CXC-Chemokine Receptor 4 Is Not a Coreceptor for Human Herpesvirus 7 Entry into CD4+ T Cells

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Human herpesvirus 7 (HHV-7) is a T-lymphotropic virus which utilizes the CD4 receptor as its main receptor to enter the target cells. Hence, HHV-7 can interfere with human immunodeficiency virus type 1 (HIV-1) infection in CD4+ T cells. It was recently suggested that the CXC chemokine receptor 4 (CXCR4), which was found to be a crucial coreceptor for T-tropic HIV-1 strains, may also play a role in the HHV-7 infection process. However, the results presented here demonstrate that CXCR4 is not involved in HHV-7 infection. The natural ligand of CXCR4, SDF-1α, was not able to inhibit HHV-7 infection in SupT1 cells or in CD8− T-cell-depleted peripheral blood mononuclear cells. Also, AMD3100, a specific CXCR4 antagonist with potent antiviral activity against T-tropic HIV strains (50% inhibitory concentration [IC50], 1 to 10 ng/ml), completely failed to inhibit HHV-7 infection (IC50 >250 μg/ml). Thus, two different agents known to specifically interact with CXCR4 were not able to inhibit HHV-7 infection. Other T-lymphoid cell lines, expressing both CD4 and CXCR4 (e.g., HUT-78 and MT-4) could not be infected by HHV-7. In addition, the CD4-transfected cell lines HOS.CD4 and U87.CD4 and the CD4/CXCR4 double-transfected cell lines HOS.CD4.CXCR4 and U87.CD4.CXCR4 were not infectable with HHV-7. Also, we found no down-regulation of surface-bound or intracellular CXCR4 in HHV-7-infected CD4+ T cells. As compared to uninfected SupT1 cells, stromal cell-derived factor 1α (SDF-1α)/CXCR4-mediated intracellular calcium flux was unchanged in SupT1 cells that were acutely or persistently infected with HHV-7. All these data argue against CXCR4 as a receptor involved in the HHV-7 infection process.
FITC) (Caltag Labs, San Francisco, Calif.). To determine CD4 expression, the cells were incubated at 4°C with phycoerythrin (PE)-conjugated Leu3a monoclonal Ab (MAb; Becton Dickinson). After washing, the cells were fixed in 1% formaldehyde solution in PBS and analyzed by flow cytometry as described before (18). In Fig. 1, the surface CXCR4 expression and the surface CD4 expression of SupT1 cells incubated with PBS, with the CXCR4 antagonist AMD3100 (4 μg/ml) or with the chemokine SDF-1α (1 μg/ml), are shown. The percentages of cells positive for CXCR4 were 95.7% (PBS), 11.2% (AMD3100), and 2.1% (SDF-1α), respectively (Fig. 1A). Thus, the binding at the cell surface of anti-CXCR4 Abs clearly decreased in the presence of SDF-1α or AMD3100. As expected, no effect on CD4 expression was observed with AMD3100 or SDF-1α (Fig. 1B).

To further study the role of CXCR4 in HHV-7 infection, the SupT1 cells were first treated with AMD3100 (4 μg/ml) and SDF-1α (1 μg/ml) at 37°C for 4 h. Then the SupT1 cells were infected with HHV-7 in the absence or presence of the same concentration of AMD3100 or SDF-1α. At day 4, half of the medium and cells were removed and fresh medium without new AMD3100 or SDF-1α was added. This procedure was repeated every 3 or 4 days. The HHV-7 infection was assayed daily by microscopy, and HHV-7 antigen expression was analyzed regularly with anti-HHV-7 MAb staining and flow cytometry. All the SupT1 cells which were inoculated with HHV-7 showed a similar cytopathic effect during the HHV-7 infection. To analyze the HHV-7 antigen expression, the cells were fixed in 1% formaldehyde solution in PBS, treated with 0.2% Tween 20 in PBS, and then incubated for 30 min with

FIG. 1. Effect of AMD3100 and SDF-1α on CD4 and CXCR4 expression in SupT1 cells. After 4 h of incubation at 37°C, the CXCR4 expression of control SupT1 cells and SupT1 cells treated with AMD3100 (4 μg/ml) and SDF-1α (1 μg/ml) was analyzed by staining with anti-CXCR4 MAb (12G5) followed by GaM-FITC (A). The CD4 expression was analyzed by staining with PE-conjugated Leu3a MAb (B). The percentages of CXCR4- and CD4-positive cells are indicated on the histograms.

FIG. 2. Effect of AMD3100 and SDF-1α on HHV-7 infection in SupT1 cells. Cells were pretreated with AMD3100 (4 μg/ml) and SDF-1α (1 μg/ml) for 4 h at 37°C. Then the cells were inoculated with HHV-7 in the absence or presence of the same concentration of AMD3100 or SDF-1α. At day 4, half of the medium and cells were replaced with fresh medium without AMD3100 or SDF-1α. This procedure was repeated every 3 or 4 days. The HHV-7 Ag expression was analyzed by RK-4 MAb as described in the text. The percentage of HHV-7 Ag-positive cells is indicated in each histogram. (A) Uninfected SupT1 cells; (B) HHV-7-infected SupT1 cells; (C) SupT1 cells infected with HHV-7 in the presence of AMD3100 (4 μg/ml); (D) SupT1 cells infected with HHV-7 in the presence of SDF-1α (1 μg/ml).
anti-HHV-7 MAb (RK-4) (Advanced Biotechnologies, Columbia, Md.) in PBS. The cells were incubated with GaM-FITC for 30 min, washed again, and analyzed by flow cytometry. The HHV-7 antigen expression at day 13 after infection is shown in Fig. 2. As indicated in Fig. 2, 1.1% of the uninfected SupT1 cells were positive for HHV-7 Ag (Fig. 2A), whereas the percentage of HHV-7 Ag-positive cells increased to 32.6% in the HHV-7-infected cell cultures (Fig. 2B). The percentages of HHV-7 Ag-positive cells in AMD3100- and SDF-1α-treated SupT1 cell cultures were 39.0 and 48.8%, respectively (Fig. 2C and D). Apparently, AMD3100 and SDF-1α were unable to inhibit HHV-7 infection.

We also studied the effect of SDF-1α and AMD3100 on CXCR4 expression during HHV-7 infection in CD8+ T-cell-depleted PBMC. PBMC were isolated by density gradient centrifugation over Lymphoprep (d = 1.077 g/ml) (Nycomed, Oslo, Norway). CD8 magnetic beads (Dynal AS, Oslo, Norway) were added to remove the CD8+ T lymphocytes. The CD8+ T-cell-depleted cells were stimulated with 10 μg of phytohemagglutinin (Sigma Chemical Co., Bornem, Belgium) per ml and 25 U of interleukin 2 (IL-2) for 3 days at 37°C. The cells were then seeded into 24-well flat-bottom tissue culture plates (IWAKI, Chiba, Japan) in the presence or absence of AMD3100 (final concentration, 250 μg/ml) or SDF-1α (final concentration, 1 μg/ml) in culture medium containing 25 U of IL-2 per ml, and HHV-7 was inoculated into each well. The HHV-7 antigen expression at day 7 is shown. (A) Uninfected primary CD8+ T-cell-depleted PBMC; (B) HHV-7-infected CD8+ T-cell-depleted PBMC; (C) CD8+ T-cell-depleted PBMC infected with HHV-7 in the presence of AMD3100 (250 μg/ml); (D) CD8+ T-cell-depleted PBMC infected with HHV-7 in the presence of SDF-1α (1 μg/ml). The percentage of HHV-7 Ag-positive cells is indicated on each histogram.
infection was analyzed regularly by microscopy and flow cytometry as described above. The HHV-7 Ag expression at day 7 is shown in Fig. 3. The percentages of HHV-7 Ag-positive cells in the uninfected and infected CD8+ T-cell-depleted PBMC cultures were 0.5 and 52.0%, respectively (Fig. 3A and B). Even at a concentration of 250 μg/ml, AMD3100 did not inhibit HHV-7 infection; 50.9% of the cells stained positive for HHV-7 Ag. Likewise, SDF-1α at a concentration of 1 μg/ml did not show any activity against HHV-7 infection: the percentage of HHV-7 Ag-positive cells remained at the high level of 56.0% (Fig. 3D).

We also monitored CXCR4 versus CD4 expression in SupT1 cells during the course of HHV-7 infection. In keeping with the findings of Secchiero et al. (15), we found that CD4 was strongly and progressively down-regulated in HHV-7-infected cell cultures. As shown in Fig. 4A, 98.7% of the uninfected SupT1 cells were CD4+/CXCR4+. In HHV-7-infected SupT1 cells, the percentage of CD4+ cells had declined to 34.7% at day 8 (Fig. 4B) and further to 4.7% at day 78 (Fig. 4C). However, unlike Secchiero et al. (16), we did not observe any effect of HHV-7 infection on CXCR4 expression (Fig. 4B and C versus Fig. 4A). We have also demonstrated that HHV-7 infection does not affect the intracellular CXCR4 expression in SupT1 cells (data not shown). Additionally, we have also seen that HHV-7-infected SupT1 cells, which have weak CD4 expression, could still be infected by HIV-1 and HIV-2 strains (HIV-1 RF, HIV-1 IIIB, HIV-1 NL4.3, and HIV-2 ROD) which use CXCR4 as coreceptor (data not shown).

Similar results were obtained with CD8+ T-cell-depleted PBMC. The staining procedures for HHV-7 Ag and surface CD4 and CXCR4 were described above. To analyze the intracellular CXCR4 expression, the mock-infected and HHV-7-
infected CD8+ T-cell-depleted PBMC were first incubated with anti-CXCR4 MAb 173 followed by fixation in 1% formaldehyde solution, washing with 0.2% Tween 20 in PBS, and incubation with anti-CXCR4 MAb followed by an FITC-conjugated secondary Ab (GaM-FITC). Figure 5 shows the expression of HHV-7 Ag, surface CD4, surface CXCR4, and intracellular CXCR4 in CD8+ T-cell-depleted PBMC at day 7 after HHV-7 infection. In uninfected cell cultures, the percentages of positive cells were 0.3, 95.0, 46.5, and 98.2% for viral Ag, surface CD4, surface CXCR4, and intracellular CXCR4, respectively (Fig. 5A).

In HHV-7-infected cell cultures, the corresponding values were 64.5, 24.6, 50.1, and 95.7% (Fig. 5B). Thus, like in SupT1 cells, the marked increase of HHV-7 antigen expression and the pronounced decrease of CD4 expression were not associated with any down-regulation of surface-bound or intracellular CXCR4 in CD8+ T-cell-depleted PBMC infected with HHV-7.

Upon binding of a chemokine to its receptor, a transient dose-dependent increase in intracellular calcium concentration occurs as an essential component of the signal transduction cascade. The intracellular calcium flux triggered in SupT1 cells by the CXCR4-specific chemokine SDF-1α is blocked by the CXCR4 antagonist AMD3100 in a concentration-dependent manner (reference 14 and data not shown). This indicates that HHV-7, in addition to CD4, requires a coreceptor other than CXCR4 to enter the target cells.

HHV-7 shows tropism toward primary human T lymphocytes and the SupT1 T-lymphoid tumor cell line (2, 12). The virus is generally known to utilize CD4 as its main receptor to enter the target cells. It has been shown that during infection, HHV-7 can inhibit CD4 gene transcription (15) and block the intracellular transport of CD4 molecules (10). A progressive and persistent down-regulation of surface CD4 expression was found in CD4+ T cells during HHV-7 infection (10, 12). Accordingly, we show here that even after several months of HHV-7 infection, CD4 expression is still markedly decreased in SupT1 cells compared to the uninfected control, although

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**TABLE 1. CD4 and CXCR4 expression in cell lines and their sensitivity to HHV-7 infection**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>% CD4+ cellsa</th>
<th>% CXCR4+ cellsa</th>
<th>Susceptibility to HHV-7 infectionb</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT-2</td>
<td>&gt;95</td>
<td>&gt;95</td>
<td>–</td>
</tr>
<tr>
<td>MT-4</td>
<td>&gt;95</td>
<td>&gt;95</td>
<td>–</td>
</tr>
<tr>
<td>SupT1</td>
<td>&gt;95</td>
<td>&gt;95</td>
<td>+</td>
</tr>
<tr>
<td>HUT-78</td>
<td>90</td>
<td>42</td>
<td>–</td>
</tr>
<tr>
<td>CEM/174</td>
<td>&gt;95</td>
<td>60</td>
<td>–</td>
</tr>
<tr>
<td>HOS.CD4</td>
<td>&gt;90</td>
<td>29</td>
<td>–</td>
</tr>
<tr>
<td>HOS.CD4.CXCR4</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td>–</td>
</tr>
<tr>
<td>U87.CD4</td>
<td>&gt;95</td>
<td>Negative</td>
<td>–</td>
</tr>
<tr>
<td>U87.CD4.CXCR4</td>
<td>&gt;95</td>
<td>88</td>
<td>–</td>
</tr>
</tbody>
</table>

a The percentages of positive cells were obtained on the basis of the cells stained with control isotype-matched MAb. CD4+ cells were stained with PE-conjugated Leu3a. CXCR4+ cells were stained with MAb 12G5 and GaM-FITC.

b HHV-7 infection was evaluated by anti-HHV-7 MAb staining at days 7, 10, and 14 after infection and microscopic evaluation of the cell cultures. –, not infectable; +, HHV-7 infectable.
very few multinucleated giant cells were present in these persistently infected cell cultures (data not shown).

In contradiction with two other reports (16, 17), we found no evidence of surface or intracellular CXCR4 down-regulation during HHV-7 infection. Furthermore, our study indicated that CXCR4 does not act as a functional coreceptor for HHV-7 entry into the host cells. First, no down-regulation of surface-bound or intracellular CXCR4 could be detected following HHV-7 infection of CD4+ T cells, not even after extended periods of time (2 to 3 months). In addition, HHV-7 infection, either short term or long term, did not influence the functional response of SupT1 cells to SDF-1α, the natural CXCR4 ligand, as ascertained by an intracellular calcium flux assay. Second, SDF-1α, which is able to block CXCR4-mediated HIV-1 infection (3), did not inhibit HHV-7 infection. Also, AMD3100, a small-molecular-weight CXCR4 antagonist that exhibits strong activity against T-tropic CXCR4-utilizing HIV strains (7, 13, 14), failed to show any inhibitory activity against HHV-7. Indeed, when preincubated with AMD3100 or SDF-1α, SupT1 cells as well as CD8+ T-cell-depleted PBMC remained equally susceptible to HHV-7 infection. Furthermore, the anti-CXCR4 MAb 12G5, which inhibits HIV infection via binding with the second extracellular domain of human CXCR4 (4), also proved inactive against HHV-7 (16, 17; our unpublished data), which further ascertains that blocking the CXCR4 receptor does not suffice to inhibit HHV-7 infection. Finally, no detectable HHV-7 Ag expression could be demonstrated in a variety of CD4+/CXCR4+ T-cell lines (e.g., MT-2, MT-4, HUT-78, CEM/X174, and CXCR4-transfected HOS, CD4 and U87, CD4 cells) following HHV-7 inoculation of the cell cultures.

Additionally, we have seen down-regulation of CXCR4 by HHV-6 strains A and B in the SupT1 cell line and in CD8+ T-cell-depleted PBMC (data not shown) in accordance with Yasukawa et al. (17). In conclusion, all the data that we obtained argue against an essential role of CXCR4 in the HHV-7 infection process. Hence, the presumed coreceptor for HHV-7 still remains to be identified.

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