The EMBO Journal vol.7 no.2 pp.513-518, 1988

Human immunodeficiency virus infection of CD4-bearing cells occurs by a pH-independent mechanism

Myra O.McClure, Mark Marsh and Robin A.Weiss

Chester Beatty Laboratories, Institute of Cancer Research, Fulham Road, London SW3 6JB, UK

Communicated by R.A.Weiss

The effect of weak bases (NH_4Cl and amantadine) and carboxylic ionophores (monensin) on the infection of CD4 (T4) positive human cell lines by HIV-1 is examined. These reagents, which raise the pH of acidic intracellular organelles, fail to inhibit HIV-1 entry and the events leading to viral protein synthesis at concentrations inhibitory for low pH-dependent fusogenic enveloped viruses. The infectivity of VSV (HIV-1) pseudotypes is unaffected by weak bases at concentrations causing 95% plaque reduction of VSV in its own envelope. HIV-1 dependent cell-cell fusion (syncytium formation) occurs in medium maintained at pH 7.4-7.6, and virions are not irreversibly inactivated by incubation in acid medium. Our results show that HIV-1 entry and membrane fusion do not require exposure to low pH. The production of infectious HIV-1 particles, however, is inhibited in cells treated with NH_4Cl.

Key words: HIV/infection/pH/membrane fusion/AIDS

Introduction

Enveloped animal viruses enter their host cells by membrane fusion (Poste and Pasternak, 1978; White *et al.*, 1983; Marsh, 1984; Kohn, 1985; Haywood, 1988). For many viruses, including representatives of the alpha-, rhabdo-, flavi-, orthomyxo- and murine retroviruses, the fusion reaction is pH-dependent (White *et al.*, 1983; Gollins and Porterfield, 1986; Redmond *et al.*, 1984). The entry mechanism has been established in detail for Semliki Forest virus (SFV) and influenza virus. With these viruses mildly acidic conditions (pH 5.3-6.5) induce conformational changes in the viral glycoproteins that render them fusogenic (Skehel *et al.*, 1982; Kielian and Helenius, 1985; Doms *et al.*, 1985). The fusion and penetration of the viral nucleocapsid into the cytoplasm occurs in acidic endocytic vesicles (endosomes) following the receptor-mediated endocytosis of intact virions (Helenius *et al.*, 1980; Marsh *et al.*, 1983; Yoshimura and Ohnishi, 1984). For other viruses, such as Sendai virus (a paramyxovirus), however, the fusion reaction is pH-independent and occurs in acidic, neutral and mildly alkaline conditions (White *et al.*, 1983; Haywood, 1988), and fusion may occur either at the cell surface or within endocytic organelles.

Weak bases, such as NH_4Cl, chloroquine and amantadine, and carboxylic ionophores, such as monensin and nigericin, reversibly raise the pH of acidic organelles including endosomes and lysosomes (Ohkuma and Poole, 1978; Galloway *et al.*, 1983; Mellman *et al.*, 1986). Because pH-dependent viruses require exposure to acid conditions, these reagents effectively block infection. SFV virions, for example, in the presence of either NH_4Cl or monensin, bind to the cell surface and are internalized but, since they do not encounter a low pH environment, they fail to undergo fusion and are not infectious (Helenius *et al.*, 1982; Marsh *et al.*, 1982; Kielian and Helenius, 1985).

The human immunodeficiency virus (HIV-1), the aetiological agent for acquired immunodeficiency syndrome (AIDS), is a retrovirus of the lentivirus subfamily (Barré-Sinoussi *et al.*, 1983; Gallo *et al.*, 1984; McClure and Weiss, 1987). The virion envelope contains one predominant species of membrane glycoprotein, the gp41-gp120 complex encoded by the viral *env* gene. The CD4 (T4) antigen, which plays a role in immune cell recognition (Gay *et al.*, 1987; Sleckman *et al.*, 1987), acts as the cell surface receptor for HIV-1 on T lymphocytes (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984; McDougal *et al.*, 1986), monocytes (Clapham *et al.*, 1987), and other cells in which CD4 is expressed following gene transfer (Maddon *et al.*, 1986). CD4 provides the cell surface binding sites recognized by the viral gp120 envelope antigen (McDougal *et al.*, 1986; Maddon *et al.*, 1986), and the epitopes of CD4 required for HIV-binding are becoming increasingly well defined (Sattentau *et al.*, 1986; McClure *et al.*, 1987). As with other enveloped viruses, the entry of HIV-1 following binding is presumed to occur by membrane fusion, and fusogenic activity has been demonstrated for the HIV-1 envelope glycoprotein (Lifson *et al.*, 1986; Sodroski *et al.*, 1986), with a putative fusogenic domain in gp41 (Kowalski *et al.*, 1987).

We have investigated the pH dependency for HIV-1 entry and show here that HIV-1 infection is not inhibited by weak bases or carboxylic ionophores. However, our results indicate that the release of infectious virus from HIV-1 producing cells is inhibited by weak bases.

Results

Cell fusion at pH 7.6

HIV-1 infects human $CD4^+$ lymphocytic and monocytic cells *in vivo* and in culture the infection can be assayed by the formation of large multinucleate syncytia (Montagnier *et al.*, 1984; Dalgleish *et al.*, 1984; Clapham *et al.*, 1987). The syncytia form as a consequence of cell-cell fusion, induced by the interaction of the viral membrane glycoprotein (gp41-gp120) with CD4 on adjacent cells (Dalgleish *et al.*, 1984; Sodroski *et al.*, 1986). The human T cell and monocytic lines, H9, C8166 and U937, express cell surface CD4 antigen and are highly susceptible to HIV-mediated cell fusion (Dalgleish *et al.*, 1984; Asjo *et al.*, 1987; Clapham *et al.*, 1987). When cells were infected with HIV-1 at a multiplicity of infection (m.o.i.) of three infectious units (IU) per cell, syncytia (Figure 1) were observed within 24 h. These results indicate that HIV-1-induced cell-cell fusion

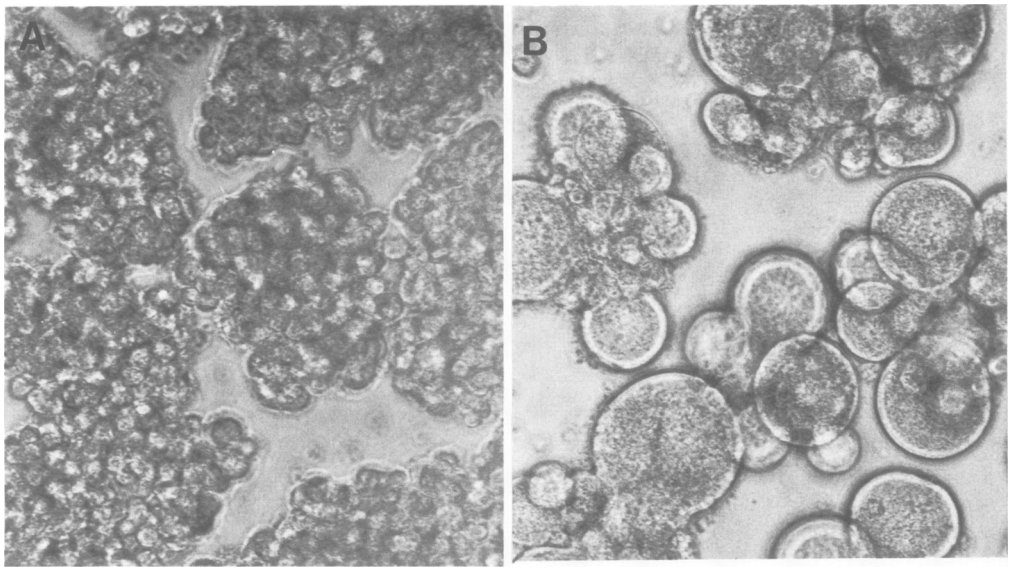

Fig. 1. Giant syncytial cell induction in $CD4^+$ cell culture by HIV-1 at pH 7.5. C8166 T cells were exposed to HIV-1 (m.o.i. 3) for 24 h. Cells were incubated in bicarbonate-free medium buffered to pH 7.6 with 25 mM Hepes throughout the culture period, and pH measurement at the beginning and end of each experiment confirmed that the pH did not fall below pH 7.4. Incubation of H9 T cells and U937 monocytes gave similar results (not shown). (**A**) Uninfected C8166 cells, ×120; (**B**) HIV-1 infected C8166 cells.

can occur in slightly alkaline conditions. The result implies that exposure to low pH is not required for entry of HIV-1. To examine this question we determined the effect of weak bases and monensin on HIV-1 entry into C8166 cells.

Effect of NH_4Cl on HIV-1 replication

HIV-1 infectivity was titrated in C8166 cells in the presence or absence of 30 mM NH_4Cl as described in Materials and methods (Table I). The $CD4^+$ C8166 cell line is highly sensitive to HIV-1 replication and may be used for HIV-1 titration. The appearance of syncytia correlates with viral antigen expression and reverse transcriptase (RT) activity (Clapham *et al.*, 1987). Table I shows that large syncytia were observed in cells cultured with the highest virus concentration, while smaller, less frequent syncytia were observed in NH_4Cl treated cells. Although the NH_4Cl was removed at 18 h, the number and size of syncytia were still reduced at 60 h, though by 84 h large syncytia were observed up to 10^{-3} virus dilution. In untreated cells, the HIV-1 end point titre was 10^5 IU/ml. These results indicate that the presence of NH_4Cl during the first 18 h of HIV-1 infection inhibits early syncytium formation and reduces the final titre of virus.

In cells treated with 30 mM NH_4Cl for 18 h, the viable cell count (by trypan blue exclusion) was reduced by 22% and [^{35}S]methionine incorporation in a 15 min pulse was down by 12% compared with non-treated control cultures.

Effect of weak bases and monensin on HIV-1 entry

To distinguish between early and late effects of NH_4Cl, amantadine and monensin on HIV-1 infection, cells were infected in the presence of the drugs and, after removal of free virus, were drug-treated for a further 5 h before being returned to normal medium. None of the drugs diminished the titre of HIV-1 (Table II), implying that early stages of infection are not dependent on the low pH of endocytic organelles.

Table I. Effect of NH_4Cl on HIV replication

HIV-1 dilution	Time post-infection (h)					
	18		60		84	
	Control	NH_4Cl	Control	NH_4Cl	Control	NH_4Cl
10^{-1}	+++	+	+++	+	+++	+++
10^{-2}	–	–	+++	+	+++	+++
10^{-3}	–	–	+++	+	+++	+++
10^{-4}	–	–	+++	–	+++	–
10^{-5}	–	–	–	–	+++	–
10^{-6}	–	–	–	–	–	–

C8166 cells were infected with serial dilutions of HIV-1, in the presence or absence of 30 mM NH_4Cl, for 1 h and subsequently incubated for 17 h in medium containing 30 mM NH_4Cl or in control medium. The cells were then transferred to NH_4Cl free medium and the development of syncytia determined immediately or at 60 and 84 h after infection. Syncytium size and number did not change after 84 h. Cultures were scored on a semi-quantitative scale, where +++ denotes the inclusion of almost all nuclei (>90%) into syncytia, + denotes ≤10% syncytia and – denotes the absence of syncytia (see Figure 1).

Effect of weak bases on pseudotype infection

The role of HIV-1 envelope glycoproteins in virus entry was investigated further by examining the effect of weak bases on infection by vesicular stomatitis virus (VSV) particles bearing the envelope glycoproteins of HIV-1. Such VSV pseudotype virions depend on the functional properties of the retrovirus glycoproteins for early stages of infection, as first demonstrated for VSV pseudotypes of avian and murine retroviruses by Zavada (1972). VSV(HIV-1) pseudotypes plate only on $CD4^+$ cells (Dalgleish *et al.*, 1984; Clapham *et al.*, 1987), and are specifically neutralized by HIV-1 antisera (Weiss *et al.*, 1986). Following penetration and uncoating, however, VSV pseudotypes replicate to produce wild-type VSV which infects neighbouring cells to produce cytopathic

Table II. Effect of weak bases and monensin on the early stages of HIV-1 infection

Inhibitors	End-point titre of HIV-1
None	5×10^5
NH_4Cl (30 mM)	5×10^5
Amantidine (2 mM)	2.5×10^5
Monensin (10 μM)	5×10^5

C8166 cells were infected with serial dilutions of HIV-1 in the presence of NH_4Cl, amantadine or monensin for 1 h. The cells were then washed and re-incubated in medium containing the drugs for a further 5 h. Subsequently the cells were transferred to inhibitor-free medium and infection was scored 5 days later by the appearance of syncytia. Results are shown for the highest concentrations of reagents without apparent toxic effects.

Table III. Effect of weak bases on VSV and VSV(HIV-1) pseudotype infectivity

Inhibitor	P.f.u. of	
	VSV	VSV(HIV-1)
None	2×10^8	2×10^4
NH_4Cl (30 mM)	1×10^7	3×10^4
Amantadine (2 mM)	8×10^6	2.5×10^4

Adherent $CD4^+$ CEM cells were infected with serially diluted VSV harvested from H9 cells producing HIV-1. The non-pseudotype VSV represents the total p.f.u. whereas the VSV(HIV-1) pseudotype fraction bearing HIV-1 envelope antigens represents the p.f.u. surviving anti-VSV treatment. Virus was adsorbed for 1 h ± inhibitor and, after washing, the cells were overlaid with mink lung cells in the presence of inhibitor. The mink cells do not express HIV-1 receptors but provide a sensitive monolayer for plaque formation following secondary infection by VSV progeny released from the CEM cells. After 2 h further incubation, the medium was replaced with medium containing 1.8% agar and no inhibitor. Plaques were counted after 48 h. The specificity of the VSV(HIV-1) envelope was confirmed by the absence of p.f.u. when anti-VSV-treated stock was plated on $CD4^-$ cells (not shown). Similar results were obtained for non-pseudotype VSV whether propagated in HIV-1-infected H9 cells (shown here) or in uninfected H9 cells (not shown).

plaques. Thus plaque formation by VSV(HIV-1) pseudotypes provides a quantitative assay for testing whether agents can inhibit early steps of infection dependent on HIV envelope functions, such as receptor binding, membrane fusion, virion penetration and uncoating.

Table III shows the effect of NH_4Cl on the plaque forming efficiency of wild-type VSV and of VSV(HIV-1) pseudotype. Whereas the infectivity of VSV particles bearing the VSV G-protein envelope was reduced by 95% in the presence of NH_4Cl, the infectivity of the VSV(HIV-1) pseudotypes was not significantly affected. Thus pseudotypes of VSV bearing the envelope glycoproteins of HIV-1 enter $CD4^+$ cells in a pH-independent manner. Similar results were obtained using 2 mM amantadine.

Low pH treatment does not irreversibly inactivate HIV-1

For influenza and SFV, incubation in low pH medium triggers an irreversible conformational change in the fusion proteins (Skehel *et al.*, 1982; Doms *et al.*, 1985; Kielian and Helenius, 1985). In association with cellular membranes the conformational change leads to fusion and infection,

Table IV. Effect of pH on HIV-1 and SFV infectivity

	pH	HIV-1		SFV
		RT (c.p.m.)	IU	[^{3}H]uridine (c.p.m.)
No virus	7.4	924		6515
NH_4Cl	7.4	NT	NT	7392
(30 mM)	7.4	168 000	5×10^5	17 600
	6.8	171 000	5×10^5	NT
	6.0	165 000	5×10^5	NT
	5.0	174 000	5×10^5	6813
	4.6	164 000	5×10^5	NT

Medium containing 5×10^5 IU/ml HIV-1 was brought to the indicated pH for 1 min at 20°C, then readjusted to pH 7.4 and serially diluted to titrate for surviving IU in C8166 cells. Before dilution aliquots were taken for RT activity. Medium containing 2×10^7 p.f.u./ml SFV was similarly treated at pH 5.0 and infectivity in BHK-21 cells was assayed by [^{3}H]uridine incorporation. Trace amounts of [^{35}S]methionine-labelled SFV were added and the specific activities of the input suspension remained the same before and after low pH treatment (not shown) indicating that SFV viral antigen was not lost at low pH by non-specific adsorption to plastic.
NT, not tested.

while in the absence of cells the virions are irreversibly inactivated.

HIV-1 was treated at the pH values indicated in Table IV for 1 min and subsequently re-neutralized. Aliquots were assayed for RT activity as a particle count and the virus stocks were titrated in C8166 cells for surviving infectivity. Equivalent titres were observed for HIV-1 treated at all pH values, indicating that the HIV-1 is not inactivated at low pH. In contrast, when SFV was treated at pH 5.0 for 1 min, there was no evidence of surviving infectivity, as background levels of uridine incorporation, equivalent to uninfected cells or cells infected in the presence of NH_4Cl, were observed (Table IV).

Synthesis of viral glycoprotein in the presence of NH_4Cl

Amantadine, NH_4Cl and monensin appear to have no effect on the early stages of HIV-1 infection. However, changes in the pH of acidic organelles induced by weak bases and carboxylic ionophores are rapid and reversible (Ohkuma and Poole, 1978; Marsh *et al.*, 1982); thus virus remaining in the cultures following the washes and removal of NH_4Cl might be infectious. We therefore examined the synthesis of HIV-1 specific proteins following infection, in the continuous presence or absence of NH_4Cl. To establish the time course of viral protein synthesis in the absence of NH_4Cl, C8166 cells were infected with HIV-1 for 1 h at 37°C, washed, and at various times aliquots of cells were removed and analysed for HIV-1 *gag* and *env* gene products by SDS–PAGE and Western blotting. From 13 h post-infection increasing amounts of viral antigens were detected (not shown). We therefore used 16 h post-infection as the earliest time to probe for newly synthesized viral antigens.

C8166 cells were infected with HIV-1 in the presence of NH_4Cl, azidothymidine (AZT) or control medium. After 16 h the cells were labelled with [^{35}S]methionine and [^{35}S]-cysteine, harvested, lysed and the gp160 immunoprecipitated with antiserum to HIV-1 env antigens. SDS–PAGE analysis shows (Figure 2) that newly synthesized viral env precursor protein, gp160, is detectable in cells infected with virus in

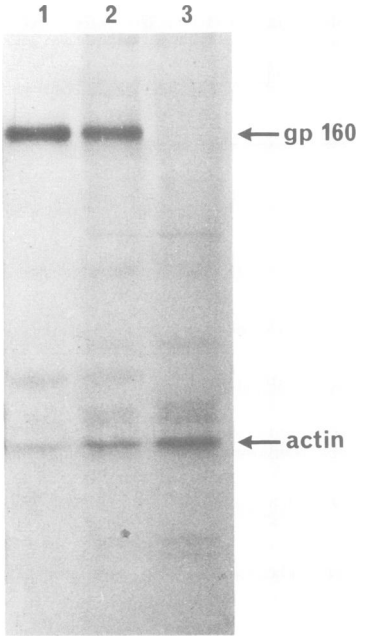

Fig. 2. Synthesis of viral gp120 following HIV-1 infection of cells in the presence of NH_4Cl. C8166 cells were infected (m.o.i. 3) with HIV-1 in control medium, the presence of 30 mM NH_4Cl, or 10 μg/ml AZT. After 1 h the cells were washed and returned to culture with or without the respective inhibitor for 16 h, when the cells were incubated for 1 h with [^{35}S]methionine and [^{35}S]cysteine. Proteins from pelleted and lysed cells were immunoprecipitated with human serum with a high anti-gp120 titre prior to SDS-PAGE and radioautography. **Lane 1**, control medium; **lane 2**, NH_4Cl containing medium; **lane 3**, AZT containing medium.

the presence or absence of NH_4Cl, but is absent from cells infected in the presence of the RT inhibitor, AZT. Actin which is precipitated non-specifically is observed in all three lanes. These results indicate that the synthesis of the viral antigens following infection is not inhibited by NH_4Cl.

Inhibition of infectious HIV-1 release by NH_4Cl

Our experiments indicate that weak bases do not inhibit the entry of HIV-1 or subsequent viral protein synthesis. However, when infected cells were maintained in NH_4Cl for 18 h syncytium formation was inhibited and the end point titration was two logs lower than the untreated cells (Table I). We therefore examined the possibility that weak bases affect late steps in viral replication.

Three cell lines (H9 T cells, Ramos B cells and U937 monocytic cells) chronically infected with HIV-1 produce infectious virus in culture. The CD4 antigen is 'down-regulated' following the initial infection, and while the cells fail to form syncytia, they readily do so when mixed with uninfected $CD4^+$ cells (Dalgleish *et al.*, 1984; Clapham *et al.*, 1987). The cells from each of the three lines were washed and resuspended in medium with or without NH_4Cl, and were incubated overnight when the medium was harvested and assayed for RT activity and infectivity. Table V shows that following incubation with NH_4Cl there is a 10–15% reduction in RT activity in the supernatant medium, this being in the same range as the reduction in viable cell number and protein synthesis (see above). In contrast, there was a 95% reduction in the titre of infectious virus in the NH_4Cl-treated cultures compared with the untreated controls.

Table V. Effect of NH_4Cl on HIV-1 production in chronically infected cells

HIV-1 producing cells	NH_4Cl treatment	Virus production	
		RT activity	IU/ml
H9 (T cell)	none	148 946	1×10^6
	30 mM	136 872	5×10^4
U937 (monocyte)	none	345 936	2×10^6
	30 mM	282 630	8×10^4
Ramos (B cell)	none	17 413	5×10^3
	30 mM	15 722	1×10^2

The cells were washed and incubated for 18 h in control medium or medium containing 30 mM NH_4Cl maintained at pH 7.4 in a CO_2-free atmosphere. Then 4 ml of medium was assayed for RT activity (c.p.m.) and 1 ml was serially diluted for end-point infectivity titration in C8166 cells.

The results indicate that NH_4Cl reduces the infectious titre of HIV-1. However, the drop in RT activity does not correspond with the loss of infectivity, suggesting that virus particle release is not inhibited but that released particles have lower infectivity. The reason for this loss of infectivity is not known but it could arise as a result of NH_4Cl-induced changes in the post-translational processing of viral membrane glycoproteins.

Discussion

Enveloped animal viruses enter their host cells by membrane fusion, either at neutral pH (e.g. paramyxoviruses) or in the acidic environment of the endosome (White *et al.*, 1983; Haywood *et al.*, 1988). We have sought to determine whether HIV-1 uses either of the pathways outlined for other enveloped viruses following binding to the CD4 receptor. Our results indicate that entry and the steps in replication leading to viral protein synthesis are not inhibited by the weak bases NH_4Cl and amantadine or by the carboxylic ionophore monensin. These endosomal pH modulators have been used at concentrations which block the entry of several viruses, including influenza, VSV, SFV and West Nile virus, all of which undergo pH-dependent fusion in the pH range 5.3–6.5. Furthermore, our results show that cell–cell fusion leading to syncytium formation occurs in media maintained at pH 7.4–7.6, and that virions are not inactivated by incubation at low pH. Thus the functional entry of HIV-1 into cells does not require exposure to low pH and does not involve an irreversible acid-induced conformational change in the viral envelope proteins.

During the completion of our experiments Stein *et al.* (1987) reported that entry of HIV-1, measured by the synthesis of proviral DNA, is not inhibited by NH_4Cl, chloroquine or monensin, and that fusion of the HIV-1 envelope with the plasma membrane could be visualized by electron microscopy. Our studies, using different assay procedures, conform with and extend these findings. Maddon *et al.* (1986), however, suggested that NH_4Cl inhibits HIV-I replication. A possible explanation for this discrepancy is our finding that weak bases inhibit the release of infectious HIV-1. Weak bases and carboxylic ionophores increase the pH in all acidic compartments of the cell. Compartments of the exocytic pathway, including the trans Golgi network

(TGN), are known to be acidic (Anderson and Pathak, 1985; Mellman *et al.*, 1986). Furthermore, weak bases and carboxylic ionophores are known to affect viral and cellular protein transport through the exocytic pathway (Matlin, 1986). Our experiments indicate that the budding and release of HIV-1 virions is not itself affected by NH_4Cl treatment, because there is no significant reduction of particles containing RT, but that the virions released in the presence of NH_4Cl have reduced infectivity. The specific nature of the defect remains to be elucidated.

Although our results demonstrate that low pH is not required for entry, they do not rule out the possibility that endocytosis is important for infection. The results reported here show that HIV-1 fuses at neutral pH, but do not indicate whether fusion also occurs at acid pH as is the case for Sendai virus. Stein *et al.* (1987) have demonstrated that HIV-1 can fuse at the cell surface and in the presence of sufficient virus, may also lead to cell fusion from without. However, it has not been demonstrated that this route leads to productive infection.

The attachment of HIV-1 to CD4 antigen at the cell surface may not be sufficient to trigger cell fusion or infection. Maddon *et al.* (1986) transfected the gene encoding the human CD4 molecule into human and murine cells of lymphoid and non-lymphoid origin. While cell surface expression of CD4 rendered the human cells susceptible to HIV-1 infection, none of the mouse cells became susceptible to HIV-1 infection or to HIV-1 induced cell fusion. The murine cells expressing CD4 bind HIV-1 virions and, as exposure to low pH is not required for entry, the block to infection remains unresolved. The replication defect does not appear to be a post-entry event because VSV(HIV-1) pseudotypes fail to plate on $CD4^+$ mouse cells, even though wild-type VSV infects these cells (Maddon *et al.*, 1986). In addition, mouse cells transfected with full length HIV-1 provirus produce infectious virus (Adachi *et al.*, 1986; Levy *et al.*, 1986).

The infectivity of pH-dependent viruses, by fusion at the cell surface with acid medium, varies according to the virus and cell under study. SFV is permissive on fusion with BHK-21 cells (Helenius *et al.*, 1982), but not when fused to the surface of CHO cells (M.Marsh and S.Swift, unpublished). Similarly, when VSV or fowl plague virus are fused with MDCK cells, or when West Nile virus is fused with murine macrophage-like cells, infection does not occur (Matlin *et al.*, 1981, 1982; Gollins and Porterfield, 1986). CD4 is known to internalize under certain conditions such as treatment by tumour promoters (Delia *et al.*, 1982; Hoxie *et al.*, 1986; Clapham *et al.*, 1987) and we have observed anti-CD4−immunogold complexes in the endocytic vesicles of HeLa−CD4 cells (unpublished). Perhaps the human CD4 molecule expressed in NIH-3T3 cells is unable to function fully as a virus receptor, because its internalization is inhibited. However, evidence favouring a role for receptor-mediated endocytosis in HIV-1 infection will require a more detailed biochemical and morphological analysis of the interactions between HIV-1 and its host cells.

Materials and methods

Cells and viruses

The human T leukaemic, $CD4^+$, non-adherent cell lines C8166 and H9, and the adherent line CEM were maintained in RPMI-1640 containing 2 mM glutamine, 10% fetal calf serum (FCS), penicillin and streptomycin as described (Clapham *et al.*, 1987). Three infected cell lines chronically producing HIV-1 (H9 infected with HTLV-IIIRF, Ramos B cells producing HTLV-IIIB, and the U937 monocyte line infected with HTLV-IIIRF) were grown as suspension cultures in the same media. Baby hamster kidney cells (BHK-21) were grown in Glasgow-modified minimal essential medium (GMEM) supplemented with 10% FCS, 10% tryptose phosphate broth (TPB), penicillin, streptomycin and 2 mM glutamine. CCL-64 mink lung fibroblasts (MLF) were grown in Dulbecco-modified Eagle's medium (DMEM) containing 2 mM glutamine, 10% FCS, penicillin and streptomycin.

HIV-1, strain HTLV-IIIRF, was propagated in chronically infected H9 cells, adding uninfected H9 cells to increase virus production 5 days before harvest. The supernatant was collected, clarified by centrifugation (2000 r.p.m. for 10 min at 4°C) and stored in aliquots in liquid nitrogen. HIV-1 stocks contained approximately 1×10^6 IU in C8166 (Clapham *et al.*, 1987) and H9 cells. SFV and [^{35}S]methionine-labelled SFV were grown in BHK-21 cells as previously described (Helenius *et al.*, 1982). VSV (Indiana strain) was propagated in MLF cells (Clapham *et al.*, 1987).

Viral assays

HIV-1 infectivity was assayed by serial dilution and plating on C8166 cells and scored by the appearance of syncytia (Clapham *et al.*, 1987) and RT activity (Hoffman *et al.*, 1985). SFV infectivity in BHK-21 cells was assayed by incorporation of [^{3}H]uridine into newly synthesized viral RNA as previously described (Helenius *et al.*, 1982). All experiments with weak bases and monensin were carried out in RPMI-1640 medium lacking bicarbonate and buffered with 25 mM Hepes at pH 7.6. The medium was monitored throughout the experiments to ensure the pH did not drop below 7.4.

Preparation of VSV(HIV-1) pseudotype viruses

VSV and VSV(HIV-1) pseudotype viruses containing the VSV nucleocapsid and the envelope glycoproteins of HIV-1 were prepared and assayed as described previously (Clapham *et al.*, 1987) using HIV-1 infected H9 cells for pseudotype propagation.

Low pH treatment of HIV-1 and SFV

HIV-1 stock virus was diluted 1:10 in RPMI-1640, containing 0.2% bovine serum albumin (BSA) and 10 mM Hepes, pH 7.4. SFV was diluted in the same medium and [^{35}S]methionine-labelled SFV was added as a tracer. The virus suspensions were adjusted to various pH values between 7.4 and 4.5 by addition of predetermined amounts of 1 M HCl, and incubated at room temperature for 5 min. The pH was readjusted to pH 7.4 by addition of an appropriate volume of 1 M NaOH. The virus samples were assayed for [^{35}S]methionine (SFV) or RT (HIV-1) activity to ensure that virions had not been lost by adsorption to the plastic surfaces during the pH adjustment, and for infectivity by [^{3}H]uridine incorporation into BHK-21 cells (SFV, 10 p.f.u./cell for control pH 7.4) and syncytium induction in C8166 cells (HIV-1) respectively.

Immunological detection of viral proteins

For immunoprecipitation, C8166 cells infected with HIV-1 were incubated in methionine-free medium for 10 min at 37°C and then transferred to methionine-free medium containing 500 μCi [^{35}S]methionine and 500 μCi [^{35}S]cysteine (Amersham International). After 1 h at 37°C the cells were spun down, washed three times in ice-cold PBS, and disrupted in 1 ml lysis buffer containing 1% Triton X-100 for 10 min on ice and then centrifuged at full speed in an Eppendorf microfuge for 15 min. The clarified supernatants were preadsorbed with a non-specific mouse immunoglobulin, followed by a goat anti-mouse immunoglobulin (Tago) and Protein A conjugated Sepharose (Sigma Chemical Co.) Subsequently the supernatants were incubated with human serum containing antibodies to HIV- env proteins, and then Protein A Sepharose beads. The immunoadsorbent beads were washed and the adsorbed proteins analysed by SDS−PAGE on 10% gels.

Acknowledgements

We are most grateful to Sally Swift for excellent technical assistance, Victoria Lewis for photography, to Gus Dalgleish and Jon Weber for advice, and to Bill Gullick and Keith Willison for their critical comments on the manuscript. We thank R.C.Gallo for cell lines H9 and C8166 and for HIV-1 strains HTLV-IIIRF and HTLV-IIIB, and J.Zavada for anti-VSV antibody. This work was supported in part by the Wellcome Foundation, Cancer Research Campaign and Medical Research Council.

References

Adachi,A., Gendelman,H.E., Koenig,S., Folks,T., Willey,R., Rabson,A. and Martin,M.A. (1986) *J. Virol.*, **59**, 284−291.

Anderson,R.G.W. and Pathak,R.K. (1985) *Cell*, **40**, 635–643.
Asjö,B., Ivhed,I., Gidlung,M., Fuerstenberg,S., Fenyo,E.M., Nilson,K. and Wigzell,H. (1987) *Virology*, **157**, 359–365.
Barré-Sinoussi,F., Chermann,J.C., Rey,F., Nugeyre,H.T., Chamaret,S., Gruest,J., Dauguet,C., Axler-Blin,C., Vezinet-Brun,F., Rouzioux,C., Rozenbaum,W. and Montagnier,L. (1983) *Science*, **220**, 868–871.
Clapham,P.R., Weiss,R.A., Dalgleish,A.G., Exley,M., Whitby,D. and Hogg,N. (1987) *Virology*, **158**, 44–51.
Dalgleish,A.G., Beverley,P.C.L., Clapham,P.R., Crawford,D.H., Greaves, M.F. and Weiss,R.A. (1984) *Nature*, **312**, 763–767.
Delia,D., Greaves,M.F., Newman,R.A., Sutherland,D.R., Minowada,J., King,P. and Goldstein,G. (1982) *Int. J. Cancer*, **29**, 23–31.
Doms,R.W., Helenius,A. and White,J. (1985) *J. Biol. Chem.*, **260**, 2973–2981.
Gallo,R.C., Salahuddin,S.Z., Popovic,M., Shearer,G.M., Kaplan,M., Haynes,B.F., Palker,T.J., Redfield,R., Oleske,J., Safai,B., White,G., Foster,P. and Markham,P.D. (1984) *Science*, **224**, 500–503.
Galloway,C.J., Dean,G.E., Marsh,M., Rudnick,G. and Mellman,I. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 3334–3338.
Gay,D., Maddon,P., Sekaly,R., Talle,M.A., Godfrey,M., Long,E., Goldstein,G., Chess,L., Axel,R., Kappler,J. and Marrack,P. (1987) *Nature*, **328**, 626–629.
Gollins,S.W. and Porterfield,J.S. (1986) *J. Gen. Virol.*, **67**, 1941–1950.
Haywood,A.M. (1988) In Wilschut,J. and Hoekstra,D. (eds), *Cellular Membrane Fusion*. Marcel Dekker, New York (in press).
Helenius,A., Kartenbeck,J., Simons,K. and Fries,E. (1980) *J. Cell Biol.*, **84**, 404–420.
Helenius,A., Marsh,M. and White,J. (1982) *J. Gen. Virol.*, **58**, 47–61.
Hoffman,A.D., Banapour,B. and Levy,J.A. (1985) *Virology*, **147**, 326–335.
Hoxie,J.A., Matthews,D.M., Callahan,K.J., Carrel,D.L. and Cooper,R.A. (1986) *J. Immunol.*, **137**, 1194–1201.
Kielian,M.C. and Helenius,A. (1985) *J. Cell Biol.*, **107**, 2284–2291.
Klatzmann,D., Champagne,E., Chamaret,S., Gruest,J., Guetard,D., Hercend,T., Gluckman,J.C. and Montagnier,L. (1984) *Nature*, **312**, 767–768.
Kohn,A. (1985) *Prog. Med. Virol.*, **31**, 109–167.
Kowalski,M., Potz,J., Basiripour,L., Dorfman,T., Chun Goh,W., Terwilliger,E., Dayton,A., Rosen,C., Haseltine,W. and Sodroski,J. (1987) *Science*, **237**, 1351–1355.
Levy,J.A., Cheng-Mayer,C., Dina,D. and Luciw,P.A. (1986) *Science*, **232**, 998–1001.
Lifson,J.D., Feinberg,M.B., Reyes,G.R., Rabin,L., Banapour,B., Chakrabarti,S., Moss,B., Wong-Staal,F., Steimer,K.S. and Engleman,E.G. (1986) *Nature*, **323**, 725–728.
McClure,M.O., Sattentau,Q.J., Beverly,P.C.L., Hearn,J.P., Fitzgerald, A.K., Zuckerman,A.J. and Weiss,R.A. (1987) *Nature*, **330**, 487–489.
McDougal,J.S., Kennedy,M.S., Sligh,J.M., Cort,S.P., Mawle,A. and Nicholson,J.K.A. (1986) *Science*, **231**, 382–385.
Maddon,P.J., Dalgleish,A.G., McDougal,J.S., Clapham,P.R., Weiss,R.A. and Axel,R. (1986) *Cell*, **47**, 333–348.
Marsh,M. (1984) *Biochem. J.*, **218**, 1–10.
Marsh,M., Wellsteed,J., Kern,H., Harms,E. and Helenius,A. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 5297–5301.
Marsh,M., Bolzau,E. and Helenius,A. (1983) *Cell*, **32**, 931–940.
Matlin,K.S. (1986) *J. Cell Biol.*, **103**, 2565–2568.
Matlin,K., Reggio,H., Helenius,A. and Simons,K. (1981) *J. Cell Biol.*, **91**, 601–613.
Matlin,K., Reggio,H., Helenius,A. and Simons,K. (1982) *J. Mol. Biol.*, **156**, 609–613.
Mellman,I., Fuchs,R. and Helenius,A. (1986) *Annu. Rev. Biochem.*, **55**, 663–700.
Montagnier,L. *et al.* (1984) In Gallo,R.C., Essex,M. and Gross,L. (eds), *Human T-Cell Leukemia–Lymphoma Viruses*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp.
Ohkuma,S. and Poole,B. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 3327–3331.
Poste,G. and Pasternak,C.A. (1978) In Poste,G. and Nicholson,G.C. (eds), *Membrane Fusion*. Elsevier/North Holland, Amsterdam, pp. 305–367.
Redmond,S., Peters,G. and Dickson,C. (1984) *Virology*, **133**, 393–402.
Sattentau,Q.J., Dalgleish,A.G., Weiss,R.A. and Beverley,P.C.L. (1986) *Science*, **234**, 1120–1123.
Skehel,J., Bayley,P., Brown,E., Martin,S., Waterfield,M., White,J., Wilson,I. and Wiley,D. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 968–972.
Sleckman,B.P., Peterson,A., Jones,W.K., Foran,J.A., Greenstein,J.L., Seed,B. and Burakoff,S.J. (1987) *Nature*, **328**, 351–353.
Sodroski,J., Goh,W.C., Rosen,C., Campbell,K. and Haseltine,W.A. (1986) *Nature*, **322**, 470–474.
Stein,B.S., Gowda,S.D., Lifson,J.D., Penhallow,R.C., Bensch,K.G. and Engleman,E.G. (1987) *Cell*, **49**, 659–668.
Weiss,R.A., Clapham,P.R., Weber,J.N., Dalgleish,A.G., Lasky,L.A. and Berman,P.W. (1986) *Nature*, **324**, 572–575.
White,J., Kielian,M. and Helenius,A. (1983) *Quart. Rev. Biophys.*, **16**, 151–195.
Yoshimura,A. and Ohnishi,S.-I. (1984) *J. Virol.*, **51**, 497–504.
Zavada,J. (1972) *J. Gen. Virol.*, **15**, 183–191.

Received on November 11, 1987