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Simian immunodeficiency virus ($SIV_{mac}251$) membrane lipid mixing with human $CD4^+$ and $CD4^-$ cell lines *in vitro* does not necessarily result in internalization of the viral core proteins and productive infection

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The cell binding site of simian immunodeficiency virus (SIV) is believed to be the CD4 molecule. Several $CD4^+$ cell lines are, however, resistant to infection by $SIV_{mac}251$ *in vitro* and additional cell membrane molecules have been implicated in $SIV_{mac}251$ entry. We investigated the binding, envelope fusion and entry of the viral core proteins (p27) of $SIV_{mac}251$ into two human $CD4^+$ cell lines (H9 and Sup-T1) which are infectible, and one $CD4^+$ (A3.01) and two $CD4^-$ cell lines (K562 and Raji) that are resistant to infection. The fusion of the viral and cellular membranes was monitored by a fluorescence assay for lipid mixing. Cell entry of the viral core was evaluated following virus–cell incubation and cell surface trypsinization. We found that $SIV_{mac}251$ can bind to and fuse (membrane lipid mixing) in a temperature-dependent but pH-independent fashion with $CD4^+$ and $CD4^-$ human-derived cell lines. In contrast, lipid mixing with CD4 expressing EL-4 mouse T cells or Mv-1-lu mink lung fibroblasts was absent or limited, suggesting that certain components of human cell membranes in addition to CD4 are involved in SIV_{mac} envelope–cell fusion. Lipid mixing with the human cells was inhibited partially by soluble CD4. Anti-CD4 antibodies inhibited the lipid inter-mixing with H9, but not with Raji cells, whereas neutralizing anti-SIV_{mac} sera inhibited fusion with both $CD4^+$ and $CD4^-$ cells. Out of the five human cell lines tested, efficient entry of p27 and productive infection took place only with H9 and Sup-T1 cells. In these two cases, the amounts of p27 internalized during virus–cell fusion correlated with the extent of infection.

Introduction

Simian immunodeficiency viruses (SIVs) are retroviruses that infect non-human primates (Allan *et al.*, 1990; Benveniste *et al.*, 1986; Daniel *et al.*, 1985; Fults *et al.*, 1986), and share common structural and biological characteristics with human immunodeficiency viruses (HIVs) (Chakrabarti *et al.*, 1987; Dietrich *et al.*, 1989; Franchini *et al.*, 1987; Kanki *et al.*, 1985; Lowenstine *et al.*, 1986; Murphey-Corb *et al.*, 1986). The primary cell targets of SIV and HIV in their respective hosts are lymphocytes and monocytes that express the CD4 antigen on their surface (Dalgleish *et al.*, 1984; Kannagi *et al.*, 1985; Klatzmann *et al.*, 1984; McDougal *et al.*, 1985). Several *in vitro* studies have indicated, however, that not all $CD4^+$ cell lines can be infected by these viruses (Clapham *et al.*, 1991). In addition, there is significant evidence from *in vitro* and *in vivo* studies that SIV and HIV can also infect $CD4^-$ lymphocytes as well as cells that are not part of the immune system (Benveniste *et al.*, 1988; Harouse *et al.*, 1991*a*; Kunsch & Wigdahl, 1991; Lackner *et al.*, 1991; Langhoff *et al.*, 1991; Moyer & Gendelman, 1991; Tateno *et al.*, 1989).

Important differences between SIV and HIV type 1 (HIV-1) have been noted regarding the mechanism(s) by which these viruses infect $CD4^+$ cell lines. It was shown previously that several $CD4^+$ lymphoid cell lines are resistant to $SIV_{mac}251$ and SIV_{Mne} (*Macaca nemestrina*) infection, although they are infectible with HIV-1 (HTLV-IIIB strain) (Agy *et al.*, 1991; Hoxie *et al.*, 1988; Koenig *et al.*, 1989).

HIV and SIV infect their target cells following viral envelope fusion with the cell plasma membrane (McClure *et al.*, 1988; Stein *et al.*, 1987). It is generally assumed that HIV and SIV will fuse only with the plasma membrane of cells that they can infect. The resistance to infection of certain $CD4^+$ cell lines could be due to inefficient virus–cell binding and/or fusion of the viral

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envelope with these cell membranes. It is also possible that, although virus–cell fusion and viral entry occur both with the 'non-infectible' and 'infectible' cells, the level of virus replication is controlled by cytoplasmic factors.

In order to examine to what extent $SIV_{mac}251$–cell interactions proceed in the case of non-infectible cells, we investigated the binding and fusion of this virus with the cell plasma membranes of three $CD4^+$ human cell lines (H9, Sup-T1 and A3.01), which have been shown previously to have a widely different capacity to support SIV_{mac} infection (Hoxie *et al.*, 1988; Koenig *et al.*, 1989), two $CD4^-$ human cells (Raji and K562), one $CD4^+$ mink cell line (Mv-1-lu) and one $CD4^+$ mouse T cell line (EL-4). To follow the kinetics of the $SIV_{mac}251$–cell fusion, we employed the octadecylrhodamine (R_{18}) fluorescence assay for the mixing of lipids between the viral envelope and the cell plasma membrane (Hoekstra *et al.*, 1984; Düzgüneş & Bentz, 1988). This method has been used extensively to examine the interaction of several enveloped viruses with cell membranes (Düzgüneş *et al.*, 1992; Gilbert *et al.*, 1990; Herrmann *et al.*, 1990; Hoekstra & Klappe, 1986; Lima *et al.*, 1991; Sinangil *et al.*, 1988; Srinivasakumar *et al.*, 1991; Volsky, 1990).

To examine whether lipid mixing is always accompanied by the delivery of the viral nucleocapsid, we monitored the entry of $SIV_{mac}251$ core proteins into the cell cytoplasm in the cases where extensive membrane fusion took place. We also examined whether $SIV_{mac}251$–cell fusion and nucleocapsid entry leads to productive infection, by determining the production of new virions in the culture medium up to 28 days post-fusion (i.e. post-infection).

We demonstrate that the envelope of $SIV_{mac}251$ could bind to and mix lipids extensively with the plasma membrane of all the human-derived $CD4^+$ and $CD4^-$ cell lines used. Minimal membrane fusion with the non-human $CD4^+$ cell lines was monitored. Efficient entry of p27 and productive infection was only detected in the case of H9 and Sup-T1 cells. In these two cases the level of infection correlated with the amount of viral core protein internalized during virus–cell fusion. Minimal membrane fusion with all the non-human cell lines was monitored. A preliminary account of our observations has been presented (Stamatatos & Düzgüneş, 1992).

Methods

Materials. Recombinant soluble CD4 (sCD4) (corresponding to the ectodomain) was a gift from Genentech. R_{18} was purchased from Molecular Probes and the non-ionic detergent $C_{12}E_9$ from Calbiochem. Goat anti-human κ light chain was from Tago, and anti-Leu3a antibodies were from Becton-Dickinson. Anti-$SIV_{mac}251$ neutralizing sera were kindly provided by Dr J. V. Torres (University of California at Davis, Calif., U.S.A.).

Cells. A3.01 cells were obtained from T. Folks (Centers for Disease Control, Atlanta, Ga., U.S.A.). Raji cells, a human Burkitt's lymphoma cell line, were obtained from Microbiological Associates through Dr P. Madden (NIH). H9 cells were obtained from Dr R. C. Gallo and Sup-T1 cells from Dr J. Hoxie, through the NIAID AIDS Research and Reference Reagent Program. The K562 cell line was obtained from the American Type Culture Collection. The percentage of $CD4^+$ cells present in each cell line was determined by flow cytometry every 4 to 6 weeks. The mouse T cell line stably expressing human CD4 (EL-4) was provided by Dr A. Koito (The Institute of Physical and Chemical Research, Tsukuba-city 305, Japan). All the above cell lines were cultured at 37 °C, with 5% CO_2 in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS). The $CD4^+$ mink lung cell line (Mv-1-lu) was obtained from Dr P. Clapham (Chester Beatty Laboratories, Institute of Cancer Research, London, U.K.) via Dr J. Levy (University of California at San Francisco, Calif., U.S.A.). They were grown in MEM supplemented with 5% FBS.

Virus production and purification. All experiments involving virus were conducted in a BSL-3 facility as recommended by the Centers for Disease Control (1988). Sucrose density gradient-purified $SIV_{mac}251$ was obtained from Advanced Biotechnologies. The virus was grown in HuT-78 cells. Cell supernatant was harvested and clarified by centrifugation. The virus was centrifuged at 19000 r.p.m. in a Beckman type 19 rotor for 90 min at 5 °C. The pelleted virus was resuspended and then banded over a 20/40% (w/w) sucrose gradient at 35000 r.p.m. for 2 h in a Beckman type 35 rotor. The purified virus was recovered from the 20/40% sucrose interface. Thereafter, the virus was resuspended in 10 mM-Tris–HCl, 150 mM-NaCl pH 7·5. The purified viral preparation contained all the major viral proteins (p14, p26, p32, gp40, p55, p66 and gp120) as determined by Western immunoblot assay. The reverse transcriptase activity was $5{\cdot}50 \times 10^8$ c.p.m./ml (rA : dT template, percentage rA activity: 99·6%). The virus was stored in small samples at −80 °C until further use.

Virus labelling. For each experiment 50 or 100 μg total viral protein was thawed and the viral envelope was labelled with R_{18} as reported previously (Konopka *et al.*, 1990; Larsen *et al.*, 1990). Briefly, the virus was incubated with an ethanolic solution of R_{18} (final ethanol concentration of less than 1% v/v) for 60 min at room temperature. The final R_{18} concentration in the viral envelope was less than 3% of the total viral lipid. The stock R_{18}-labelled virus was kept on ice for the duration of each experiment. The presence of R_{18} did not alter the fusion properties of our viral preparation since similar quantities of p27 were internalized during fusion whether the viral envelope was labelled with R_{18} or not (data not shown). The virus remained infectious following R_{18} labelling (see Results).

Determination of p27. To quantify the amount of viral core proteins introduced into the cytoplasm during virus–cell fusion, we determined the amounts of p27 internalized by using the SIV core antigen capture assay kit from Coulter Immunology. Cell lysates were added to wells coated with a murine monoclonal antibody to p27. A second, human biotinylated antibody was added, which reacted with conjugated streptavidin–horseradish peroxidase. Colour developed upon addition of hydrogen peroxide in the presence of 3,3′,5,5′-tetramethylbenzidine. The intensity of the colour is dependent upon the amount of p27 present. In control wells, lysates from cells that had not been incubated with virus were added. The same assay was also used to evaluate the production of new virions in the cell culture medium during productive cell infection.

Binding of $SIV_{mac}251$ to cells. All binding and fusion studies were performed in RPMI-1640 medium without phenol red, containing 25 mM-HEPES (Medium A). Increasing amounts of R_{18}-labelled SIV_{mac} were incubated with 2×10^6 cells in 0·1 ml of Medium A for

30 min at 4 °C. The mixture was then diluted 20-fold with the same medium and centrifuged at 200 *g* for 10 min at 4 °C. The cell pellet was washed twice. During the first centrifugation, almost 95% of the unbound virus was removed from the cell pellet (determined from the extent of R_{18} fluorescence recovered in the cell supernatant following each wash). The R_{18} fluorescence in the cell pellet and in the combined supernatants was measured, and the amount of cell-associated SIV_{mac} was evaluated. The amount of bound virus was quantified in parallel by measuring the amount of p27 associated with the cells. Both assays gave similar results.

Virus–cell fusion. All fusion experiments were performed in Medium A. The R_{18} fluorescence assay was used to monitor the intermixing of the viral envelope with the cell plasma membrane (Hoekstra *et al.*, 1984). The fluorescence of R_{18} depends on the concentration of the probe in the membrane. Initially, the probe is introduced into the viral envelope at a concentration that results in the quenching of its fluorescence. Upon virus–cell fusion, the fluorophore is diluted in the much larger area of the cell plasma membrane, resulting in an increase of fluorescence. All fluorescence measurements (555 nm excitation, 595 nm emission, with a 590 nm low end cut-off filter) were carried out with a Perkin-Elmer LS-5B fluorescence spectrophotometer. In order to minimize the effects of light scattering produced by high cell concentrations, the front face configuration was used. The cells were washed twice with PBS, once with Medium A and then kept on ice until use. Virus and suspension cells, at a ratio of 1 μg viral protein/2×10^6 cells, were pre-incubated at 4 °C for 30 min and then the temperature of the mixture was raised to 37 °C. Samples of 100 μl were taken at various times and diluted 20-fold with Medium A. Their fluorescence was measured before and after the addition of detergent to a final concentration of 1% (v/v). Since R_{18} is diluted maximally in the presence of detergent, the fluorescence in the presence of detergent is taken as 100% fusion. In the case of the adherent cells (Mv-1-lu), following the incubation of the virus with the cells for various periods of time, the cell supernatant was removed and the cells were recovered from the plate following a 10 min incubation with calcium- and magnesium-free PBS (containing 5 mM-EDTA). The cells and the virus-containing supernatant were then re-combined, and the extent of fusion was determined as described below.

The extent of lipid mixing (given as the percentage of maximal mixing) was evaluated according to the following formula: $100 \times (F_t - F_0)/(F_d - F_0)$. Here, F_0 is the R_{18} fluorescence of the virus–cell mixture starting at time zero (immediately following the addition of the virus to the cells), F_t is the R_{18} fluorescence at time t, and F_d is the R_{18} fluorescence in the presence of detergent.

Glutaraldehyde treatment of R_{18}-labelled SIV_{mac}. R_{18}-labelled virus was incubated with 0·025% glutaraldehyde for 30 min at 37 °C. The glutaraldehyde-treated viral stock was then diluted 38-fold with Medium A and incubated with cells at 4 °C for 30 min. Subsequently the temperature of the mixture was raised to 37 °C. The extent of R_{18}-dequenching was determined 30 min later. When glutaraldehyde was added directly to the virus–cell mixture at the same final concentration, it had no effect on the R_{18}-dequenching.

Effect of pH and temperature on SIV_{mac}–cell fusion. To evaluate the effect of pH on the fusion of SIV with cells, the virus (1 μg) was first incubated with the cells (2×10^6) at 4 °C for 30 min at neutral pH in Medium A (0·1 ml). The pH was then lowered to the desired value by the addition of appropriate amounts of acetate (2 M-stock solution pH 1·5). The temperature of the mixture was then raised to 37 °C for 30 min and the extent of lipid mixing was evaluated as described above. Cell viability, determined by trypan blue exclusion, was not affected during this period. The effect of temperature on the fusion potential of the virus was determined as follows: R_{18}-labelled virus (1 μg) was pre-incubated with cells (2×10^6) in 0·3 ml of Medium A for 30 min at 4 °C. The temperature was then raised rapidly to the value desired and the extent of lipid mixing was determined 30 min later.

Inhibition of fusion by recombinant soluble CD4. The virus (1 μg) was pre-incubated at 37 °C with various concentrations of CD4 for 30 min. The cells (2×10^6) were then added and the extent of lipid mixing within 60 min was determined. The volume of the interaction was 0·3 ml. In control experiments, the virus was pre-incubated alone at 37 °C and then added to the cells.

Effect of anti-SIV_{mac} neutralizing sera and anti-Leu3a antibodies on the fusion of SIV_{mac} with $CD4^+$ and $CD4^-$ cells. The cells (2×10^6) and the virus (1 μg) were incubated for 60 min at 37 °C in the presence of different concentrations of sera. In the case of anti-Leu3a antibodies, the cells (2×10^6) were first incubated with the antibody (25 μg/ml) for 30 min at room temperature and then R_{18}-labelled virus (1 μg) was added. The mixture (0·3 ml Medium A) was incubated for 30 min at 4 °C and then the temperature was raised to 37 °C for 30 min. The extent of lipid mixing was determined as above.

Cytoplasmic entry of viral core proteins (p27). The amount of viral core proteins introduced into the cytoplasm during the virus–cell fusion experiments, was determined by quantifying the amount of trypsin-resistant, cell-associated p27. Samples from the virus–cell mixture were removed following the 30 min incubation at 4 °C and then at the 30, 60 and 180 min time points at 37 °C. Usually, 0·5 μg total viral protein (10% of which represents p27) were used in order to reliably quantify the amount of p27 that became cell-associated or internalized. At each time point, the amount of p27 in the supernatants and in the cell pellet was determined, in order to evaluate the percentage of p27 that became cell-associated (bound and internalized). The internalized amount of p27 was determined subsequently, by subjecting the washed cells to trypsinization (25 μg of trypsin/10^5 cells in 1 ml of PBS for 15 min at 37 °C), to remove any cell-bound but unfused virus. The cells were then placed on ice and an equal volume of FBS was added. Following centrifugation at 200 *g* for 10 min in 30% FBS, the cell pellet was washed twice with Medium A. The number of cells in each sample was then determined. The cells were lysed with 0·5% (v/v) Triton X-100 and the amount of viral core protein introduced into the cytoplasm was quantified by measuring the amount of p27 in the cell lysates.

Infection. During the SIV_{mac}–cell fusion experiments, following 1 h incubation at 37 °C samples containing 0·5 μg total viral protein of R_{18}-labelled SIV_{mac} and 2×10^6 cells were withdrawn. They were diluted 20-fold and centrifuged at 4 °C for 10 min at 200 *g*. The cells were washed three times in order to remove the unbound virus, resuspended in 4 ml RPMI-1640 medium supplemented with 10% FBS, and incubated in 12-well tissue culture plates (Lincoln Park). Every 3 or 4 days post-fusion (infection), 2 ml samples from the cell supernatant were collected and 2 ml of fresh medium was added without further manipulation of the cells. These samples were used for SIV_{mac} core antigen (p27) determination.

Results

SIV_{mac} binds to both $CD4^+$ and $CD4^-$ membranes

The results shown in Fig. 1 demonstrate that, under the conditions used, $SIV_{mac}251$ could bind at 4 °C both to $CD4^+$ and $CD4^-$ human cell plasma membranes. However, the number of SIV_{mac}-binding sites on the surface of Raji cells appeared to be saturated at lower concentrations of virus compared to the H9 and Sup-T1 cells. At a low virus-to-cell ratio, more virus was actually

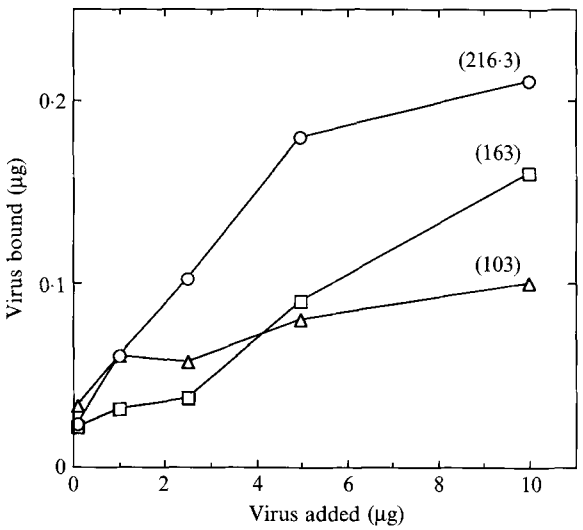

Fig. 1. Binding of SIV_{mac} to cells. Increasing amounts of R_{18}-labelled virus were incubated at 4 °C for 30 min with 2×10^6 (△) Raji, (□) H9 and (○) Sup-T1 cells in 0·1 ml of Medium A. The mixture was then diluted 20-fold and centrifuged for 10 min at 200 *g* at 4 °C. The pelleted cells were resuspended and washed twice with Medium A. The amount of virus that became cell-associated was evaluated by measuring the R_{18} fluorescence of the combined supernatants and of the cellular pellet. Results represent the average values of three experiments. The maximum deviation from the mean was within 20% of the mean. The numbers in parentheses indicate the calculated number of virions bound per cell at the highest virus-to-cell ratio.

bound to Raji than to Sup-T1 or H9 cells. However, as this ratio increased, the Sup-T1 and H9 cells bound more virus than the Raji cells. The estimated number of virions bound per cell is indicated for the highest virus-to-cell ratio used (10 μg $SIV/2 \times 10^6$ cells/0·1 ml Medium A) (Fig. 1). The number of virions was calculated assuming a protein-to-lipid ratio similar to that for HIV-1 and an average virion size of 130 nm (Aloia *et al.*, 1988).

The viral envelope fuses with both CD4⁺ and CD4⁻ human cell plasma membranes, but not with CD4⁺ non-human cell lines

The incubation of R_{18}-labelled virus with cells at 4 °C for as long as 1 h resulted in negligible R_{18}-dequenching, an indication that although virus is bound to the cells (Fig. 1 and Table 3), virus–cell fusion does not occur (Fig. 2). When the temperature was raised to 37 °C, only a minimal increase in R_{18}-dequenching was observed in the case of the non-human cell lines. However, an increase in the R_{18}-dequenching was observed as a function of time, with $CD4^+$ and $CD4^-$ human cells (Fig. 2). Following an initial rapid phase of 30 min, the rate of R_{18}-dequenching decreased, but lipid mixing proceeded even after 60 min at 37 °C. The extent of lipid intermixing observed did not correlate with the percentage of $CD4^+$ cells present in each cell line. Lipid mixing was less with Sup-T1 cells (over 99% $CD4^+$) than with H9 (about 50% $CD4^+$) or A3.01 cells (over 90% $CD4^+$). Extensive virus–cell membrane fusion was also observed when SIV_{mac} was incubated with Raji and K562 cells, which were $CD4^-$ according to fluorescence-activated cell sorting analysis.

Control experiments to assess possible R_{18} exchange

It is possible that upon virus–cell incubation, a fraction of the virus-associated R_{18} molecules are transferred to the cell plasma membrane by simple exchange in the absence of membrane fusion. It is important, therefore, to adjust the value for R_{18}-dequenching monitored during virus–cell fusion for possible 'probe exchange'. Several control experiments were performed and the results are presented in Table 1. When 1 μg R_{18}-labelled virus was incubated with 2×10^6 H9 or Raji cells up to 1 h at 4 °C in 0·1 ml of Medium A a minimal dequenching of the R_{18} fluorescence was found. Longer incubations (up to 5 h) resulted in less than 4% dequenching. The dequenching of R_{18} was mediated by the viral proteins, since a negligible dequenching of the R_{18} fluorescence was found when R_{18}-labelled liposomes, of a lipid composition similar to that of the viral envelope, were incubated with 2×10^6 cells for 1 h at 37 °C. Glutaraldehyde (0·025% v/v) treatment of 1 μg R_{18}-labelled virus with 2×10^6 cells in 0·3 ml of Medium A for 30 min at 37 °C, had a drastic effect on the lipid mixing ability of our viral preparation. Although the binding to H9 cells was unaffected by the glutaraldehyde treatment, a 25% increase in binding was found in the case of Raji cells. The R_{18}-dequenching was completely inhibited in H9 cell samples, and was reduced by 75% in the Raji cells. It was evident that glutaraldehyde treatment did not affect the ability of R_{18} to dequench as shown by the observation that detergent addition could indeed cause the maximal dequenching expected from a similar amount of untreated labelled virus. The extent of inhibition of dequenching by glutaraldehyde in our experiments was similar to or higher than that reported for several other enveloped viruses such as Rous sarcoma virus (Gilbert *et al.*, 1990) and respiratory syncytial virus (Srinivasakumar *et al.*, 1991).

Effect of sCD4 and anti-Leu3a antibodies on virus–cell fusion

Soluble CD4 inhibits infection of $CD4^+$ lymphocytes and monocytes by HIV-1, HIV-2 and SIV in a concentration-dependent fashion (Clapham *et al.*, 1989). We determined whether sCD4 would block the fusion of the viral

Table 1. *R_{18}-exchange control experiments*

Type of control	Cell line	Fluorescence-dequenching (%)
Incubation at 4 °C	H9 or Raji	< 1
Liposomes*	H9 or Raji	< 2
Glutaraldehyde†	H9	< 1 (20)
	Raji	5 (20)

* The liposomes were composed of cholesterol/phosphatidylcholine/sphingomyelin/phospatidylethanolamine and phosphatidylserine at the molar ratios reported for HIV clinical isolates (Aloia *et al.*, 1988). The concentration of the liposomes was 0·48 nmol of lipid, corresponding approximately to 1 μg of total viral protein.

† Values in parentheses indicate the percentage of R_{18}-dequenching measured when the virus was not treated with glutaraldehyde.

Table 2. *Effect of sCD4 and anti-Leu3a on virus–cell membrane lipid mixing*

Cell line	Concentration of agent (μg/ml)	Inhibition of lipid mixing (%)* sCD4	κ-chain	Anti-Leu3a
H9	0·1	19±15	0	ND†
	1	36±6	ND	ND
	5	68±14	5±2	ND
	10	ND	17±10	ND
	25	ND	ND	30±6 (35)
Raji	0·1	13·5±6	0	ND
	1	35±12	ND	ND
	5	58±25	ND	ND
	10	68±8	27±10	ND
	25	ND	ND	−8±3 (< 1)

* Mean values and the standard deviation from the mean of three experiments are given. Negative values indicate an enhancement of membrane lipid mixing. Values in parentheses indicate the percentage inhibition of virus–cell binding.

† ND, Not determined.

envelope with the cell plasma membranes (Table 2). The virus (1 μg) was pre-incubated with sCD4 for 30 min at 37 °C, and the cells (2×10^6) were added to the mixture, while keeping the blocking agent concentration constant. This incubation was performed in 0·3 ml of Medium A and the extent of virus–cell lipid mixing was evaluated 1 h later. As indicated in Table 2, sCD4 inhibited R_{18}-dequenching, and the inhibition, determined by comparing the extent of lipid mixing in the presence or absence of these agents, was concentration-dependent. Pre-incubation with a non-specific protein (goat anti-human κ light-chain) resulted in inhibition only at high, but not low, concentrations, indicating that sCD4 inhibition is specific. We did not investigate whether sCD4 inhibited cell infection in a similar fashion. When the virus was added to H9 cells that had been pre-incubated with anti-Leu3a antibodies for 30 min at 22 °C, a 30% inhibition of R_{18}-dequenching, and a 35% inhibition of binding was found. In contrast, anti-Leu3a treatment of Raji cells had no effect on the extent of

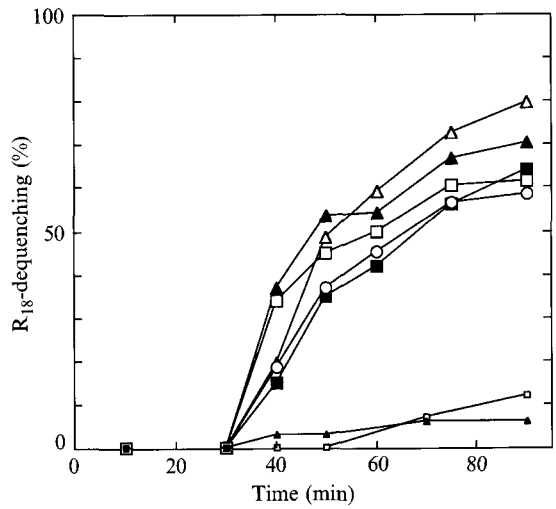

Fig. 2. Lipid mixing of the viral envelope with various CD4$^+$ and CD4$^-$ cell plasma membranes. One μg of R_{18}-labelled SIV$_{mac}$ was added to 2×10^6 (■) A3.01, (△) Raji, (○) Sup-T1, (▲) K562, (□) H9, (□) EL-4 and (▲) Mv-1-lu cells in 0·1 ml of Medium A. At the starting time the mixture was placed at 4 °C for 30 min and then the temperature was raised to 37 °C. Samples were withdrawn from the mixture and diluted 20-fold with the same medium. In the case of the adherent cells (Mv-1-lu), the cells were collected from the plate at the indicated time points, as described in Methods. The R_{18} fluorescence of each sample was then measured in the presence and absence of 1% (v/v) detergent. The extent of virus–cell fusion was evaluated as described in Methods. Points represent the mean value of three to five independent experiments, each done in triplicate. The maximum deviation from the mean was within 10% of the mean.

Table 3. *Effect of neutralizing anti-SIV$_{mac}$251 sera on virus–cell membrane mixing*

Cell line	Serum dilution	Inhibition of R18-dequenching (%)
H9	1:500	95±5
	1:1000	23±10
Raji	1:500	95
	1:1000	50±11

virus–cell binding and produced a slight enhancement of R_{18}-dequenching.

Inhibition of virus–cell membrane fusion by anti-SIV$_{mac}$251 sera

When the virus (1 μg) was incubated for 1 h with 2×10^6 H9 or Raji cells in the presence of different concentrations of anti-SIV$_{mac}$251 sera (in 0·3 ml of Medium A), an inhibition of membrane fusion, as measured by the extent of virus–cell lipid mixing, was found (Table 3) both in the case of the CD4$^+$ and CD4$^-$ cells. The percentage of inhibition was dependent on the concentration of the sera.

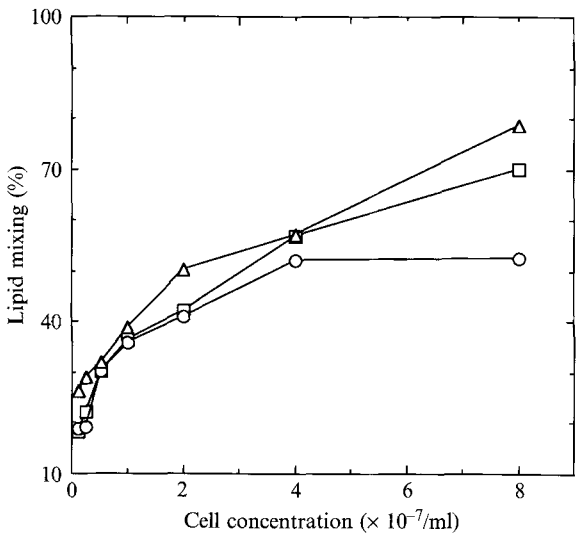

Fig. 3. Effect of cell concentration on the extent of fusion of SIV_{mac} with (○) Sup-T1, (□) H9 and (△) Raji cells. R_{18}-labelled SIV_{mac} (0·1 μg) was incubated for 30 min at 4 °C with an increasing number of cells in 0·1 ml of Medium A. The temperature was then raised to 37 °C. The extent of fusion was evaluated 1 h later, by measuring the R_{18} fluorescence of each sample in the presence and absence of 1% (v/v) detergent. Points represent the mean value of three independent experiments. The maximal deviation from the mean was 20% of the mean.

Cell concentration affects the extent of fusion

R_{18}-labelled $SIV_{mac}251$ (0·1 μg) was incubated with increasing cell concentrations at 4 °C for 30 min and the temperature of the mixture (0·1 ml total volume) was raised to 37 °C for 1 h. The extent of lipid mixing was then determined for each cell concentration. At low cell concentrations, the R_{18}-dequenching exhibited a sharp increase as the number of cells increased (Fig. 3). As predicted from mass action kinetics, this result indicates that the number of virions that fuse with the cells increases as the target cell concentration increases. The slightly lower extent of fusion observed with 2×10^6 cells and 0·1 μg of virus, compared to that obtained with 1 μg of virus (Fig. 2), is most likely due also to mass action kinetics. At high cell concentrations ($> 2 \times 10^6$ cells/ml), the extent of R_{18}-dequenching did not increase with increasing cell number as much as at lower cell concentrations, suggesting that a limited number of fusion sites per cell is available on the cell surface. This observation also suggests that the increase of R_{18} fluorescence is not an artefact due to simple exchange of the fluorescent probe into the cell membranes in the absence of lipid mixing. Since the total cell surface area is much larger than the viral surface and a large excess of target membranes was used during our experiments, we would have expected that the slope of the curve of R_{18}-dequenching plotted against cell concentration would be constant over the cell concentration range studied, if the probe were easily exchangeable.

SIV_{mac}–cell fusion is pH-independent

Certain enveloped viruses such as influenza and vesicular stomatitis viruses infect cells by taking advantage of the endocytic machinery of the cells (Matlin *et al.*, 1981; Marsh & Helenius, 1989). The acidification of the endosomal fluid causes conformation changes in the viral fusion proteins, which then mediate fusion of the viral envelope with the endosomal membranes (Hoekstra, 1990; Stegmann *et al.*, 1989). However, other enveloped viruses, such as Sendai virus and HIV-1, can fuse with their target membranes with a pH-independent mechanism (Lima *et al.*, 1991; Sinangil *et al.*, 1988). It is generally accepted that, like HIV (Maddon *et al.*, 1988), SIV does not require endocytosis for infection. We examined the effect of pH on the membrane fusion ability of $SIV_{mac}251$ by following the extent of R_{18}-dequenching occurring between the viral envelope and the cell plasma membrane (Fig. 4). Our results indicate that the fusion measured in this way was neither promoted nor inhibited by acidification of the medium.

The fusion of the viral envelope with the cell plasma membrane is temperature-dependent

Several studies have shown that enveloped viruses can fuse with target membranes in a temperature-dependent fashion (Gilbert *et al.*, 1990; Hoekstra *et al.*, 1989; Srinivasakumar *et al.*, 1991; Düzgüneş *et al.*, 1992). It was therefore of interest to examine the temperature dependence of SIV–cell fusion. As can be seen in Fig. 5, at temperatures below 15 °C no R_{18}-dequenching was found. An increase of R_{18} fluorescence was observed only when the temperature was raised above 15 °C. The temperature dependence of membrane fusion was similar for both $CD4^+$ and $CD4^-$ cells.

Infection of cells by $SIV_{mac}251$ as determined by virus production in the cell culture medium

Our results on lipid mixing between the virus and human cells were unexpected. The extent of lipid mixing was independent of the percentage of $CD4^+$ cells present in the incubation mixture (Fig. 2, 3, 4 and 5). Although similar observations were reported by others examining the fusion between cells expressing gp120/gp41 (HIV-1) and cells expressing different amounts of CD4 (Dimitrov *et al.*, 1991), the fact that extensive lipid mixing was observed with both $CD4^+$ and $CD4^-$ cells was intriguing. Therefore, we investigated whether SIV_{mac}–cell fusion

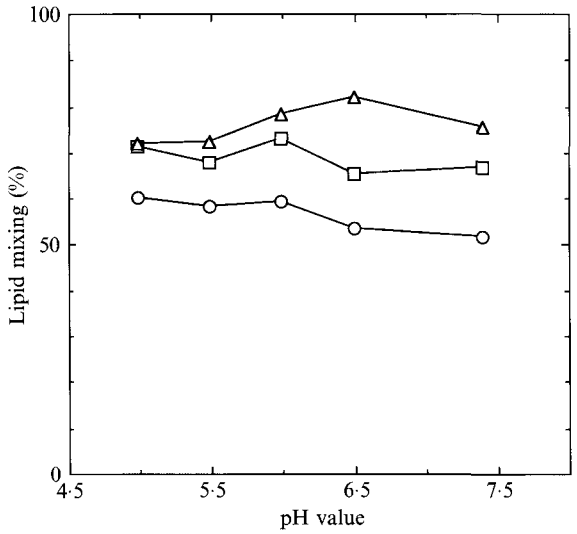

Fig. 4. Effect of pH on the fusion of SIV$_{mac}$ with (△) Raji, (○) Sup-T1 and (□) H9 cells. One μg of R_{18}-labelled SIV$_{mac}$ was added to 2×10^6 cells in 0·1 ml of Medium A for 30 min at 4 °C. The pH of the medium was then lowered to the desired value upon addition of a predetermined amount of concentrated acetate solution (2 M, pH 1·5). The temperature of the mixture was then raised to 37 °C for 30 min. The extent of virus–cell fusion was evaluated as described in Methods. Results represent average values of three independent experiments (performed within a period of 3 months after virus purification). The maximum deviation from the mean was within 15% of the mean.

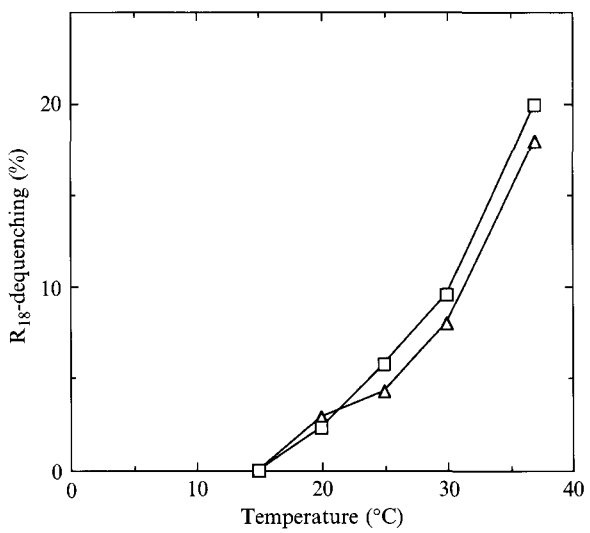

Fig. 5. Effect of temperature on the fusion of SIV$_{mac}$ with Raji (△) and H9 (□) cells. One μg of R_{18}-labelled SIV$_{mac}$ was added to 2×10^6 cells in 0·3 ml of Medium A for 30 min at 4 °C and the temperature of the mixture was raised to the value indicated. The extent of R_{18}-dequenching was evaluated 30 min later.

resulted in productive infection, by evaluating the production of virus in the cell culture medium several days post-fusion (Fig. 6). Fusion between SIV$_{mac}$251 and

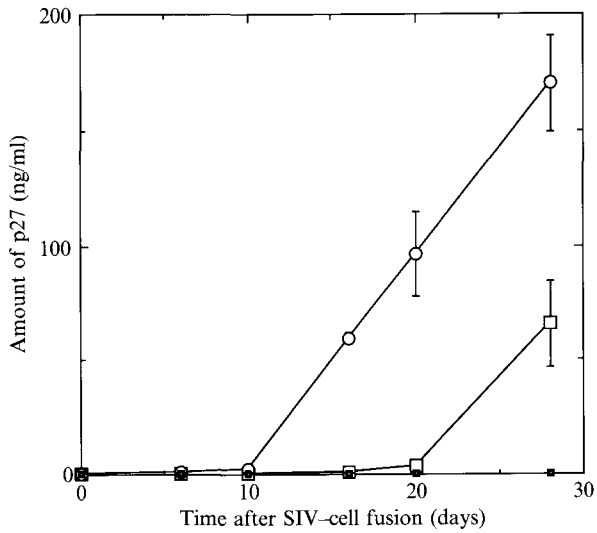

Fig. 6. Infection of cells by SIV$_{mac}$251 as determined by virus production in the cell culture medium. Following virus–cell fusion (30 min at 4 °C and 1 h at 37 °C) the (□) H9, (○) Sup-T1 and (■) K562/A3.01/Raji cells were washed by centrifugation three times, resuspended in 4 ml of culture medium and cultured up to 28 days. Every 3 to 4 days post-fusion (i.e. post-infection) 2 ml of medium was replaced and the presence of the viral core protein p27 was evaluated, as described in Methods. The results are the mean and standard deviation of three independent experiments.

the different human cell lines was allowed to proceed for 1 h. The cells were then washed three times to remove the unbound virus and resuspended in normal medium. The presence of p27 in the cell culture medium was monitored every 3 to 4 days, up to 28 days post-fusion. Productive infection was monitored only in the case of Sup-T1 and H9 cells (Fig. 6). The third CD4$^+$ cell line (A3.01) and the two CD4$^-$ cell lines were not infected productively, according to this assay. Large syncytia could be observed in the H9 cultures 16 days post-infection. Syncytia were not observed in the case of Sup-T1 cells, but very large cell aggregates began to form 15 days post-infection. In the cases of A3.01, Raji and K562 cells no such changes were observed.

Virus association and internalization of the nucleocapsid during virus–cell fusion

The fact that we could observe fusion (lipid mixing) of the viral envelope with the plasma membranes of A3.01, Raji and K562 cells, although these cells remained uninfected, prompted us to investigate in more detail the initial interactions between the cells and the virus. The R_{18} analysis reveals solely the rate and extent of lipid intermixing occurring during virus–cell membrane fusion. Therefore, it is possible that in some cases, lipid mixing occurs only (or primarily) between the outer lipid monolayers of the viral and cell membranes without

Table 4. *Virus–cell association and entry of p27 into CD4⁺ and CD4⁻ cells*

Conditions	Cell line	Percentage of cell-associated p27	Percentage of total p27 added that becomes internalized
30 min at 4 °C	H9	29·4 (4)*	0
30 min at 37 °C		17·5 (4·3)	1 (0·1)
60 min at 37 °C		11 (5)	2 (0·3)
30 min at 4 °C	Sup-T1	32 (4·5)	0
30 min at 37 °C		19 (5)	2 (0·5)
60 min at 37 °C		13 (6)	7 (1)
30 min at 4 °C	Raji	27·5 (5)	0
30 min at 37 °C		16·5 (6)	0·1 (0·1)
60 min at 37 °C		17 (6)	0·2 (0·1)
60 min at 37 °C	K562	12·3	0·3 (0·1)
30 min at 4 °C	A3.01	17 (5)	0
60 min at 37 °C	A3.01	21 (9)	0·4 (0·2)

* The values represent the mean and standard deviation (in parentheses) from the mean of three to five independent experiments.

entry of the nucleocapsid into the cytoplasm, a type of fusion called 'semi-fusion' (Düzgüneş, 1985). This possibility led us to examine whether, concurrently with lipid intermixing, viral core proteins are introduced into the cytoplasm.

Two-million cells were incubated with R_{18}-labelled virus (15 ng of p27) for 30 min at 4 °C and then the temperature was raised to 37 °C to examine p27 internalization (Table 4). At different times the cells were washed to remove the unbound virus and the amount of cell-associated p27 was determined. The amount of p27 internalized, out of the total added, was evaluated following trypsinization of the washed cells, as described in Methods. The results of these experiments are presented in Table 4. Incubation of virus with cells at 4 °C resulted in association of the virus with the cell surface measured by the amount of p27 that became cell-associated. Following trypsinization of the cell surface, we could not detect any p27 (or R_{18}) associated with the cell pellets, an indication that all the virus associated with the cells remained on the cell surface at 4 °C and was not internalized. This result correlates well with the results obtained with the R_{18} assay, which showed no lipid mixing occurring between the virus and the cells at this temperature (Fig. 2 and 5). Upon raising the temperature to 37 °C, the amount of cell-associated p27 decreased, because at high temperatures some dissociation of the virus from its binding sites occurs (Hoekstra & Klappe, 1986; Nir *et al.*, 1986). At the same time we could observe an increase in the amount of p27 that was internalized (trypsin-resistant), in the case of H9 and Sup-T1 cells. The amounts of p27 internalized increased with the incubation time at 37 °C. In the case of A3.01, Raji and K562 cells, however, a very low proportion of the total p27 amount that was added became internalized. This indicates that the virus is unable to enter these cells efficiently by directly fusing either with the plasma membranes or with endosomes following endocytosis.

Discussion

Enveloped viruses introduce their genetic material into host cells by binding to and subsequently fusing with cellular membranes (Hoekstra, 1990; Marsh & Helenius, 1989). Virus–cell fusion is a complex event involving several viral and cellular molecules (Marsh & Helenius, 1989; Stegmann *et al.*, 1989; Hoekstra, 1990). The binding of HIV and SIV to CD4⁺ cells is thought to be mediated by gp120–CD4 interactions (Dalgleish *et al.*, 1984; Kannagi *et al.*, 1985; Klatzmann *et al.*, 1984; McDougal *et al.*, 1985). Several CD4⁻ cells, however, can be infected by HIV-1 (Dalgleish *et al.*, 1984; Kannagi *et al.*, 1985; Klatzmann *et al.*, 1984; McDougal *et al.*, 1985), and alternative pathways of HIV-1 entry not involving the CD4 molecule have been proposed (Tateno *et al.*, 1989; Harouse *et al.*, 1991 *a*, *b*). Although the CD4 molecule is an important factor in SIV infection, several CD4⁺ cells are not capable of being infected *in vitro* by $SIV_{mac}251$ and SIV_{Mne} (Agy *et al.*, 1991; Hoxie *et al.*, 1988; Koenig *et al.*, 1989). This implies that in addition to CD4, the expression of one or more, as yet unidentified, cellular surface molecules could be required for SIV infection (Hoxie *et al.*, 1988; Koenig *et al.*, 1989). These molecules could be involved in the binding, membrane fusion or the immediately subsequent events that lead to entry of the viral nucleocapsid into the cytoplasm. To elucidate the extent of the interaction of $SIV_{mac}251$ with non-infectible cells, we examined virus–cell binding and fusion, intracellular entry of viral core proteins and productive infection of three human and two non-human CD4⁺ and two human CD4⁻ cell lines, which have widely differing capacities for infection by $SIV_{mac}251$.

The amount of SIV_{mac} bound to the cell surface at 4 °C was determined by quantifying the amount of either R_{18} or p27 associated with the cells and remaining in the cell supernatant. Both assays gave similar results (data not shown). The fact that, at any SIV_{mac}/cell ratio, more virus is bound on the surface of Sup-T1 cells (100% CD4⁺ by flow cytometry) than on the surface of H9 cells (55 to 65% CD4⁺) (Fig. 1) suggests the involvement of CD4 in the binding of SIV_{mac} to the surface of these CD4⁺ cells. The virus also bound to the surface of CD4⁻ cells (Raji and K562). It appears that the binding sites on the CD4⁻ cells (Raji) are saturated at low concentrations of virus, and that saturation of the binding site(s) on CD4⁺ cells requires higher amounts of virus. It is

possible, although unlikely, that SIV_{mac} binds to a small number of $CD4^+$ cells present in the Raji and K562 (Table 3) population, this being undetectable by flow cytometric analysis, or that the virus uses alternative binding sites on the surface of these $CD4^-$ cells. Additionally, SIV_{mac} may bind to $CD4^-$ cell surfaces via non-specific interactions, under the binding conditions used. It has been demonstrated previously that $HIV\text{-}1_{SF33}$ can also associate strongly with the surface of $CD4^-$ cells, including Raji cells (Tang & Levy, 1991). Anti-Leu3a antibodies partially inhibited the binding and fusion of the viral envelope with the plasma membrane of H9 cells (Table 2) but had no effect on the binding of SIV_{mac} to Raji cells and slightly promoted the extent of R_{18}-dequenching in this latter case. Under identical conditions, 70% inhibition was found in the case of HIV-1 and H9 cells (L. Stamatatos *et al.*, unpublished). It is possible that SIV_{mac}–cell binding occurs through domains on the cell surface that are composed of cellular membrane lipids, as was reported for HIV-1–cell binding (Harouse *et al.*, 1991*a*, *b*; Yahi *et al.*, 1992). Binding achieved through such lipid domains could result in lipid mixing, once the temperature is increased to 37 °C, and be responsible for the lipid mixing found with the $CD4^-$ cells.

The extent of lipid mixing, as measured by the R_{18}-dequenching assay, did not correlate with the percentage of $CD4^+$ cells present in the cell culture (Fig. 2). The membrane of SIV_{mac} could mix with the membranes of both $CD4^+$ and $CD4^-$ human cells. It was demonstrated previously, using the same fluorescence technique, that $HIV\text{-}1_{N1T\text{-}E}$ could fuse with the plasma membrane of human-derived $CD4^+$ T cell lines and with $CD4^-$ brain-derived cells, but not with a gibbon $CD4^-$ T lymphocyte line (Sinangil *et al.*, 1988; Volsky, 1990). Our results with the EL-4 and Mv-1-lu cells indicate that no appreciable membrane lipid mixing occurred with the membranes of these non-human cells. Thus, membrane lipid mixing was essentially confined to the human cell lines tested in this study. This observation suggests that certain, as yet undetermined, components of human cell membranes are likely to be involved in sustaining the fusion of the SIV_{mac} envelope.

The extent of SIV_{mac}–cell lipid mixing was independent of the pH of the medium (Fig. 4). In this respect the pH profiles of SIV_{mac}– and HIV-1–cell membrane fusion are similar (Sinangil *et al.*, 1988). Protonation of the viral envelope proteins does not seem to be required for fusion of SIV_{mac} with cell membranes, while the fusion of SIV_{mac} with liposomes containing primarily negatively charged lipids was shown to be enhanced at low pH (Larsen *et al.*, 1990). Our results, however, demonstrate that SIV_{mac} does not lose its overall fusion ability at low pH and it is possible that virions can introduce their nucleocapsid into the cytoplasm following endocytosis or phagocytosis.

The temperature dependence of fusion was shown previously for Sendai virus–erythrocyte ghost fusion (Hoekstra *et al.*, 1989), and for the fusion of respiratory syncytial virus (Srinivasakumar *et al.*, 1991), Rous sarcoma virus (Gilbert *et al.*, 1990) and influenza virus (Düzgüneş *et al.*, 1992) with cultured cells. Here the fusion of SIV_{mac} with both $CD4^+$ and $CD4^-$ cells was shown to be temperature-dependent (Fig. 5). Below 15 °C no R_{18}-dequenching was found. The extent of membrane fusion does not have a linear relationship with increasing temperature. It appears that the extent of fusion increases more rapidly at temperatures above 25 °C.

Soluble CD4 and neutralizing anti-SIV_{mac} sera inhibited SIV_{mac}–cell lipid mixing in a concentration-dependent manner (Tables 2 and 3). The fact that lipid mixing was inhibited with both $CD4^+$ and $CD4^-$ cells suggests that gp120 is involved in the interaction of SIV_{mac} with both cell types. It is known that pretreatment of several laboratory strains of HIV-1 with sCD4 results in the removal of gp120 from the viral surface (Moore *et al.*, 1990). This could also occur with $SIV_{mac}251$ and impair the ability of the virus to bind to the cell surface. Alternatively, the loss of gp120 molecules from the viral surface exposes underlying gp40 molecules that in the absence of target membranes might result in the inactivation of the viral fusion capacity. A similar type of inactivation was reported for influenza virus. Although acidification of the medium is required for activation of the fusion potential of influenza virus, exposure of the N-terminal fusion peptide of haemagglutinin in the absence of target membranes (by pre-incubation of the virus at low pH), results in virus inactivation (Stegmann *et al.*, 1987; Düzgüneş *et al.*, 1992).

As expected from the results of the lipid mixing assay (Fig. 2), incubation of SIV_{mac} with cells at 4 °C did not result in p27 internalization (Table 4). At 37 °C, however, fusion of the viral envelope with the plasma membranes of human cells resulted in efficient entry of p27 into certain $CD4^+$ cells (H9 and Sup-T1) but not into others (A3.01). No significant entry into $CD4^-$ cells was observed. In the cases of H9 and Sup-T1 cells, the percentage of cell-associated p27 (cell-bound and internalized) decreased with time, due to dissociation of a fraction of the cell-bound virus at 37 °C, as reported previously for Sendai virus (Hoekstra & Klappe, 1986). In the case of H9 and Sup-T1 cells, the amount of p27 internalized (determined following cell surface trypsinization) increased with increasing time of incubation at 37 °C, due to fusion of virions with the cells and cytoplasmic entry of the viral nucleocapsid. The fact that extensive lipid mixing takes place between the virus and

all the human cells tested, although p27 enters primarily into the cytoplasm of only two $CD4^+$ cells (Sup-T1 and H9), suggests that CD4 is necessary, but not sufficient, for viral core entry. Additional cell surface molecules may therefore be required for efficient cytoplasmic entry of the nucleocapsid. In the case of A3.01 and the $CD4^-$ human cells, these molecules seem to be absent (or inactive) and virus–cell interactions probably result in the formation of small areas of contact through which mostly lipid molecules can intermix. Alternatively, it is possible that in the case of H9 and Sup-T1 cells, but not in the other cases, SIV_{mac} was internalized by endocytosis, and this would imply that endocytosis was required for infection. Another explanation for the non-entry of the nucleocapsid could be the presence of molecules on the surface of the $CD4^-$ and the A3.01 cells that inhibit viral core entry.

In the case of H9 and Sup-T1 cells, lipid mixing proceeds at a much faster rate than the rate at which the nucleocapsid is internalized (Fig. 2 and Table 3). Similar observations were reported recently with influenza virus–cell fusion. The rate of lipid mixing was much faster than the rate of nucleocapsid entry when the temperature of the reaction was below 37 °C (Lowy *et al.*, 1992). In order for the viral nucleocapsid to enter the cytoplasm several events, such as re-organization of cell surface proteins and of the cytoskeleton, may have to take place and these events may require more time than does lipid mixing. The observed quantitative differences between the p27 entry and the R_{18}-dequenching assays could be due to additional factors. A small proportion of dead cells (between 0 and 10% depending on the experiment) which were not separated from live cells during our experiments could have contributed to R_{18}-dequenching. In addition, some virions could begin to fuse with cell membranes but owing to structural abnormalities in their envelope the fusion process may be incomplete, resulting in lipid mixing but not p27 entry. Electron microscopic observations of HIV-1 fusing with H9 cells have indeed revealed structures which have been interpreted as the fusion of the outer leaflets of the viral and cellular membranes (Grewe *et al.*, 1990).

Although some of the R_{18}-dequenching may be due to transfer (exchange) of the fluorescent probe from the viral envelope to the cell plasma membranes, our control experiments (Table 1) indicate that only a very small fraction (if any) of the R_{18}-dequenching monitored was attributable to 'probe-exchange'. R_{18}-dequenching was observed only when the temperature was raised above 15 °C, although the virus could associate with the cell surface below that temperature. Glutaraldehyde treatment of R_{18}-labelled virus resulted in a drastic reduction of the R_{18}-dequenching, while not inhibiting the binding capacity of the virus for H9 cells. These observations strongly support the interpretation that the R_{18}-dequenching occurring is due to membrane lipid mixing. Similar inhibition of virus–cell membrane fusion upon glutaraldehyde treatment of the virus was previously reported for Rous sarcoma virus (75%; Gilbert *et al.*, 1990) and for respiratory syncytial virus (25%; Srinivasakumar *et al.*, 1991). No R_{18}-dequenching was observed when R_{18}-labelled liposomes with a lipid composition similar to that of the viral envelope were incubated with the cells. The observation that lipid mixing with two non-human cells expressing the CD4 molecule was minimal (Fig. 2) provides further evidence that the fluorophore does not transfer at a molecular level to just any target membrane. In addition, although p27 entry was very inefficient in the case of the human $CD4^-$ cells, the fact that some p27 was present in the cytoplasm of these cells (Table 4) is evidence for the occurrence of membrane lipid mixing of the viral envelope with the surface of these cells. Others (Potash *et al.*, 1992) have reported that HIV-1–cell membrane fusion does not necessarily result in productive infection of alveolar macrophages. It is possible, therefore, that although virus–cell fusion takes place, entry of the viral nucleocapsid is very limited and does not result in productive infection.

Our infection studies (Fig. 6) confirm the previously reported observations on the different potential for infection by $SIV_{mac}251$ of H9 and A3.01 cells (Hoxie *et al.*, 1988; Koenig *et al.*, 1989). Our finding that Sup-T1 cells are infected by $SIV_{mac}251$, in contrast to that reported by Koenig *et al.*, (1989) is probably due to the use of a more sensitive assay for virus production and to a prolonged (up to 28 days) examination of the cell supernatant for the presence of virus. Our Sup-T1 infection results are in general agreement with those reported by Hoxie *et al.* (1988). The p27 internalization (Table 4) and infection experiments (Fig. 6) demonstrate a correlation between the quantities of viral core proteins (and probably RNA) internalized during fusion and the level of infection (p27 production in the cell culture medium). The faster rate and the higher quantities of p27 internalized in the case of Sup-T1, compared to values for H9 cells, resulted not only in earlier signs of productive infection but also in higher levels of infection (Fig. 6). In the case of the A3.01 and the $CD4^-$ cells, the entry of the nucleocapsid is very inefficient and does not result in productive infection. It may be possible, however, to 'rescue' virus present in very small quantities in these cells, by co-cultivation with H9 or Sup-T1 cells. In addition to the inefficient entry of SIV_{mac} into certain $CD4^+$ and $CD4^-$ cells, cytoplasmic factors may determine the fate of the initially internalized virion nucleocapsid, by either allowing or inhibiting viral replication and productive infection.

Our results also support the hypothesis that other molecules in addition to CD4 are required for efficient entry of SIV$_{mac}$251 into CD4$^+$ cell lines. We believe that these molecules play a role in steps following virus binding, probably during the formation of pores that the nucleocapsid passes through as it enters the cytoplasm. It is important to identify these molecules and elucidate the mechanism by which they participate in virus entry, in order to understand better the mechanisms of retrovirus–cell interactions.

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