Critical involvement of human T cell leukaemia virus type I virions in mediating the viral mitogenic effect

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Human T cell leukaemia virus type I (HTLV-I) is a direct activator of human resting T lymphocytes. The present study was undertaken to delineate further the role of viral particles and to define the involvement of envelope glycoproteins in the induction of T cell mitogenic stimulation. Virus-producing cells treated with paraformaldehyde (PFA) were found to be unable to induce the formation of syncytia, but still able to trigger the proliferation of resting T cells. Likewise, PFA-treated virus particles were still mitogenic. These results suggest that the mitogenic event is triggered before the fusion of the envelope with the cell membrane. Furthermore, HTLV-I envelope-expressing cells obtained after infection of C8166/45 cells (HTLV-I-transformed, but defective in virion production) with an HTLV-I envelope recombinant vaccinia virus were unable to activate normal T cells. Human immunodeficiency virus type 1 particles produced by C8166/45 cells were also devoid of mitogenic ability. However, when HTLV-I viral preparations were purified by chromatography, only the virion-containing fractions were found to be mitogenic for human resting T lymphocytes. This mitogenic activity was partially abolished by preincubating the purified virus with a monoclonal antibody directed to the surface envelope glycoprotein. Finally, treatment of HTLV-I-transformed cells by tunicamycin, an inhibitor of N-linked glycosylation, led to the production of virus particles with a decreased mitogenic activity. Collectively, these observations suggest that the HTLV-I mitogenic activity is triggered by the contact of HTLV-I virions with T cells.

Introduction

The interaction of viruses with their target cells is critical with regard to the initiation of the infectious process. Binding of virus particles to cell surface receptors sets the stage for entry into the cell, either by direct fusion with the plasma membrane or by receptor-mediated endocytosis. Therefore, recognition of receptors by virus particles is a key event for viral tropism in the pathogenesis of infection (Lentz, 1990; Paulson, 1985). As virus receptors are cell surface molecules that are of importance in the physiology of a normal cell, the binding step may represent an early stage interfering with the life of the target cell. A well studied example is the reovirus type 3 interaction with receptors that bear structural and antigenic similarity to mammalian β-adrenergic receptors. Binding of virus particles and subsequent aggregation of reovirus receptor molecules was shown to be necessary and sufficient to provide the signal for the inhibition of host cell DNA synthesis, within 8 to 10 h after infection (Gaulton & Greene, 1989).

We have previously shown that virus particles prepared by high-speed centrifugation of human T cell leukaemia virus type I (HTLV-I)-containing supernatants induce the expression of the α chain of the interleukin-2 (IL-2) receptor and the synthesis of IL-2 by human peripheral T lymphocytes, thus triggering their proliferation (Duc Dodon & Gazzolo, 1987; Gazzolo & Duc Dodon, 1987). Likewise, human peripheral blood mononuclear cells infected in vitro with human immunodeficiency virus type 1 (HIV-1) can be induced to produce large quantities of HIV-1 after mitogenic stimulation with gradient-purified virions (Zack et al., 1988). The observation that even HTLV-I particles rendered infectious after u.v. irradiation are still mitogenic indicates that the mitogenic stimulation may be restricted to the attachment and/or fusion steps, as both steps are mediated by the virus envelope glycoproteins (Gazzolo & Duc Dodon, 1987).

As with other retroviruses, the envelope glycoprotein complex of HTLV-I is translated from a singly spliced env gene mRNA into a precursor that is cleaved after glycosylation into two polypeptides, an external hydrophobic polypeptide, gp46 (SUgp) and a transmembrane protein, gp21 (TMgp). The SUgp is thought to express
the binding domain to the receptor on the membrane of the host cell and the TMgp is thought to mediate the fusion of the virus envelope with the cellular membrane, thus allowing the delivery of the virus capsid into the cytoplasm (Cann & Chen, 1990; Pique et al., 1992).

The aims of this study were to delineate further the role of virus particles and to define the involvement of envelope glycoproteins in the induction of T cell mitogenic stimulation. We first showed that HTLV-I mitogenic stimulation is independent of the fusion process. We then performed a functional evaluation of these glycoproteins, either associated with virus particles or obtained from recombinant expression vectors. The results indicate that the contact of HTLV-I virions with T cells appears to be critical in triggering the mitogenic event.

Methods

**Cell culture.** C91/PL (Popovic et al., 1983) and C8166/45 (Salahuddin et al., 1983) cells were cultivated in RPMI 1640 medium supplemented with 10% fetal calf serum. T lymphocytes were obtained after Ficoll-Hypaque centrifugation of peripheral blood from normal healthy donors (at the Centre de Transfusion Sanguine, Lyon, France) and prepared as described previously (Duc Dodon et al., 1989: Duc Dodon & Gazzolo, 1987).

**Virus preparation and neutralization.** The culture medium of HTLV-I-producing cells (C91/PL) was harvested 48 h after cell seeding and centrifuged at low speed to remove cells. The virus-containing supernatant was concentrated 20-fold by centrifugation (3500 r.p.m. for 3 h at 32000 g) in a type 35 rotor (Beckman) and by resuspending the viral pellet in an appropriate volume of RPMI 1640 medium; 50 µl samples of these virus preparations were added to each well. In neutralization experiments, the virus preparation was incubated with the anti-gp46 human 0.5e monoclonal antibody (Matsushita et al., 1986) which was kindly provided by Dr S. Broder.

**IV-I (LAI strain) was propagated in CEM cells. Supernatant of the culture (incubated for 24 h) was clarified by centrifugation (3500 r.p.m. for 20 min) and 1 ml reverse transcriptase level of 8 x 10<sup>6</sup> c.p.m./ml; Rey et al., 1984) was used to infect 2 x 10<sup>6</sup> C8166/45 cells. When cells were chronically infected, culture media (reverse transcriptase level of 8 x 10<sup>6</sup> c.p.m./ml) were collected and virus was prepared as described above.**

**HTLV-I purification.** HTLV-I was prepared from cell-free supernatant media of C91/PL cells seeded at a concentration of 1.5 x 10<sup>6</sup> cells/ml and incubated for 15 h. Media were first centrifuged at low speed and then at 5000 r.p.m. for 10 min. These cell-free supernatants were then concentrated 100-fold on an Amicon ultrafiltration membrane. The concentrate was purified on a Sepharose CL-4B (Pharmacia) column in 10 mM-phosphate buffer pH 7.2 (McGrath et al., 1978) at a flow rate of 0.5 ml/min. The absorbance of each fraction was measured at 260 and 280 nm. Fractions with an A<sub>260</sub>/A<sub>280</sub> ratio of equal or greater than 1.2 were pooled and diluted in RPMI 1640 medium.

**Tunicamycin treatment.** C91/PL cells were incubated in complete medium at 37 °C in the presence of tunicamycin (Sigma) at different concentrations. Cells were then washed and fixed with 0.2% paraformaldehyde (PFA) for 20 min, washed once in PBS containing 0.2% glycol-glycine and four times in RPMI 1640, resuspended in complete medium and incubated overnight at 37 °C in a 5% CO₂ atmosphere. Before co-culture, these PFA-fixed cells were washed and resuspended in complete medium.

**Mitogenic assays.** T lymphocytes were seeded in triplicate in 96-well flat-bottomed microdilution plates at a ratio of 1 x 10<sup>5</sup> cells per well in 100 µl of medium in the absence or presence of HTLV-I virions. Five days later, [³H]thymidine (1 µCi per well with a sp. act. of 6.7 Ci/mm; NEN) was added. Cells were collected 6 h later with a Skatron harvester. Thymidine incorporation was measured by standard liquid scintillation counting techniques.

**Syncytium formation assays.** The ability of the HTLV-I expressing cells to mediate the formation of syncytia was assessed in co-cultures of C91/PL cells expressing the envelope glycoproteins with C8166/45 indicator cells at a ratio of 1:1. Cells were then incubated in a 5% CO₂ incubator at 37°C for 12 h. They were examined at magnification of 200 under an inverted microscope for the emergence of envelope-induced syncytia. The same observations were performed with C8166/45 cells infected 36 h earlier with a recombinant vaccinia virus, harbouring the envelope gene of HTLV-I. This recombinant virus, referred to as WRproenv1 (Shida et al., 1987) and kindly provided by H. Shida (Kyoto University, Japan) was used at a m.o.i. of 2 p.f.u./cell. Control cultures were infected at the same m.o.i. with the corresponding parental strain of vaccinia virus (WT). After infection, C8166/45 cells were extensively washed in RPMI 1640 medium, fixed in 4% PFA for 20 min, washed four times in PBS containing 0.2% glycol-glycine and once again in RPMI 1640 medium, then resuspended in complete medium and incubated overnight at 37°C. These PFA-fixed cells were then X-ray-irradiated (6500 rads) to inactivate the vaccinia virus.

**Monoclonal antibodies (MAbs) and immunofluorescence analysis.** The following unconjugated and conjugated MAbs were used in this study: MAb 104, specific for the B7/BB1 antigen (Valle et al., 1990), kindly provided by J. Blanchereau (Scherin Corporation Laboratories, Dardilly, France); MAbs IOL58 (anti-CD58), specific for the LFA-3 antigen, and IOT14 (anti-CD25), specific for the α chain of the IL-2 receptor (purchased from Immunotech, Marseille, France); MAb IC11, raised to affinity-purified gp46 and the polyepolonal anti-SP-11 antiserum raised to gp21 (Cann & Chen, 1990; Palker et al., 1992), both a kind gift of T. Palker (Duke University Medical Center, Durham, N.C., U.S.A.). Cells were incubated with saturating amounts of MAbs in saline buffer for 1 h at 4°C. Binding was thereafter detected by addition of a fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG. Cells were then analysed on a FACScan (Becton-Dickinson).

**Immunoprecipitation of cell-associated viral glycoproteins.** C91/PL cells (10<sup>6</sup>/ml) were treated with 2 µg/ml tunicamycin or left untreated, and incubated in complete RPMI 1640 medium at 37 °C for 90 min. After 1 h of starvation in cysteine-free culture medium, cells were metabolically labelled using 1 ml of cysteine-free RPMI 1640 containing 125 µCi/ml of [³5S]cysteine (Amersham) and 10% dialysed fetal calf serum, for 1 h followed by a chase with an excess of unlabelled cysteine for 4 h. Tunicamycin was present throughout the labelling period. After washing, cells were lysed in RIPA buffer (10 mM-Tris-HCl pH 7.4, 150 mM-NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM-EDTA and 0.25 mM-PMSE). The lysates were cleared for 1 h at 80000 g. Cleared lysates were incubated with the polyclonal rabbit anti-SP-11 antiserum recognizing the HTLV-I envelope precursor gp21. Immune complexes were precipitated using Protein A-Sepharose (Calbiochem). After being washed in RIPA buffer, immunoprecipitates were subjected to electrophoresis on a 12% SDS-polyacrylamide gel followed by autoradiography.
Results

The HTLV-I-induced mitogenic event occurs before the fusion process

Binding of retroviral particles to receptor-expressing cells is controlled by the surface envelope glycoproteins, whereas the transmembrane glycoproteins are responsible for the fusion between the viral envelope and the host cell membrane. Thus, when virus-producing C91/PL cells either exposed to u.v. irradiation (which inhibits virus infectivity) or treated with mitomycin C (which preserves it, as shown by co-cultivation of treated cells with activated T lymphocytes) were added to C8166/45 cells (HTLV-I-transformed but not virus-producing T cells), large multinucleated cells could be observed as early as 3 to 4 h after the onset of the co-culture. In contrast, C91/PL cells treated with PFA were found to lose the ability to form syncytia. Furthermore, these PFA-treated cells induced the proliferation of human resting T lymphocytes at the same extent as u.v.-exposed or mitomycin C-treated cells (Table 1). In control experiments, PFA-treated C8166/45 cells were unable to increase thymidine incorporation by T lymphocytes. Virus particles obtained by ultracentrifugation of cell-free culture medium and fixed with PFA were still able to induce the proliferation of normal T lymphocytes (Table 2). Taken together, these results indicate that inhibiting the fusogenic ability does not interfere with the mitogenic ability of virus particles, and that the mitogenic effect is solely due to the contact of virions with T cells.

Recombinant envelope glycoproteins are not mitogenic

To determine whether the HTLV-I envelope glycoproteins that mediate binding and penetration are involved in the mitogenic activity, C8166/45 cells were infected with a recombinant vaccinia virus (WRproenv1) encoding the envelope glycoproteins of HTLV-I. About 50% of C8166/45 cells infected with this recombinant vaccinia virus (at an m.o.i. of 2 p.f.u./cell) expressed envelope glycoproteins, 24 h after infection (Fig. 1c). As expected, in cultures infected with the recombinant virus, numerous syncytia were observed as early as 36 h after infection (data not shown). Neither the expression of the envelope glycoproteins nor the infection with the vaccinia virus modified the expression of CD25 (the z chain of the IL-2 receptor), B7 (a B cell activation antigen, which is expressed on the membrane of HTLV-I-transformed T cells; Vallé et al., 1990) and LFA-3 (lymphocyte function-associated antigen type 3) antigens (Fig. 1b and c). These antigens were also expressed on C91/PL cells (Fig. 1a). To explore the mitogenic ability of C8166/45 cells infected with the recombinant vaccinia virus, they were treated with PFA and then exposed to X-rays in order to destroy the cytolitic activity of the vaccinia virus, prior to their co-culture with T lymphocytes. The same treatment when applied to C91/PL cells did not inhibit their mitogenic ability (Fig. 2). In contrast, C8166/45 cells, either uninfected or infected with the wt vaccinia virus or the recombinant vaccinia virus and expressing the envelope glycoproteins of HTLV-I were

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Table 1. Effect of different treatments of HTLV-I-transformed cells on T cell proliferation

<table>
<thead>
<tr>
<th>Cells</th>
<th>Treatment</th>
<th>Addition of T lymphocytes*</th>
<th>Thymidine incorporation (c.p.m. × 10^{-3}) ± s.d.†</th>
</tr>
</thead>
<tbody>
<tr>
<td>C91/PL</td>
<td>PFA‡</td>
<td>−</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>PFA§</td>
<td>+</td>
<td>613 ± 82</td>
</tr>
<tr>
<td></td>
<td>u.v.§</td>
<td>−</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>u.v.</td>
<td>+</td>
<td>564 ± 90</td>
</tr>
<tr>
<td></td>
<td>Mitomycin‖</td>
<td>−</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Mitomycin</td>
<td>+</td>
<td>233 ± 3.2</td>
</tr>
<tr>
<td>C8166/45</td>
<td>PFA</td>
<td>−</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>PFA</td>
<td>+</td>
<td>3.7 ± 0.8</td>
</tr>
</tbody>
</table>

* T lymphocytes (1.5 × 10^5 cells) were cultured with treated cells (2 × 10^5 cells) in a 96-well flat-bottomed microdilution plate, in a final volume of 100 µl of complete medium.
† Thymidine incorporation was assessed after 5 days of co-culture, during the last 6 h of incubation. Incorporation by triplicate cultures is reported as the arithmetic mean ± S.D.
‡ Cells were treated with 0.2% PFA for 20 min, washed and resuspended in complete medium (see Methods).
§ Cells were irradiated for 10 min with u.v. light (4 J/m².s) in complete medium and then used for co-culture.
‖ Cells were treated with mitomycin C at a final concentration of 25 µg per 5 × 10^5 cells for 20 min, then washed three times in complete medium and used for co-culture.

Table 2. Effect of HTLV-I and HIV-I viral particles on DNA synthesis by T lymphocytes*

<table>
<thead>
<tr>
<th>Virus</th>
<th>Treatment</th>
<th>Thymidine incorporation (c.p.m. × 10^{-3}) ± S.D.†</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTLV-I‡</td>
<td>None</td>
<td>15.0 ± 1.1</td>
</tr>
<tr>
<td>HIV-1‡</td>
<td>None</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>HIV-1‡</td>
<td>PFA</td>
<td>10.5 ± 28</td>
</tr>
<tr>
<td>HIV-1‡</td>
<td>u.v.</td>
<td>16.3 ± 4.3</td>
</tr>
</tbody>
</table>

* T lymphocytes (1.5 × 10^5 cells) were cultured with appropriate virus in a 96-well flat-bottomed microdilution plate, in a final volume of 100 µl of complete medium.
† Thymidine incorporation was assessed after 5 days of culture, during the last 6 h of incubation. Incorporation by triplicate cultures is reported as the arithmetic mean ± S.D.
‡ HTLV-I particles were prepared from culture media of C91/PL cells and obtained as described in Methods.
§ Virus was treated with 1% PFA for 20 min, washed and resuspended in complete medium.
‖ Virus was irradiated for 10 min with u.v. light (4 J/m².s) in complete medium.
¶ HIV-1 particles were prepared from culture media of C8166/45 chronically infected by HIV-1 and obtained as described in Methods.
unable to induce the proliferation of T lymphocytes. The latter were still able to proliferate upon addition of phytohaemagglutinin (PHA) to the co-cultures (Fig. 2). These results showed that recombinant envelope glycoproteins expressed on the membrane of HTLV-I-transformed T cells are not mitogenic.

**Purified HTLV-I particles are mitogenic**

Previous experiments have demonstrated the mitogenic ability of HTLV-I particles concentrated by the high-speed centrifugation of cell-free growth medium from HTLV-I-transformed C91/PL cells (Gazzolo & Duc Dodon, 1987; Zack et al., 1988). To confirm this mitogenic ability, virus particles, first incubated with different concentrations of the human 0.5α MAb recognizing an epitope of gp46 were added to resting T lymphocytes prepared from the peripheral blood of normal healthy individuals. A decrease in T cell proliferation was observed in a concentration-dependent manner (Fig. 3). Similar results were obtained with virus preparations incubated with sera from HTLV-I-infected individuals (data not shown).

C91/PL cells were cultivated in the absence or presence of tunicamycin, an inhibitor of N-linked glycosylation. To ascertain the effect of tunicamycin, extracts of cells labelled with [35S]cysteine were immunoprecipitated with the anti-envelope (anti-SP-11) antiserum (Palker et al., 1992). The glycosylated envelope precursor (gp63) was detected in untreated C91/PL cells, but only the
HTLV-I mitogenic ability

Fig. 2. Mitogenic activity of the HTLV-I envelope glycoproteins expressed by C8166/45 cells infected with recombinant vaccinia virus. Cells infected with vaccinia virus (2 x 10⁴; see Methods) were incubated with T lymphocytes (1.5 x 10⁵) in a 96-well flat-bottomed microdilution plate in a final volume of 100 µl of complete medium, in the presence (Ξ) or absence (Ξ) of 1 µg/ml PHA. C91/PL cells (■) having undergone the same treatment as C8166/45 cells were also co-cultivated with T lymphocytes. Thymidine incorporation was assessed after 5 days of co-culture, during the last 6 h of incubation. Results are expressed as the mean of triplicate experiments.

unglycosylated precursor (p46) was observed in tunicamycin-treated cells (Fig. 4). The p19 content of viral pellets obtained from the cell-free medium of C91/PL cells treated with tunicamycin was about half as much as that of viral pellets obtained from untreated cells. As shown in Fig. 5(a), only a 38% inhibition in cell-mediated stimulation was observed. This partial inhibition may correlate with the amount of glycoproteins present at the onset of the tunicamycin treatment. Likewise, virion-mediated stimulation was inhibited by 63% (Fig. 5b).

To evaluate further the involvement of HTLV-I virions in mediating the mitogenic stimulus, HTLV-I virions were purified by a chromatography method that conserves envelope glycoprotein integrity (McGrath et al., 1978). Fractions 3 to 9 (with a $A_{260}/A_{280}$ ratio between 1.2 and 1.3) corresponding to the virus-containing fractions, as assessed by Western blot analysis (data not shown) were consistently found to trigger the proliferation of resting T lymphocytes (Fig. 6a). This
proliferation was significantly inhibited by preincubating them with the 0.5x MAb (Fig. 6b). In contrast, fractions 16 to 20 (with an $A_{260}/A_{280} < 1.2$) were found to be non-mitogenic.

Finally, no mitogenic activity of any fraction obtained after chromatography of a cell-free culture medium of C8166/45 cells could be revealed. HIV-1 particles produced by C8166/45 cells, concentrated by high-speed centrifugation and then treated with PFA, were unable to increase $[^{3}H]$thymidine incorporation by normal T lymphocytes (Table 2). Collectively, these results indicate that HTLV-I viral particles are involved in conveying a mitogenic signal to T lymphocytes.

**Discussion**

We have previously reported that incubation of quiescent human T lymphocytes with HTLV-I virions triggered the proliferation of these cells (Duc Dodon & Gazzolo, 1987; Gazzolo & Duc Dodon, 1987). The present study provides lines of evidence that suggest that HTLV-I virions are critically involved in mediating the mitogenic effect. Prevention of the binding to T cells of viral particles preincubated with an antibody to the surface envelope glycoprotein leads to a significant decrease in...
the mitogenic activity. Furthermore, contact of either virus-producing cells or virions with T cells appears to be necessary or sufficient to stimulate the proliferation of these cells. Indeed, virus-producing cells fixed with PFA lose the ability to fuse with indicator cells, but keep the ability to induce the proliferation of T lymphocytes. Likewise, viral particles fixed with PFA were still found to be mitogenic for T cells. As demonstrated for other enveloped viruses, such as Sendai and Rous sarcoma viruses (Chejanovsky et al., 1986; Gilbert et al., 1990), treatment with low concentrations of PFA leads to an inhibition of fusion between the virus envelope and the cell membrane. These results suggest that no structural rearrangement occurring as a consequence of virion interaction with the T cell membrane is required for the mitogenic event to take place.

The activity of surface envelope (gp46) glycoproteins was investigated by infecting C8166/45 cells with a recombinant vaccinia virus harbouring the envelope gene of HTLV-I (Shida et al., 1987). These cells were able to form syncytia when co-cultivated with receptor-bearing cells, but were not mitogenic for T cells. This is in contrast with virus-producing T cells and raises the possibility that the envelope glycoproteins are mitogenic for T cells only when expressed on virus particles. A theoretical explanation could be that gp46 molecules are present at a higher density on the envelope of virus particles than on the membrane of cells infected with the envelope recombinant vaccinia virus. The mitogenic event will therefore take place because multiple gp46 molecules on virus particles could coordinate membrane antigens on T cells. Alternatively, our observations do not exclude the possibility that other viral proteins might be involved in the mitogenic event. A direct interaction between the outermost protein of the Gag complex, the matrix (MA) protein, and the envelope glycoproteins has been demonstrated for murine, avian and human retroviruses (Gebhardt et al., 1984; Satake & Luftig, 1983; Yu et al., 1992). Accordingly, the interaction of the transmembrane envelope (gp21) glycoprotein with the underlying MA (p19) protein of HTLV-I should produce an ordered organization of the surface glycoproteins, allowing them to induce the mitogenic event. Furthermore, it is very unlikely that this MA protein directly triggers the mitogenic process since, as indicated above, virus particles are still mitogenic, even after treatment with PFA which freezes structural proteins in their native conformation and does not allow any structural re-arrangement.

It has been reported (Kimata et al., 1993; Wucherpfennig et al., 1992) that the mitogenic activity of HTLV-I might be due to cellular membranes contaminating virus preparations. Evaluating the mitogenic ability of two HTLV-I-transformed cell lines, C91/PL, which is virus-producing, and C8166/45 which is defective in viral production, demonstrates that the mitogenic activity is restricted to the former, suggesting that cellular components released by these HTLV-I-transformed cell lines are not responsible for the induction of the mitogenic stimulus. However, since cellular antigens could bind to retroviral particles (Arthur et al., 1992), the possibility that, when present on HTLV-I virions, these cellular antigens may act as cofactors in mediating the mitogenic effect cannot be excluded.

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