Host cell membrane proteins on human immunodeficiency virus type 1 after in vitro infection of H9 cells and blood mononuclear cells. An immuno-electron microscopic study

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Human immunodeficiency virus type 1 (HIV-1)-infected H9 and blood mononuclear cells (MNCs) were studied by immunogold electron microscopy for the presence of HIV-1 gag p24 protein, env gp41 and gp120 proteins, and host cell molecules CD4, CD11a, CD25, CD54, CD63, HLA class I and HLA-DR. Uninfected H9 cells and MNC membranes labelled for CD4, HLA class I and class II, and, at low density, CD11a and CD54; lysosomal structures in the cytoplasm labelled for CD63. The infected cell surface showed immunolabelling for HIV-1 proteins, as did budding particle-like structures. Immunogold labelling of the cell membrane for CD4 was almost non-existent. The level of immunolabelling for CD11a and CD54 on infected cells was greater than that on uninfected cells; this is presumably related to a state of activation during virus synthesis. Budding particle-like structures and free virions in the intercellular space were immunogold-labelled for all host cell markers investigated. This was confirmed by double immunogold labelling using combinations of HIV-1 gag p24 labelling and labelling for the respective host cell molecule. We conclude that virions generated in HIV-1-infected cells concentrate host-derived molecules on their envelope. Also molecules with a prime function in cellular adhesion concentrate on the virion.

Infection of cells by human immunodeficiency virus type 1 (HIV-1) is followed by the disappearance of the virus receptor molecule CD4 from the cell membrane (Gelezinus et al., 1991; Gielen et al., 1989; Hoxie et al., 1986). This phenomenon has also been observed for other surface molecules including HLA antigens (Eales et al., 1988; Gelderblom et al., 1987b; Henderson et al., 1987; Kerkau et al., 1989; Schols et al., 1992) and the CD3, CD8 and CD11 antigens (Stevenson et al., 1987). By using immuno-electron microscopy we have previously demonstrated the complete absence of CD4 antigen and the partial absence of HLA-DR and CD5 antigen on H9 cells 2 days after HIV-1 infection (Meerloo et al., 1992). The CD3 and CD25 antigens remained detectable on the cell surface at similar density, and the CD63 antigen, a lysosomal membrane glycoprotein, became detectable at higher density on cells after HIV-1 infection. In addition, CD3, CD4, CD5, CD25, CD30 and CD63 antigens, and HLA-DR are detected on budding figures and free virions in intercellular areas (Meerloo et al., 1992). Thus, during the first phase after infection of H9 cells, host cell-derived molecules concentrate on newly generated HIV-1 virions. This phenomenon might contribute to the disappearance of these molecules from the cell membrane after infection. The present study focuses on the presence of adhesion molecules of the CD11a [x chain of leukocyte function-associated antigen-1 (LFA-1)] and CD54 [intercellular adhesion molecule-1 (ICAM-1)] family on H9 cells after HIV-1 infection. In addition, we analysed blood mononuclear cells (MNCs) after in vitro infection with HIV-1 for the presence of host-derived molecules on budding particles and newly generated virions.

H9 cells were infected with HIV-1 IIIB strain 2 days before harvest by mixing one part infected cells with four
Table 1. **Antibodies used in this study**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>CD*</th>
<th>Subclass</th>
<th>Source†</th>
<th>Reciprocal dilution ‡</th>
<th>Reactivity</th>
</tr>
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<tbody>
<tr>
<td>Anti-Leu-3a</td>
<td>CD4</td>
<td>G1</td>
<td>B&amp;D</td>
<td>10</td>
<td>T helper cell phenotype</td>
</tr>
<tr>
<td>ADP 336</td>
<td>CD4</td>
<td>G2a</td>
<td>MRC</td>
<td>100</td>
<td>T helper cell phenotype</td>
</tr>
<tr>
<td>Anti-LFA-1α</td>
<td>CD11a</td>
<td>G1</td>
<td>AB</td>
<td>Undiluted</td>
<td>χ-Chain of LFA-1</td>
</tr>
<tr>
<td>Anti-IL2R §</td>
<td>CD25</td>
<td>G1</td>
<td>B&amp;D</td>
<td>10</td>
<td>Activated T cells</td>
</tr>
<tr>
<td>Anti-ICAM-1</td>
<td>CD54</td>
<td>G1</td>
<td>AB</td>
<td>12</td>
<td>ICAM-1</td>
</tr>
<tr>
<td>RUU-SP 5-15</td>
<td>CD63</td>
<td>G1</td>
<td>MM</td>
<td>3000</td>
<td>Platelets, macrophages and granulocytes</td>
</tr>
<tr>
<td>Anti-HLA-class I</td>
<td>Polyclonal</td>
<td></td>
<td>HP</td>
<td>300</td>
<td>Heavy chain of class I molecule</td>
</tr>
<tr>
<td>Anti-HLA-DR</td>
<td>G2a</td>
<td>B&amp;D</td>
<td>10</td>
<td>HIV-1 DR</td>
<td></td>
</tr>
<tr>
<td>Anti-p24</td>
<td>G2a</td>
<td>Abbott</td>
<td>1000</td>
<td>HIV-1 env gp41</td>
<td></td>
</tr>
<tr>
<td>10.15.64</td>
<td></td>
<td></td>
<td></td>
<td>500</td>
<td>HIV-1 env gp120</td>
</tr>
</tbody>
</table>

* CD, Cluster of differentiation.
‡ Reciprocal dilution used in immunogold electron microscopy.

Parts uninfected H9 cells. MNCs, isolated from heparinized blood from healthy donors by conventional Ficoll-Hypaque density gradient centrifugation and subjected to in vitro stimulation with phytohaemagglutinin in medium supplemented with polybrene and recombinant interleukin-2, were infected using a virus-containing cell-free supernatant of HIV-1 IIIB-infected H9 cells. Cells were harvested 3 weeks after infection, when virus production was maximal as determined by capture ELISA for HIV-1 gag p24 in the supernatant.

For conventional transmission electron microscopy (TEM), cell pellets were fixed in 2% glutaraldehyde (GA) in 0.1 M cacodylate buffer pH 7.4, followed by embedding in Epon. Ultrathin sections were contrasted with uranyl magnesium acetate and Reynolds' lead citrate.

For immunogold labelling, cell pellets were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 for 2 h at 4°C, embedded in 10% gelatin and impregnated overnight at 4°C with 2.3 M sucrose. Ultrathin cryosections (80 nm) cut at --100°C were subjected to the immunogold labelling procedure (Meerloo et al., 1992). Post-sectioning immunogold labelling enables the detection of both intra- and extracellular determinants. The procedure included incubation with a first antibody (listed in Table 1), followed by a rabbit anti-mouse Ig antibody (diluted 1:400) or a rabbit anti-sheep Ig antibody (diluted 1:500) (Dakopatts) and finally with protein A-gold complex (15 nm gold particle size). Double immunogold labelling (Meerloo et al., 1992) was done for HIV-1 gag p24 antigen (sheep antibody), and for HIV-1 env gp120 [using a monoclonal antibody (MAB), HLA class I (rabbit antibody), HLA-DR (MAB), CD11a (MAB), CD54 (MAB) and CD63 antigen (MAB)]. In this procedure, ultrathin cryosections were incubated with mouse or rabbit antibody, in the case of a mouse MAB followed by rabbit anti-mouse Ig. Sections were incubated with protein A–gold complex (15 nm particle size), and fixed for 10 min in 1% GA to prevent binding of the Protein–A gold complex used in the subsequent labelling. The subsequent incubation was done with sheep anti-HIV-1 p24 antibody followed by rabbit anti-sheep antibody and Protein A–gold complex (10 nm particle size). The sections were embedded and contrasted in 1.8% methylcellulose containing 3% uranyl acetate pH 7.0. The sections were examined in a Jeol 1200 EX electron microscope.

The optimal dilution for each antibody was determined in preliminary titration experiments. Controls included incubation of the anti-HIV-1 antibody with uninfected H9 cells or uninfected MNCs. In addition, primary or secondary antibodies were omitted or applied at higher dilutions in single or double labelling experiments. To exclude labelling as a result of non-specific Ig isotype binding to virus particles, we incubated H9 cells with MABs anti-Leu-5b (IgG2a, CD2) and anti-Leu-2a (IgG1, CD8), or a polyclonal rabbit anti-fluorescein isothiocyanate antiserum, which did not label cells. Labelling was not observed in any control experiment.

The preparations of blood MNCs after culture showed a mixed cell population including lymphoblastoid cells and macrophages. Conventional TEM of infected H9 cells and MNCs revealed free virions and virions attached to the cell surface, with ultrastructural features similar to those described previously (Meerloo et al., 1992; Gelderblom et al., 1987a; Hausmann et al., 1987; Marx et al., 1988; Palmer & Goldsmith, 1988; Timár et al., 1986).
Budding particles were observed on about 80% of H9 cells and about 5% of cells in MNC preparations. The incidence of budding particles on H9 cells 2 days post-infection was higher than that on chronically infected cells (about 40%), indicating that budding figures are derived from newly infected cells. In cryosections of infected MNCs the typical characteristics of virions were lost, but virions could be identified easily by immunogold labelling for HIV-1 antigens. Free virions were labelled, as well as structures resembling budding particles (Fig. 1). The anti-p24 antibodies labelled a product located mainly in the core of virions and budding particle-like structures. The immunogold labelling for env antigens gp41 and gp120 was localized more to the membrane part of the virus. Immunolabelling for viral antigens was also observed scattered in the membrane region and in the cytoplasm of cells (Fig. 1). The density of p24 labelling was greater than that for gp41 or gp120 (data not shown). These data were similar for H9 cells and MNCs (Fig. 1), but the number of immunolabelled cells in preparations of MNCs was much lower (about 5%).

Immunogold labelling for HLA class I and HLA-DR was found on the membrane of uninfected H9 cells and MNCs; labelling was also observed in the cytoplasm of the cells. On infected H9 cells and MNCs (Fig. 2a), the density of labelling on the cell surface was somewhat lower. Labelling was observed on budding particle-like structures and free virions in the intercellular space. In double immunogold labelling experiments, co-localization of HLA class I (Fig. 3a) or HLA-DR label and HIV-1 p24 label was observed on virus particles and budding particle-like structures. In the cytoplasm, HLA class I (Fig. 3a) or II immunolabelling segregated from that for p24. The preservation of cytoplasmic components was such that it was not possible to identify which structures were labelled by either anti-p24 or anti-HLA antibody.

Immunogold labelling for CD4 was readily visible on the membrane of uninfected cells. This was observed for port of MNCs and for H9 cells. Infected cells, either MNCs or H9 cells, showed no CD4 immunogold labelling of the cell membrane. Immunolabelling was observed on budding particle-like structures and on free virions in the intercellular space. This was confirmed by double labelling for CD4 and p24 (Fig. 2b). The data were similar for antibodies anti-Leu-3A and ADP 336.

CD25 cell surface immunolabel was observed on H9 cells, and at a higher density on some MNCs. Infected
cells showed CD25 immunogold label on budding particle-like figures and virus particles in the intercellular space (data not shown). The CD63 antibody labelled cytoplasmic structures in uninfected H9 cells and some MNCs. The label was associated with lysosomal and vesicular structures in the cytoplasm. On infected H9 cells and MNCs, CD63 immunogold labelling of budding particle-like structures and free virions was seen. In double immunogold labelling experiments, the CD63 and HIV-1 p24 label co-localized to the same site in the cytoplasm (Fig. 3b), presumably in lysosomal structures. Co-localization on virions was also observed (Fig. 3c).

In immunogold labelling experiments for CD11a (Fig. 4a, b, c) and CD54 (Fig. 4d), the CD54 reagents F10.2 and F10.3 gave similar results. Uninfected H9 cells showed only a low density of CD11a on the cell membrane (Fig. 4a). Similarly, there was low density immunolabelling by anti-CD54 antibodies. On infected H9 cells the density of CD11a or CD54 was greater. This observation confirms the data of Weeks et al. (1991) who documented enhanced expression of \( \alpha_\beta \) integrin on T lymphocytes after HIV-1 infection. Presumably this is related to a state of activation of the cells in the first period after infection. The CD11a and CD54 labelling also localized to budding particle-like figures and free virions in the intercellular space (illustrated for CD11a in Fig. 4b, for CD54 in Fig. 4d). This was confirmed by double labelling experiments with a combination of anti-p24 antibody and either an anti-CD11a (Fig. 4c) or CD54 antibody. In preparations of MNCs only a few cells were immunolabelled by the anti-CD11a and anti-CD54 antibodies. Virion structures on these cells were also labelled; this labelling co-localized with HIV-1 p24 immunolabelling.

The presence of host-derived molecules on newly generated virions has been demonstrated for CD3, CD4, CD5, CD25, CD63 (Fig. 3c), HLA-DR and HLA class I (Fig. 3a) (Gelderblom et al., 1987b; Henderson et al., 1987; Kerkau et al., 1989; Meerloo et al., 1992; Schols et al., 1992). Both H9 cells and blood MNCs after \textit{in vitro} infection show host-derived molecules on budding particle-like figures and virions in the intercellular spaces (Fig. 2). From these results we conclude that the uptake
of host-derived molecules by forming virions may be a
general phenomenon in HIV-1-infected cells actively
producing virus. In addition, this study has demonstrated
that virions carry adhesion molecules LFA-1 and ICAM-
1 (Fig. 4b, c, d), that appear to be up-regulated on the
surface of H9 cells after infection. There is no apparent
selectivity in the insertion of host-derived cell surface
molecules to concentrate on virions during the budding
process. This conclusion contrasts with the previous
results showing non-random association, e.g. for HLA-
DR but not HLA-DP and HLA-DQ using flow
cytometry (Schols et al., 1992), and for HLA-class I and
II and β2-microglobulin, but not 11 other cell surface
components, using ELISA of solubilized virions (Hoxie
et al., 1987). Selectivity can be ascribed to differences in the
methods applied. Quantitative data on the number of host-
derived molecules on virions, in relation to that on the
host cell membrane, may give additional information on
this discrepancy, but immunoelectron microscopy is not
directly suitable for such measurements.

The relevance of the presence of host-derived mole-
cules on newly synthesized virions should be considered
while bearing in mind that cells were infected in vitro. To
investigate this phenomenon in vivo infection, we examined blood MNCs from HIV-1-infected patients
after in vitro stimulation with mitogens. This approach
was not successful; the proportion of cells actively
producing virus, as judged by examining budding
structures, was too low to make reliable observations
(data not shown).

The presence of host-derived molecules on virions is
not unique for HIV-1. It has also been documented for
Friend leukaemia virus (Chen & Lilly, 1979), avian
leukosis virus (Young et al., 1990), VSV (Hecht &
Summers, 1976; Calafat et al., 1983), MLV (Calafat et
al., 1983), Sindbis virus (Strauss, 1978) and influenza
virus (Holland & Kiehn, 1970). Conversely, Simons &
Garoff (1980) have shown that budding structures of
Semliki Forest virus do not contain host-derived mole-
cules. The relevance of the presence of host-derived
molecules remains subject to speculation. Immuno-
electron microscopy does not distinguish between pro-
teins inserted into the envelope membrane of the virus
and proteins attached to the viral envelope.

Our findings may have implications for the interaction
between virions and cells. The virus may use host-derived
molecules in addition to env protein in binding and
subsequent infection of other (CD4-negative) cells, and
in this way contribute to the spread of infection. This
phenomenon is particularly relevant for cellular adhesion
molecules, which have a prime function in intercellular
contacts (Dustin & Springer, 1991). In this study, the
interaction between ICAM-1 and LFA-1, which mediate
leukocyte adhesion and signalling, has been examined.
The CD54 antibodies used (F10.2 and F10.3) recognize
epitopes involved in cellular adhesion (Bloemen et
al., 1992). The LFA-1 molecule has been shown to be
involved in in vitro syncytium formation by HIV-1-
infected cells (Hildreth & Orentas, 1989; Valentijn et
al., 1990), but not in HIV spread and virus replication
(Pantaleo et al., 1991).

This use of host-derived molecules adds to the range of
potential mechanisms by which the cell binds HIV-1 and
may subsequently become infected. These mechanisms
include the CD4-env gp120 interaction (Meltzer et al.,
1990; Tersmette & Miedema, 1990) and interactions
between HIV-1-containing complexes and the receptor
for the Fc part of Ig (Homsy et al., 1989; Takeda et
al., 1988) and that for the C3 component of complement
(June et al., 1991). When designing in vivo therapies to
prevent adherence and spread of HIV-1 infection in the
body, one should be aware of the possibility that the
virus may not only use its own envelope components, but
also may use host-derived adhesion molecules in binding
and subsequent infection.

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