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Fusion of simian immunodeficiency virus with liposomes and erythrocyte ghost membranes: effects of lipid composition, pH and calcium

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Simian immunodeficiency virus from macaques (SIV_{mac}) is closely related in its structure and biological activity to human immunodeficiency virus, and is the best animal model for the acquired immunodeficiency syndrome. We investigated the kinetics of membrane fusion between SIV_{mac} and phospholipid vesicles and the effects of various parameters on this process. Purified SIV_{mac} was labelled with octadecyl rhodamine B chloride, and fusion was continuously monitored as the dilution of the probe in target membranes. These studies show that SIV_{mac} fusion is strongly dependent upon the liposome composition. Fusion with pure cardiolipin (CL) liposomes is significantly faster than with CL/dioleoylphosphatidylcholine (DOPC) (3:7), phosphatidylserine (PS) or disialoganglioside (G_{D1a})/DOPC (1·5:8·5) vesicles. SIV_{mac} does not fuse appreciably with pure DOPC liposomes. Reduction of pH from 7·5 to 4·5 greatly enhances the rate of SIV_{mac} fusion with CL, CL/DOPC and PS membranes, but does not affect fusion with DOPC or G_{D1a}/DOPC membranes. Calcium stimulates viral fusion with CL liposomes, but not with CL/DOPC or DOPC liposomes. SIV_{mac} fuses with human erythrocyte ghost membranes only slowly at reduced pH. Our results indicate that SIV_{mac} can fuse with membranes lacking the known viral receptor, CD4. Although the mechanism of SIV_{mac} fusion with model and biological membranes remains to be determined, the fusion activity of SIV_{mac} shares similarities with other lipid-enveloped viruses such as Sendai and influenza viruses.

Introduction

The aetiological agents of AIDS are human immunodeficiency virus 1 (HIV-1) (Barré-Sinoussi *et al.*, 1983; Gallo *et al.*, 1984; Levy *et al.*, 1984) and HIV-2 (Clavel *et al.*, 1986). Simian immunodeficiency virus from macaques (Asian old world monkeys) (SIV_{mac}) causes simian AIDS (Letvin *et al.*, 1985) and is closely related in its genetic sequence to HIV-2 (Chakrabarti *et al.*, 1987) and HIV-1 (Chakrabarti *et al.*, 1987; Desrosiers, 1988). Infection of macaques by SIV_{mac} represents the best animal model for human AIDS. Electron microscopy (Munn *et al.*, 1985; Hockley *et al.*, 1988) and lipid composition (Aloia *et al.*, 1988) studies have revealed that the envelopes of HIV and SIV_{mac} are acquired by budding from the infected cell surface after membrane insertion of virus-encoded proteins.

The infectious route of immunodeficiency virus entry into host cells is unclear. Whether endocytosis of these viruses precedes viral–cell membrane fusion is controversial (Maddon *et al.*, 1986, 1988; Stein *et al.*, 1987; McClure *et al.*, 1988; Pauza & Price, 1988) and, although most reports suggest that HIV infection is not dependent on CD4 internalization or acidification of endocytic vesicles, receptor-mediated endocytosis leading to HIV infection remains a possibility. Whatever the route, there are two essential steps for the delivery of the nucleocapsid of lipid-enveloped viruses into target cells. First, the virus must bind to the cell membrane and, second, viral and cellular membranes must fuse to deliver the viral genetic material into the cytoplasm. Viral envelope proteins are thought to participate in both steps, binding and fusion, of the entry process (White *et al.*, 1983; Ohnishi, 1988; Hoekstra & Kok, 1989). The molecular basis of the fusion step is far less understood than that of the binding step.

The identified cellular receptor for HIV (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984; McDougal *et al.*, 1985) and SIV_{mac} (Kannagi *et al.*, 1985) is the CD4 protein, an antigenic marker most commonly found on circulating helper T cells, monocytes and macrophages. These cells

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appear to be the most common targets of HIV and SIV_{mac}. Although CD4 is the only known cell surface receptor for HIV and SIV_{mac}, it has been suggested that CD4 may not be required for HIV infection of some cell types (Harouse *et al.*, 1989; Tateno *et al.*, 1989; Weber *et al.*, 1989). Others have suggested that CD4 may not be sufficient for HIV (Maddon *et al.*, 1986) or SIV_{mac} (Koenig *et al.*, 1989) infection.

HIV-1 contains two envelope-associated glycoproteins, gp120 and gp41, thought to mediate viral fusion with cellular membranes (Lifson *et al.*, 1986*a*; Sodroski *et al.*, 1986; Kowalski *et al.*, 1987). HIV-2 and SIV_{mac} also contain two related envelope-associated glycoproteins, gp120. The external gp120 binds to the CD4 receptor (McDougal *et al.*, 1986) and the transmembrane protein that ranges in size from 32K (gp32) to 40K (gp40) (Chakrabarti *et al.*, 1987) bears some similarity to the fusion proteins of other lipid-enveloped viruses (Gallaher, 1987; Marsh and Dalgleish, 1987). By analogy to these viruses, the putative role of gp41 (gp32/40) is to disrupt the membranes and possibly act as a molecular bridge for the viral and cellular lipids. Although the CD4 binding site on gp120 (Lasky *et al.*, 1987) and a 'fusion peptide' portion of SIV_{mac} gp32 (Bosch *et al.*, 1989) have been identified, the molecular mechanism of the viral fusion process is unknown.

Most immunodeficiency virus entry studies published to date are in reality secondary event assays. These assays are rate-dependent on events, such as viral replication and/or antigen production, which take place hours or days after the initial viral entry. For example, cell-associated or cell-free reverse transcriptase levels (Hoffman *et al.*, 1985; Koenig *et al.*, 1989), viral DNA (Stein *et al.*, 1987) or p24 antigen production and syncytium (giant cell) formation (Lifson *et al.*, 1986*b*; Koenig *et al.*, 1989) cannot be detected for several hours after initial HIV exposure, and are often followed for days. Giant cell formation (cell–cell fusion) probably requires the expression of viral proteins on the cell surface (Lifson *et al.*, 1986*a*; Sodroski *et al.*, 1986). These measurements are rate-dependent on viral replication or antigen production, as well as viral entry. Proposed mechanisms of HIV and SIV_{mac} entry (Marsh & Dalgleish, 1987; Gallaher, 1987; Koenig *et al.*, 1989) therefore remain unproven.

Development of a direct SIV_{mac}/HIV fusion assay using fluorescently labelled lipids will allow clear delineation of the viral entry step, differentiated from viral replication or antigen production. The development of such assays for other viruses (for example, Sendai and influenza) has been critical to understanding viral entry mechanisms (Hoekstra *et al.*, 1984; Nir *et al.*, 1986*a*, *b*; Bentz *et al.*, 1988; Düzgüneş & Bentz, 1988; Loyter *et al.*, 1988; Hoekstra & Kok, 1989). One recent report of a quantitative lipid mixing assay for HIV fusion with cells (Sinangil *et al.*, 1988), showed the CD4 dependence of HIV fusion with T lymphocyte cell lines. However, the cell entry mechanism of HIV and SIV_{mac} is still unresolved.

The use of well defined target membranes, such as liposomes and/or erythrocyte ghosts, can be helpful in identifying molecules critical for virus entry. For example, the requirement for cholesterol in Semliki Forest virus fusion with target membranes was shown by the use of liposomes (White & Helenius, 1980; Kielian & Helenius, 1984). Liposomes allow rigorous kinetic characterization of the fusion process (Nir *et al.*, 1986*a*), enable the target composition to be manipulated to identify the dependence of viral fusion on target membrane lipids and incorporated receptors (Hsu *et al.*, 1983; Stegmann *et al.*, 1985, 1989; Klappe *et al.*, 1986), and allow for the direct comparison of the fusion characteristics of different viruses. Here, we investigated the fusion of SIV_{mac} with liposomes of different composition and with erythrocyte ghost membranes, using a fluorescence lipid-mixing assay based on the dequenching of octadecyl rhodamine B chloride (R_{18}) (Hoekstra *et al.*, 1984) to monitor the fusion reaction continuously. A preliminary report of our observations has been presented (Larsen *et al.*, 1989).

Methods

Chemicals. R_{18} was purchased from Molecular Probes. Cardiolipin from bovine heart (CL), dioleoylphosphatidylcholine (DOPC) and phosphatidylserine from bovine brain (PS) were obtained from Avanti Polar-Lipids. Disialoganglioside (G_{D1a}) and $C_{12}E_8$ detergent were from Calbiochem.

Virus and target membrane preparation. All handling of infectious virus or infected cell lines was performed in P-2 facilities in accordance with guidelines recommended by the Centers for Disease Control (1988). SIV_{mac} was grown in the human cell line HuT-78 using RPMI 1640 medium/5% foetal bovine serum in stationary cultures at 37 °C (5% CO_2). The virus and the cell line tested negative for *Mycoplasma* contamination (Gen-Probe) immediately prior to infection. Medium from chronically infected cell cultures was collected twice in 1 week and the pooled harvests were clarified by centrifugation at 1000 *g* for 10 min. The virus was precipitated using an equal volume of cold saturated ammonium sulphate, adjusted to pH 7·4 with Tris base. The precipitate was kept overnight at 4 °C and pelleted at 6000 *g* for 30 min. The pellet was resuspended in 10 mM-Tris–HCl, 1 mM-EDTA, 100 mM-NaCl, pH 7·4 (TEN) buffer, layered onto a 30 to 65% sucrose step gradient and centrifuged at 275000 *g* (4 °C) for 30 min in a Beckman SW41 rotor. The band containing virus was collected at the 30 to 65% interface by bottom puncture, diluted 1:4 with TEN buffer, layered onto a 4 ml 15 to 65% continuous sucrose gradient and centrifuged at 275000 *g* (4 °C) for 60 min. The virus band was collected by bottom puncture, assayed for protein by the BCA method (Smith *et al.*, 1985), diluted to 0·2 mg/ml in sucrose/TEN buffer and stored at −70 °C. Western blot analysis showed that purified SIV_{mac} contained all viral antigens, including gp32 and gp120. Purified virus was infectious as

determined by its ability to induce syncytium formation and cell-free reverse transcriptase activity in HuT-78 cell culture.

HIV-1 (LAV-1) (provided by Dr Myra Jennings, U.C. Davis, Ca., U.S.A.) was cultured, purified and stored under similar conditions (except that initial virus concentration after harvest was obtained by centrifugal pelleting). Purified HIV-1 contained all viral antigens, including gp41 and gp120 (Western blot), and was infectious as determined by its ability to induce syncytium formation and cell-free p24 antigen production (ELISA) in $CD4^+$ A3.01 cell culture.

Liposomes (large unilamellar vesicles) were prepared in 150 mM-NaCl, 10 mM-TES and 10 mM-citrate (pH 7·5) (fusion buffer) using lipids of various compositions by a modification (Düzgüneş *et al.*, 1983) of the reverse phase evaporation procedure (Szoka *et al.*, 1980). Vesicles were extruded three times under argon through polycarbonate membranes of 0·08 µm pore diameter (Poretics) to achieve a uniform size distribution of vesicles (Szoka *et al.*, 1980). Lipid concentration was determined by phosphate assay (Bartlett, 1959).

Erythrocyte ghost membranes were generated by hypotonic lysis, washing and resealing of fresh whole human red blood cells (Dodge *et al.*, 1963). The membranes were stored in fusion buffer.

Virus labelling. A well established fluorescence assay was used to monitor the intermixing of viral and target membranes during fusion, using R_{18} as the fluorescent lipid marker (Hoekstra *et al.*, 1984; Loyter *et al.*, 1988). SIV_{mac} or HIV (100 µg) was labelled with R_{18} at 3 to 5% of the total viral lipid by incubating the purified virus with an ethanolic solution of R_{18} (final ethanol concentration of less than 1%) for 0·5 to 1·0 h at room temperature in the dark. The virus was subsequently kept on ice. Free fluorophore was not present. An excess of added unlabelled virus did not result in redistribution of R_{18} as measured by fluorescence dequenching (data not shown). Labelled virus chromatographed on Sephadex G-75 gave similar results, but resulted in low yields of labelled virus. Previously reported HIV lipid to protein ratios (Aloia *et al.*, 1988) were assumed for determining the amount of R_{18} with which to label the viruses.

R_{18}-labelled virus remained infectious as determined by its ability to induce syncytium formation and cell-free reverse transcriptase activity (SIV_{mac}), or syncytium formation and cell-free p24 antigen production (HIV-1), in $CD4^+$ cell culture. Binding of unlabelled or R_{18}-labelled HIV-1 to CL or DOPC multilamellar vesicles (MLV) was measured after 30 min incubation at 0 °C in fusion buffer. Free virus (supernatant) was separated from MLV (pellet) by ultracentrifugation at 27000 *g* (15 min, 4 °C). A p24 antigen capture assay was used to detect virus in the supernatant and pellet. R_{18} did not alter the binding of HIV-1 to CL or DOPC liposomes (data not shown).

Fusion experiments. The dilution of R_{18} in the target membranes was measured by following the increase of rhodamine fluorescence, which results from the relief of self-quenching upon membrane fusion (Hoekstra *et al.*, 1984). Fluorescence measurements were performed with an SLM-4000 fluorimeter (SLM Instruments) (555 nm excitation, 590 nm low end cut-off filter) linked to a strip chart recorder or a Perkin-Elmer LS-5B fluorimeter (Perkin Elmer) (555 nm excitation, 595 nm emission and a 590 nm low end cut-off filter), linked through a MacAdios analog:digital signal converter using Manager II software (GW Instruments) to a Macintosh SE/30 computer to collect raw data. All experiments were performed at 37 °C in a stirred cuvette. Liposomes were rapidly injected into a temperature-equilibrated cuvette containing labelled virus in fusion buffer set to various pH values and calcium concentrations. The final reaction volume was 1·0 ml (SLM) or 2·0 ml (Perkin Elmer). Complete fusion (100%) was set by addition of 0·1 to 0·5% (final volume) of $C_{12}E_8$ detergent to the reaction mixture to disperse the R_{18} completely. All SIV_{mac} data were collected within 3 months of virus preparation.

Results

Fusion dependence on target membrane composition

Fusion of SIV_{mac} with liposomes was highly dependent on the lipid composition of target membranes (Fig. 1). Fusion at neutral pH was fastest and most extensive with pure CL liposomes. Fusion of the virus with liposomes composed of CL:DOPC (3:7) and pure PS was significantly slower than with pure CL liposomes. The virus did not fuse appreciably with pure DOPC membranes. The preferential fusion of SIV_{mac} with negatively charged liposomes at neutral pH (CL and PS have net negative charges whereas DOPC is neutral) is qualitatively similar to that seen with other lipid-

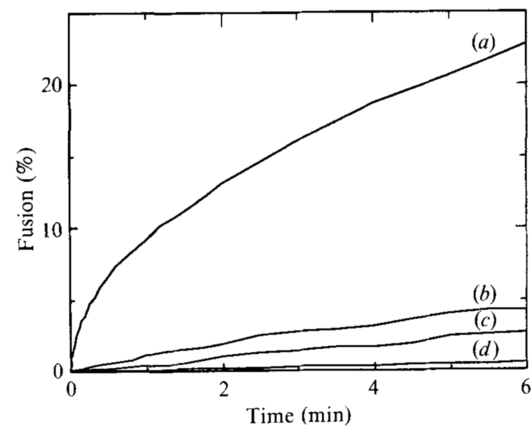

Fig. 1. Kinetics of fusion between SIV_{mac} and liposomes of various compositions. Fusion between R_{18}-labelled SIV_{mac} (3 µg/ml) and liposomes (50 µM lipid) (which corresponds to an approximate 30:1 liposome : virus lipid ratio) was monitored continuously at pH 7·5 as the increase of R_{18} fluorescence due to lipid mixing. At 50 µM liposomes, concentrations of virus down to 0·5 µg/ml (below which light scattering interferes with R_{18} fluorescence) gave essentially the same results. (*a*) CL, (*b*) CL:DOPC (3:7), (*c*) PS, (*d*) DOPC.

Table 1. *Comparison of the initial rate of fusion of SIV_{mac} with target membranes**

Target membrane	pH 7·5	pH 5·0
DOPC	0·3	0·4
Erythrocyte ghost	0·3	1·7
CL:DOPC (3:7)	4·8	16
CL	21	112
CL, 0·5 mM-Ca^{2+}	31	ND†

* Initial fusion rates were calculated from the slopes of fluorescence dequenching per unit time (values are given as % fusion/min). The results for SIV_{mac} fusion with DOPC liposomes and erythrocyte ghosts are averaged over several min. The rates of fusion in these cases did not change appreciably with time. Virus concentration was 1 µg/ml and the liposome concentration was 50 µM lipid. Calcium and pH conditions were set prior to injection of target membranes. For the experiments with a Ca^{2+}, fusion buffer did not contain citrate. Citrate had no effect on fusion. All experiments were performed at 37 °C.

† ND, Results not determined.

enveloped viruses, including Sendai (Klappe *et al.*, 1986) and influenza (Stegmann *et al.*, 1989) viruses. As shown in Table 1, there was little, if any, fusion of SIV_{mac} with erythrocyte ghosts at neutral pH.

Effect of pH on SIV_{mac} fusion

To examine the effects of pH on SIV membrane fusion, we compared the fusion activity of SIV_{mac} with various liposomes in the pH range 4·5 to 7·5 (Fig. 2). SIV_{mac} fusion with CL, CL:DOPC (3:7) or PS liposomes was enhanced by reduced pH, whereas fusion with DOPC liposomes was not enhanced. Although there was a steady increase in fusion activity with the negatively charged liposomes as the pH was lowered, the change between pH 5·0 and 4·5 was the most dramatic.

Sialic acid is the only known essential component of cellular receptors for influenza type A (Wiley & Skehel, 1987) or Sendai (Hoekstra & Kok, 1989) viruses. These viruses fuse with liposomes composed of neutral lipids if the liposomes contain a ganglioside such as G_{D1a}, which contains two sialic acid groups, as a receptor for the virus. SIV_{mac} fusion with DOPC liposomes containing 15 mol% G_{D1a} was similar to its fusion with PS liposomes at pH 7·5, but the rate of fusion did not increase at pH 5·0 (data not shown). These results suggest that G_{D1a} does not act as a receptor for SIV_{mac}. Reduced pH also enhanced the fusion activity of SIV_{mac} toward erythrocyte ghost membranes (Table 1), although the activity was lower than that with all liposome compositions tested except pure DOPC. In contrast to SIV_{mac} fusion with liposomes, where the fusion rate was reduced over time, the initial fusion rate with erythrocyte ghosts at pH 5·0 was maintained over a 10 min period (data not shown).

Effect of pH on HIV-1 fusion

The fusion activity of HIV with negatively charged liposomes was also enhanced by reduced pH. Fig. 3 shows the effect of reduced pH on both SIV_{mac} and HIV-1 fusion with CL liposomes. Although the qualitative effect of reduced pH was the same, HIV was not as dramatically affected by reduced pH as SIV_{mac} in its fusogenic activity. The two viruses may exhibit different pH profiles in their fusion with particular phospholipid membranes. Alternatively, even different strains of one virus (e.g. HIV-1) may exhibit differences of this magnitude. Essentially no fusion was observed between HIV-1 and DOPC liposomes at pH 7·5 or 5·0 (data not shown). The effect of reduced pH on HIV fusion with CL:DOPC (3:7) liposomes was the same as that seen with SIV_{mac} (data not shown).

Calcium effect on SIV_{mac} fusion with pure CL liposomes

Fig. 4(*a*) shows the effect of added calcium ions on SIV_{mac} fusion with CL liposomes. The dependence of SIV_{mac} fusion on calcium concentration is presented in Fig. 4(*b*). Calcium stimulated SIV_{mac} fusion with pure CL but not with DOPC vesicles, in which fusion activity was the same with or without calcium up to 5 mM-Ca^{2+} (data not shown). Fusion of SIV_{mac} with CL:DOPC (3:7) or erythrocyte ghost membranes was not affected by calcium (data not shown). The effect of calcium on the fusion of HIV-1 with these liposomes was similar. Whereas HIV-1 fusion with DOPC or CL:DOPC (3:7) liposomes was unaffected by the presence of calcium (up

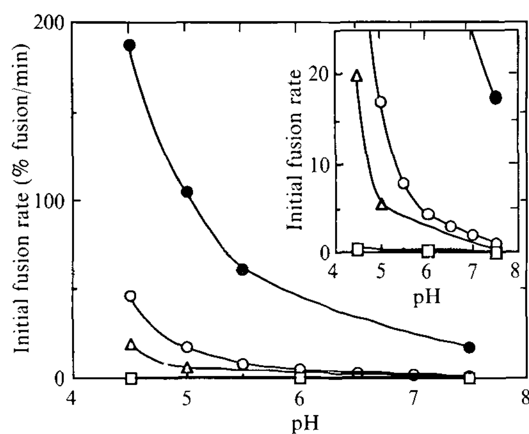

Fig. 2. Effect of pH on the fusion rate of SIV_{mac} with liposomes of various compositions. R_{18}-labelled SIV_{mac} (3 μg/ml) was incubated in fusion buffer at the stated pH values for 2 to 5 min at 37 °C. Liposomes (50 μM lipid) were then injected to initiate the fusion reaction. The initial fusion rates were calculated and plotted as a function of pH. (●), CL; (○) CL:DOPC (3:7); (△), PS; (□), DOPC. Inset: the data plotted on a different scale to highlight the changes at reduced pH.

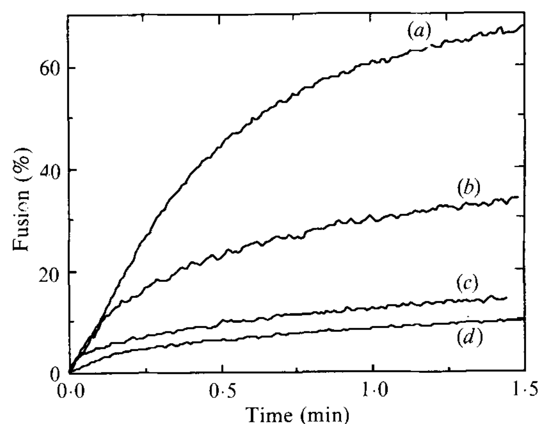

Fig. 3. Comparison of SIV_{mac} and HIV-1 fusion with cardiolipin liposomes. Labelled SIV_{mac} or HIV-1 (1 μg/ml) was temperature-equilibrated (37 °C) in fusion buffer at pH 7·5 or pH 5·0 for 2 to 5 min. Cardiolipin liposomes (50 μM lipid) were injected to initiate the fusion reaction, which was monitored continuously. (*a*) SIV_{mac}, pH 5·0; (*b*) HIV-1, pH 5·0; (*c*) HIV-1, pH 7·5; (*d*) SIV_{mac}, pH 7·5.

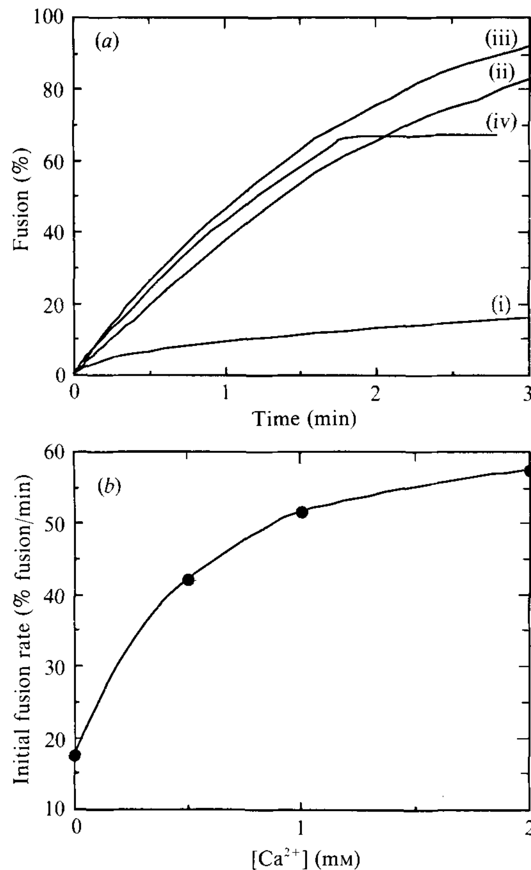

Fig. 4. Enhancement of SIV_{mac} fusion with CL liposomes in the presence of Ca^{2+}. (*a*) R_{18}-labelled SIV_{mac} (3 µg/ml) was incubated in fusion buffer (minus citrate) for 2 to 5 min at 37 °C in the presence of (i) no calcium, (ii) 0·5 mM-Ca^{2+}, (iii) 2·0 mM-Ca^{2+} or (iv) 1 mM-Ca^{2+}. Liposomes (50 µM lipid) were then injected to initiate the fusion reaction. (iv) At 1·7 min after initiating the reaction, EDTA was added from a 400 mM stock (pH 7·5 in TES buffer) to a final concentration of 5 mM. (*b*) The initial fusion rates were calculated and plotted as a function of calcium concentration.

to 0·5 mM), 0·5 mM-Ca^{2+} enhanced the initial fusion rate of HIV-1 with CL liposomes by 1·5- to 2-fold). As with SIV_{mac}, the presence of calcium resulted in a significantly higher degree of HIV-1 fusion after several min.

When a saturating concentration of EDTA was added during the fusion of SIV_{mac} with CL liposomes in the presence of calcium, the reaction stopped (Fig. 4*a*). Calcium may have been stimulating multiple fusion events, causing some otherwise inactive virus particles to become fusogenic, enhancing viral binding to the target membrane, or altering a rate-limiting step in the fusion reaction. Further kinetic studies are required to answer these questions. Calcium (up to 1 mM, the highest concentration tested) and EDTA do not affect influenza virus fusion with pure CL liposomes (Stegmann *et al.*, 1985). This observation suggests that calcium affects the viral envelope and not the target membrane.

Discussion

Fusion mechanisms of even simple model membranes are difficult to delineate and, owing to the biochemical complexity of viral and cellular membranes, the fusion mechanisms utilized by lipid-enveloped viruses to enter their target cells are even less understood. Unlike previously studied lipid-enveloped viruses that can fuse with a large variety of mammalian cells (Poste & Pasternak, 1978; Hoekstra & Kok, 1989), HIV and SIV_{mac} seem to have a restricted host cell range, preferentially infecting CD4-expressing peripheral blood cells (Sattentau & Weiss, 1988). Yet, expression of CD4 on a target membrane is insufficient for infection of the cell (Maddon *et al.*, 1986; Koenig *et al.*, 1989). These facts suggest that the nature of the target membrane plays an unusually critical role during HIV or SIV_{mac} entry. On the other hand, HIV-1 can infect certain CD4-negative neural cells (Harouse *et al.*, 1989) and some other CD4-negative cell types (Tateno *et al.*, 1989), suggesting that this virus may use more than one entry mechanism. Studies of these possibly novel fusion mechanisms should, therefore, offer insights into viral infection generally.

To obtain clear and direct biochemical information regarding this complex process, it has proven useful to study the fusion characteristics of these viruses with pure lipid membranes (often of a simple lipid composition). For example, liposomes were used to show that Semliki Forest virus fusion requires cholesterol in the target membrane (White & Helenius, 1980; Kielian & Helenius, 1984) and acidic pH to induce the fusion reaction (Helenius *et al.*, 1980; White & Helenius, 1980). Stegmann *et al.* (1985) used liposomes to show that the low pH-induced conformational change in the haemagglutinin (HA) protein, in itself, is not sufficient to trigger fusion activity. Doms *et al.* (1985) demonstrated the amphiphilic properties of influenza virus HA at low pH by studying the interaction of the protein with liposomes. Using liposomes, Klappe *et al.* (1986) supported the idea that there is a direct relationship between the ability of viral envelope proteins to penetrate into the target membrane and their ability to trigger fusion.

The fusion of influenza virus with several model membranes (Stegmann *et al.*, 1986, 1989) shows a pH dependence similar to that of its cell–cell fusion activity (White *et al.*, 1981), and may represent a physiological model for *in vivo* influenza virus membrane fusion. Influenza viruses, which fuse *in vivo* with endocytic vesicle membranes at reduced pH (Marsh *et al.*, 1983), optimally fuse with erythrocyte ghost membranes and liposomes containing a receptor at pH 4·5 to 5·5 (Stegmann *et al.*, 1986, 1989). Additionally, Stegmann *et al.* (1985) used pure CL liposomes to confirm earlier work

(White *et al.*, 1982) showing that divalent cations do not play a role during influenza virus fusion.

Of the target membranes tested with influenza virus, only pure CL liposomes show some fusion properties that are different from those observed with erythrocyte ghost membranes (the biological target membrane often considered to be physiologically relevant) (Stegmann *et al.*, 1986, 1989). One difference is that preincubation of influenza virus (X-47 strain) in mildly acidic medium results in the loss of fusogenic activity toward erythrocyte ghost membranes but not toward pure CL membranes (Stegmann *et al.*, 1986). However, recent results in this laboratory show that influenza virus preincubated in mildly acidic medium retains fusogenic activity toward at least two human cell lines (HL-60 and CEM) even though activity is lost toward erythrocyte ghost membranes (N. Düzgüneş, M. Pedroso de Lima, D. Flasher, D. Alford, D. Friend & S. Nir, unpublished results). These observations indicate that pure CL liposomes exhibit some properties of physiological significance as target membranes for influenza virus.

Studies on the fusion of Sendai virus with phospholipid membranes have been more controversial. Sendai virus, which fuses with the cellular plasma membrane at neutral pH, exhibits an optimum rate of fusion with erythrocyte ghost membranes near neutral pH (Hoekstra *et al.*, 1985) but in its fusion with CL and PS liposomes has an optimum pH of about 4·0 (Klappe *et al.*, 1986). Liposome fusion with Sendai virus should be extrapolated to biological systems, but with caution. Our recent studies indicate that the pH profile of the fusion activity of Sendai virus toward human cells is more like that toward CL or PS liposomes than that toward erythrocyte ghost membranes (M. Pedroso de Lima, S. Nir, D. Flasher, K. Klappe, D. Hoekstra & N. Düzgüneş, unpublished results). Such liposomes may provide valuable insights that might be missed using erythrocyte ghost membranes, which are convenient but also somewhat artificial.

Whether the enhanced fusogenic activity of SIV_{mac} and HIV with liposomes at lowered pH is of physiological significance is not proven in the present study. SIV_{mac} does not fuse with erythrocyte ghost membranes at neutral pH in our system but a slow fusogenic activity is seen at pH 5·0. However, a study of HIV fusion with $CD4^+$ cells using the same fluorophore used in the present study indicates that virus entry does not require low pH and is most efficient at pH 7, although membrane fusion also occurs at pH 5 (the lowest pH tested) (Sinangil *et al.*, 1988). Furthermore, most reports suggest that HIV entry occurs at the plasma membrane and is not dependent on acidification of endocytic vesicles. Although we show that both viruses exhibit enhanced fusogenic activity with some membranes at lower pH, reduced pH may not be required for viral entry into cells.

The pH profile of SIV_{mac} fusion with liposomes is generally more like that of Sendai than influenza virus, and Sendai virus fuses with the plasma membrane at neutral pH. The fusion with erythrocyte ghost target membranes, although different from either Sendai or influenza virus, shows a dependence upon reduced pH that is more like that of influenza virus (but not nearly as dramatic). Taken together, these results suggest that SIV_{mac} and HIV exhibit novel pH effects in their fusion activity toward target membranes.

It is unclear at present whether reduced pH enhances the binding capacity and/or the fusogenic properties of the viral membrane proteins. Our working assumption is that protonation of viral proteins is responsible for the low pH enhancement of SIV_{mac} and HIV fusion activity. It is doubtful that the increased fusion activity of SIV_{mac} with negatively charged liposomes is caused by protonation of the acidic lipid head groups of the target membranes. The pK_a of the acidic group in CL is around 4·0 (Boggs, 1980) and 3·6 in PS (Tsui *et al.*, 1986). At pH 4·5 to 5·5, there would not be sufficient change in the surface charge of liposomes containing these lipids to explain the observed enhancement in the fusion rate.

The data also show that HIV and SIV_{mac} exhibit fusogenic activity toward pure CL liposomes that is stimulated by the presence of low levels of calcium ion. This is unlike influenza virus (Stegmann *et al.*, 1985) or Sendai virus (D. Hoekstra, personal communication) fusion with pure CL liposomes. The difference between the dependence on calcium of the HIV and SIV_{mac} fusion activities toward CL liposomes may exist even between different HIV or SIV_{mac} isolates. The results suggest that the calcium effect is on viral envelope constituents.

The present study does not attempt to prove that the lipid compositions investigated represent a physiological model for SIV_{mac} fusion with host cell membranes. It is significant, however, that SIV_{mac} is capable of fusing with target membranes that do not contain CD4. These observations, at the very least, should confirm the finding that CD4-independent cellular entry of SIV_{mac} and HIV occurs (Harouse *et al.*, 1989; Tateno *et al.*, 1989; Weber *et al.*, 1989). The recent observations that activated peripheral blood mononuclear cells stain with anti-CL antibodies (Misra *et al.*, 1989), and that such cells are particularly prone to infection with HIV (Gowda *et al.*, 1989), suggest that SIV_{mac} or HIV may interact with molecules on the cell surface that mimic CL.

We demonstrate here the use of a quantitative lipid-mixing assay to determine which target membrane structures and environmental conditions promote HIV and SIV_{mac} fusion. To investigate some of these, it may

be necessary to create an artificial model system that mimics SIV_{mac} fusion with $CD4^+$ cells by incorporation of the CD4 molecule in DOPC or mixed composition liposomes. If CD4 liposomes show enhanced binding and/or fusion activity, they would improve our understanding of the molecular mechanisms of fusion of SIV_{mac} and HIV with cell membranes. Of course, it will ultimately be necessary to compare fusion activity toward these modified liposomes with the activity toward living human cells. The CD4-containing liposomes may also prove useful as carriers of antiviral or cytotoxic compounds that could be directed preferentially toward free-cell virus or infected cells expressing viral membrane glycoproteins on their surface. The potential of this approach is demonstrated by our recent observations that incubation of HIV-1 with CL liposomes inhibits the production of viral antigen in cultures of A3.01 cells (K. Konopka, B. Davis, C. Larsen, D. Alford, R. Debs & N. Düzgüneş, unpublished results).

In conclusion, we have demonstrated membrane fusion of SIV_{mac} and HIV-1 with liposomes lacking the identified virus receptor, CD4. SIV_{mac} fusion with human erythrocyte ghost membranes lacking CD4 also occurs but only slowly and at reduced pH. Viral fusion activity is dependent upon target liposome composition, pH of the reaction mixture and, with pure CL liposomes, calcium ion concentration. Using the R_{18} fusion assay, we have shown that SIV_{mac} and HIV-1 have qualitatively similar fusion activities toward artificial membranes. This assay system can be used to define the kinetics of SIV_{mac} and HIV fusion with model membranes, to compare fusion activity with liposomes containing putative receptors and with biological membranes, to investigate the novel calcium stimulation observed with CL liposomes and to test potential inhibitors of immunodeficiency virus fusion and infectious cell entry.

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