of other cytokines or chemokines, in lymphoid tissue. Using the Luminex technology, we measured the secretion of 12 cytokines (IL-1α, IL-1β, IL-2, IL-6, IL-8, IL-16, MIP-1α, MIP-1β, RANTES, IP-10, TNF-α, and SDF-1α) in HHV-6-infected and control human lymphoid tissue. As previously documented (9), HHV-6A induced a marked upregulation of the CC chemokine RANTES. Similarly, we found that this chemokine is also upregulated by HHV-6B, although to a consistently lesser extent. As shown in Fig. 5, the secretion of RANTES in tissue infected with HHV-6A and -6B was approximately (6 ± 2)-fold (P < 0.01) and (2 ± 0.3)-fold (P = 0.03) higher, respectively, than in uninfected controls. Among the other CCR5-binding chemokines, both MIP-1α and MIP-1β were upregulated by HHV-6A [(4 ± 1.0)-fold and (1.9 ± 0.5)-fold, respectively], but the difference did not reach statistical significance due to the high experimental variability. By contrast, HHV-6B caused only modest enhancement (<1.5-fold). Similarly, no significant increase was observed in the secretion of SDF-1, the CXCR4 ligand, despite a moderate upregulation with HHV-6A [(2.2 ± 0.6)-fold]. The levels of three other chemotactic cytokines, IP-10, IL-8, and IL-16, were virtually unaltered in HHV-6-infected tissues, relative to uninfected controls (Fig. 5 and data not shown). Likewise, HHV-6 infection did not significantly modify the production of any of the other cytokines tested, namely, IL-1α, IL-1β, IL-2, IL-6, and TNF-α. The amount of secreted RANTES correlated with the levels of HHV-6 replication (r = 0.81 [P = 8.9 × 10−7] and r = 0.7 [P = 3.9 × 10−5] for HHV-6A and -6B, respectively) (Fig. 6) while no correlation was observed for any of the other chemokines tested (not shown). The correlation between HHV-6 expression and chemokine production was also investigated at the single cell level using intracellular immunostaining. HHV-6 infection increased the number of RANTES-producing cells relative to uninfected controls [(2.3 ± 0.3)-fold and (1.5 ± 0.2)-fold in HHV-6A and HHV-6B infected tissue, respectively; n = 6]; such increases were ob-

FIG. 5. Modulation of cytokine and chemokine secretion by HHV-6 in human lymphoid tissue ex vivo. Production of cytokines and chemokines accumulated in tissues infected with HHV-6 over 13 days of infection. The results represent the mean ± SEM (error bars) from 8 to 11 experiments with HHV-6A and 3 to 6 experiments with HHV-6B, each performed with 54 tissue blocks derived from an individual tonsil donor. The production of chemokines in infected tissues is expressed as percent of the production in matched uninfected tissues.

FIG. 6. Correlation between RANTES production and HHV-6 replication in ex vivo-infected tissues. Log regression analysis of data obtained from tissues of five donors infected ex vivo with HHV-6A (A) or HHV-6B (B) over a 12-day period is shown. The regression analysis was performed by plotting the concentration of RANTES and the number of HHV-6 genome equivalents per microliter of culture supernatant (log-transformed).
served in both CD4⁺ and CD8⁺ T cells. Interestingly, a significant fraction of RANTES-producing cells was HHV-6 pp41/38 antigen-negative (15% ± 4% and 40% ± 6%, in tissues infected with HHV-6A and -6B, respectively; n = 6), suggesting that HHV-6 infection induces RANTES secretion in both infected and bystander T cells.

**DISCUSSION**

In this study, we characterized a novel experimental model to study the biology and pathogenic effects of HHV-6, an emerging viral agent of increasing importance in human pathology (17). The cellular tropism and the pathogenic effects of HHV-6 were investigated using human lymphoid tissue ex vivo, a system that has previously been used to investigate the pathogenic mechanisms of HIV-1 infection (5, 6). We found that both major subgroups of HHV-6, A and B, efficiently replicate in lymphoid tissue, inducing significant alterations in cellular viability and immunological phenotype. At variance with previous studies using suspension mononuclear cell cultures (21), the present model presents a critical methodological novelty since the lymphoid tissue allows the growth of HHV-6 without the addition of exogenous stimuli, whereas mononuclear cell cultures require exogenous polyclonal activation as a necessary precondition for HHV-6 replication. Thus, the ex vivo lymphoid tissue system seems to mimic more closely the physiological conditions of natural infection in vivo.

Although in vitro HHV-6 shows a broad cellular tropism (17), as also suggested by the widespread cellular expression of its main receptor, CD46 (25), in most cell types the viral life cycle fails to progress to the lytic phase, as documented in mononuclear phagocytic cells (14) and epithelial cells (3). In fact, it has been reported that productive infection by both HHV-6 subgroups, both in vitro and in vivo, is essentially restricted to T lymphocytes, particularly those of the CD4⁺ subset (21, 28). Interestingly, this observation was confirmed in human lymphoid tissue, where T lymphocytes were found to be the predominant target cells for both HHV-6A and -6B replication, while other cell types were minimally, if at all, affected. However, no attempts were made in our system to document the presence of nonproductive or latent HHV-6 infection. Simultaneous staining with antibodies against HHV-6 late antigens and cellular antigens demonstrated productive infection in both CD4⁺ and CD8⁺ T cells, although HHV-6B was markedly less efficient than HHV-6A in targeting CD8⁺ T cells, as previously reported using suspension T-cell cultures (18, 20). In addition, we demonstrated that both HHV-6 subgroups preferentially infect nonnaive T cells and that such a preference does not simply result from the lower prevalence of naive T cells in ex vivo-cultured lymphoid tissue. Nevertheless, naive T cells can also be infected, thus broadening the spectrum of potential suppressive effects of HHV-6 on T cells.

Our study provides the first evidence in a physiologically relevant system that HHV-6 infection can cause dramatic alterations in the expression of surface antigens of physiological relevance to normal T-cell function. Thus, this virus has the potential capability of profoundly affecting both cellular and humoral immune responses. While some effects were directly linked to events occurring inside individual infected cells, others affected uninfected bystander cells as well, implying a mechanism that operates in trans. For example, downregulation of the CD46 receptor was documented in the vast majority of the cells present in infected cultures, irrespective of their infection status. One of the possibilities is that some of the cells that downmodulated CD46 might be nonproductively infected. However, the expression of CD46 is not modified in the course of nonproductive infection in vitro, as documented in latently infected HeLa cells (F. Santoro and P. Lusso, unpublished data) harboring multiple copies of the viral genome per cell (3). Thus, the downmodulation observed in HHV-6 antigen-negative cells is most likely the result of an authentic bystander effect. The most obvious explanation for this phenomenon is that, with the progression of the infection, CD46 molecules on both infected and uninfected cells become increasingly engaged by either cell-to-cell contact with infected cells, free virions released in the extracellular space, or soluble viral envelope components. However, a role of soluble trans-acting factors released by infected cells cannot be ruled out.

Downmodulation of CD46 may have important pathological consequences in lymphoid tissue as it can expose autologous cells to spontaneous complement activation and cytotoxicity (26). As a result of CD46 downmodulation in bystander cells, the effect of HHV-6 infection in a minority of tissue lymphocytes may be considerably amplified, rendering a wide range of neighboring cells potentially more sensitive to autologous complement attack. Moreover, this phenomenon may be particularly relevant for HHV-6 pathogenesis in light of the recent demonstration of the critical role of CD46 as a costimulatory molecule for T-cell-regulatory type 1 induction (12). Besides CD46, a second surface molecule that plays a critical role in T-cell physiology, CD3 (23), was dramatically downmodulated in HHV-6-infected tissue-residing T cells, as previously seen in T-cell suspension cultures (21). However, at variance with CD46, CD3 is downmodulated only in HHV-6-infected cells but it remains unaltered in bystander cells, suggesting a differential mechanism of viral modulation of cell surface markers. Future systematic analyses will reveal whether other important costimulatory and immunomodulatory antigens for T cells (e.g., CD40L, OX40, LFA-1, Fas ligand, CD25, CD28, and major histocompatibility complex molecules, etc.) are affected by HHV-6 infection in the context of lymphoid tissue.

We observed that HHV-6 strains of both subgroups induced cell depletion in lymphoid tissues. This was directly shown in our experiments by measuring the absolute cell loss in HHV-6 infected cultures, which could not be attributed to inhibition of cell proliferation by HHV-6 since replication of both CD4⁺ and CD8⁺ T cells in ex vivo lymphoid tissue was shown to be negligible (10). Thus, any putative antiproliferative effect of HHV-6 would not exert any influence on the cell counts recorded at the end of the experiment. Likewise, the enumeration of T lymphocytes in our experiments could not be confounded by modulation of cell surface markers by HHV-6, since we based our analysis on the expression of CD2, a marker not affected by HHV-6 infection, at least in vitro (20).

As previously documented using purely in vitro cell culture systems (18, 20), we found that the expression of the CD4 glycoprotein was upregulated in HHV-6-infected tissues. The mechanism underlying HHV-6-mediated CD4 upregulation seems to involve direct activation of the CD4 promoter in HHV-6-infected cells (4), as indirectly confirmed by the lack of
bystander effect observed in our system. The ability of HHV-6 to upregulate CD4 expression was fingered as one of the major mechanisms of positive interaction of HHV-6 with HIV, as CD4 is the primary HIV receptor molecule, corroborating the hypothesis of a putative cofactorial role of HHV-6 in AIDS (15, 19). Despite the evidence hitherto accumulated, however, definitive proof of the role played by HHV-6 in AIDS is still wanting. Previous data on the interaction between HHV-6 and HIV-1 in the heterochimeric SCID-hu thy/liv model have been inconclusive (7). The development of novel experimental systems, such as the ex vivo lymphoid tissue model described herein, provides a unique opportunity for investigating the pathological effects of HHV-6 and its interactions with HIV in a physiologically relevant context. In a previous report (9), we observed a dramatic suppression of CCR5-using strains, those commonly transmitted in vivo and predominating during the early phases of HIV infection, while CXCR4-using variants, those emerging only during the progression toward full-blown AIDS, were not affected or even enhanced in tissue from some donors. This phenomenon was associated with an augmented production of the CC chemokine RANTES (9), a potent natural inhibitor of HIV-1 strains that use CCR5 (1). Here, we confirmed the upregulation of RANTES upon infection with subgroup A of HHV-6 and demonstrated that this phenomenon also occurred with a viral strain belonging to subgroup B. Moreover, in this report we systematically studied a set of 13 cytokines, showing that most of them were only marginally affected by HHV-6 infection.

It is still conceivable that some of these or other cytokines may be upregulated in HHV-6-infected tissues, although only in small subset of cells without significantly affecting cytokine concentrations in the medium. A more thorough analysis at the single-cell level will be necessary to detect potential subtle chemokine modulation in infected tissues. Nevertheless, the dramatic increase in RANTES production described here appears to be a highly specific phenomenon that cannot be simply explained as a consequence of generalized cell activation. In addition, analysis of RANTES expression at the single cell level showed that uninfected cells also upregulate their RAN-

tes production in the context of HHV-6 infected lymphoid tissue. This increased secretion of RANTES in HHV-6-infected tissues may profoundly influence the physiology of the immune responses, as well as, more specifically, the replication of different HIV-1 biological variants.

In conclusion, the dramatic effects that we documented in the lymphoid tissue model infected with HHV-6 of both A and B subgroups provide compelling evidence, in a physiologically relevant system, that this agent can efficiently infect both memory and naive T cells, causing downmodulation of physiologically relevant cell surface receptors in both infected and bystander cells, and thereby, potentially, immune dysregulation and immunosuppression. The availability of this new model system for the study of HIV-6 infection may permit a deeper understanding of the complex relation between this viral agent and the immune system. Moreover, it provides a unique experimental model to investigate the interactions between this agent and other human pathogens, including HIV, under controlled experimental conditions.

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