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Human immunodeficiency virus type 1 (HIV-1) fusion with model membranes: kinetic analysis and the role of lipid composition, pH and divalent cations

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The kinetics and extent of HIV-1 fusion with model membranes was studied. HIV-1 was labeled with octadecyl rhodamine B chloride, and fusion was monitored continuously as the dilution of the probe into target membranes. The results were analyzed by a mass action model which yielded good simulations and predictions for the kinetics and final extents of fluorescence increase. The model determined the percents of virions capable of fusing and rate constants of fusion, aggregation and dissociation. Ultrastructural analysis of the virus and reaction products by electron microscopy also provided evidence of HIV-1 fusion with membranes lacking CD4. HIV-1 fusion activity depends on the target membrane lipid composition according to the sequence: cardiolipin (CL) >> phosphatidylinositol > CL/dioleoylphosphatidylcholine (DOPC) (3 : 7), phosphatidic acid > phosphatidylserine (PS), PS/cholesterol (2 : 1) > PS/PC (1 : 1), PS/phosphatidylethanolamine (1 : 1) > DOPC, erythrocyte ghosts. Reduction of pH from 7.5 generally enhances the rate and extent of HIV-1 fusion. Physiologically relevant concentrations of calcium stimulate HIV-1 fusion with several liposome compositions and with erythrocyte ghost membranes. The fusion products of HIV-1 with liposomes consist of a single virus and several liposomes. The mass action analysis revealed that, compared to intact virions, the fusion products show a striking reduction in the fusion rate constant. Like influenza and Sendai viruses, HIV-1 fusion with membranes containing its own envelope glycoprotein(s) is strongly inhibited. Unlike these viruses, HIV-1 fusion is promoted by physiological levels of calcium. HIV-1 fusion with liposomes is qualitatively similar to simian immunodeficiency virus fusion.

Introduction

Several lines of evidence have shown that CD4 is the high-affinity receptor for the HIV envelope glycoprotein gp120 [1]. The primary target cells for HIV in vivo, helper/inducer T lymphocytes [2,3], the monocyte/macrophages [4] and microglial cells of the brain [5], all show a CD4-dependence for infection. In a study employing a fusion assay for lipid mixing, HIV only fused with human cells expressing CD4 [6]. How-

ever, CD4 is not required for HIV infection of some cell types [7–12], and HIV-1 infection has been detected upon autopsy in human tissues which do not normally contain CD4-positive cells [12–15]. Alternative receptor mechanisms have been proclaimed for certain special circumstances [11,16], but there is no identified alternative high-affinity binding molecule for most susceptible CD4-negative cells. In our laboratory, studies with model membranes have suggested that no specific protein or lipid molecule in the target membrane is required for the fusion of immunodeficiency viruses [17–19].

There are two essential steps for the delivery of the nucleocapsid of lipid enveloped viruses into target cells [20–23]. The virus must first bind to the cell membrane, and then the viral and cellular membranes must fuse. Viral envelope proteins are thought to participate

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in both of these steps, binding and fusion [24–26]. The molecular basis for the fusion step is far less understood than for the binding step.

Most immunodeficiency virus “entry” studies have used assays that measure infection. Infection is a more complicated process, however, and such 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 [26–28]. Cell-cell fusion can be assayed directly, by mixing cells expressing the viral envelope glycoprotein complex, gp120/gp41, on their cell surface with target cells that express CD4 [29,30]. Although it is commonly assumed that HIV-1 entry and cell-cell fusion utilize a similar fusion mechanism, this has not been proven [26]. Several factors that inhibit cell-cell fusion have been found not to affect virus entry [31,32], and the reverse is also true [26].

Here, we used a direct fusion assay and electron microscopy to investigate whether HIV-1 virions could fuse with artificial membranes lacking the CD4 receptor. We monitored quantitatively fusion between HIV-1 and liposomes or erythrocyte ghost membranes, using a fluorescence assay for lipid-mixing. We determined the effects of environmental parameters on this process, and analyzed the kinetics and extents of HIV-1 fusion with liposomes of several compositions using a mass action model. The results of these studies were used to compare, both qualitatively and quantitatively, characteristics of HIV-1 fusion with those of other well characterized enveloped viruses.

Materials and Methods

Chemicals

Octadecyl rhodamine B chloride (R_{18}) was purchased from Molecular Probes (Eugene, OR). Cardiolipin from bovine heart (CL), dioleoylphosphatidylcholine (DOPC), phosphatidylinositol (PI), phosphatidic acid (PA), *trans*-phosphatidylated phosphatidylethanolamine (PE), egg PC, egg PE and bovine brain phosphatidylserine (PS) were obtained from Avanti Polar-Lipids, (Alabaster, AL). Cholesterol and octaethylene glycol dodecyl ether detergent were from Calbiochem (La Jolla, CA). Trypsin and soybean trypsin inhibitor were purchased from Sigma (St. Louis, MO).

Virus and target membrane preparation

All handling of infectious virus or infected cell lines was performed in BSL-2 and BSL-3 facilities in accordance with guidelines recommended by the Centers for Disease Control [33]. HIV-1 (LAV-1/BRU) was obtained from Dr. F. Barré-Sinoussi (Pasteur Institute, Paris) and propagated in the human cell line CEM (Pasteur Institute, Paris). All virus-producing cell lines tested negative for *Mycoplasma* contamination, using the Gen-Probe Mycoplasma Detection System (Gen-

Probe, San Diego, CA). Culture supernatants were clarified at $2000 \times g$ at 4°C for 0.5 h to remove cell debris. The virions were concentrated by pelleting at $100\,000 \times g$ at 4°C for 2 h. Viral pellets were gently resuspended in 10 mM Tris/100 mM NaCl, pH 7.4 (TN buffer), layered onto a continuous 20–50% sucrose gradient and centrifuged at $200\,000 \times g$ (4°C) for 3 h. Fractions having peak reverse transcriptase activity were diluted in TN buffer and centrifuged at $150\,000 \times g$ at 4°C for 1.25 h. The viral pellets were resuspended in TN buffer, assayed for protein by the Bradford [34] method, adjusted to a final concentration of 1 mg/ml and stored at -70°C . Western blot analysis showed that purified HIV-1 contained all viral antigens, including gp41 and gp120. Purified virus was infectious as determined by its ability to induce syncytium formation and cell-free p24 antigen production assayed by ELISA in CD4^+ A3.01 cell culture [35,36]. Electron microscopy showed a relatively homogeneous population (with respect to size and overall shape) of intact virions (data not shown).

Liposomes (large unilamellar vesicles) were prepared in 150 mM NaCl with 10 mM *N*-(tris(hydroxymethyl)methyl)-2-aminoethanesulfonic acid (Tes) (“fusion buffer”) containing 0.1 mM EDTA (pH 7.5), using lipids of various compositions, by a modification of the reverse phase evaporation procedure [37,38]. For lipid-mixing experiments, vesicles were extruded three times through polycarbonate membranes of $0.08\text{-}\mu\text{m}$ pore diameter (Poretics, Livermore, CA) to yield vesicles with an average diameter of 100 nm [37]. For electron microscopy, liposomes were extruded through $0.2\text{ }\mu\text{m}$ membranes. Lipid concentration was determined by phosphate assay [39].

Erythrocyte ghost membranes were generated by hypotonic lysis, washing and re-sealing of fresh whole human red blood cells [40]. Membrane concentration was quantified by protein assay [41]. The membranes were stored in fusion buffer at 4°C prior to use.

Labeling of virus with R_{18}

A previously described lipid-mixing assay, using R_{18} as the fluorescent lipid marker, was used to monitor membrane fusion directly and continuously [17,42,43]. Previously reported HIV lipid to protein ratios [44] were assumed for determining the amount of R_{18} with which to label the virus. HIV-1 (100 μg) was labeled 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 0.5–1.0 h at room temperature, and then maintained at 4°C . Labeled virus chromatographed on Sephadex G-75 gave similar results of labeling levels and fluorescence dequenching during fusion experiments, but this method resulted in low virus recovery. Free fluorophore was not present (as monomer or in micellar

form): in both procedures, the addition of unlabeled virus in excess did not result in redistribution of R₁₈ as measured by fluorescence dequenching. R₁₈-labeled virus remained infectious as determined by its ability to induce syncytium formation and cell-free p24 antigen production in CD4⁺ cell culture (data not shown). R₁₈ did not affect the binding of HIV-1 to CL or DOPC liposomes [17].

Fluorescence measurements

Membrane fusion was monitored by following the increase of rhodamine fluorescence, which results from the relief of self-quenching upon the dilution of R₁₈ into the target membranes [42]. Fluorescence measurements were performed with a Perkin-Elmer LS-5B fluorometer (Perkin-Elmer, Beaconsfield, UK) (555 nm excitation, 595 nm emission) linked through a Mac-Adios analog:digital converter using Manager II software (GW Instruments, Somerville, MA) to a Macintosh SE/30 computer. Unless stated otherwise, labeled virus (0.75 µg/ml viral protein, which corresponds to 0.36 µM viral lipid) was pre-equilibrated to 37°C in fusion buffer with or without additional factors. Target membranes (liposomes or ghosts) were injected into a stirred cuvette containing the virus. The initial fluorescence of the virus and vesicle suspension was set to 0% fluorescence. Complete fusion (100% fluorescence) was set by addition of 0.1–0.5% (final volume) of octaethylene glycol dodecyl ether detergent to the reaction mixture to disperse the R₁₈ completely. R₁₈ in the virion membrane was self-quenched at 82% on average (range = 74% to 89%) as determined by the method of Hoekstra et al. [42]. The initial fusion rates were calculated from the slopes of fluorescence dequenching per min. Slopes of less than 5%/min were averaged over the first minute (in these cases, the rates of fusion did not change appreciably with time in the first minute). The others were averaged over the first 0.2 min.

Electron microscopy (EM)

Liposomes were mixed with HIV-1 at a 4:1 (target/virus) lipid ratio at 37°C (pH 7.5). DOPC liposomes were incubated with virus for 5 min (negative stain samples) or 4 h (freeze-fracture samples). CL liposomes were incubated with virus for 5 min. The mixtures were then fixed with 1% paraformaldehyde for 1 h at 37°C (to inactivate HIV-1, as required by BSL-3 protocol). Negative staining was with 2% ammonium molybdate [45]. For freeze-fracture, one part of glycerol was added to one part of the fixed virus-liposome mixture. A small aliquot of this suspension was frozen by liquid nitrogen slush and fractured using standard techniques in a JEOL FTD 9000. The negative stain preparation and freeze-fracture replica were examined in a JEOL CX100 electron microscope.

Trypsinization

R₁₈-labeled HIV-1 was trypsinized in fusion buffer (pH 7.5 ± 5 mM Ca²⁺) at a trypsin:virus protein ratio of 1:1. Trypsinization was allowed to proceed for 1 h at 37°C. Soybean trypsin inhibitor was then added, at a concentration (inhibitor/trypsin = 1:2) known to inhibit trypsin completely, before lipid mixing was measured. Control experiments were also performed, in which the inhibitor was mixed with trypsin prior to addition to the virus.

Theoretical analysis

Final extents of fusion and fluorescence increase. The method of analysis has been previously described in detail [20,46,47]. Briefly, we first established that the fusion products of HIV-1 and liposomes consist of a single virus and any number of liposomes (see Results, 'A kinetic model can predict the final extents of HIV-1 fusion with liposomes' and Fig. 6), as was previously found for the cases of influenza [47] and Sendai virus [46,48]. Consequently, only the equations corresponding to fusion products consisting of a single virus and any number of liposomes are presented (see Ref. 47 for other cases).

The analysis assumed that a fraction, $0 < \alpha < 1$, of virus particles would not fuse with the particular liposomes at the given pH, whereas all liposomes could fuse unless they were bound irreversibly to fusion-inactive virus particles [46]. We denote the initial molar concentrations of liposomes and virus particles by L_0 and V_0 , and the concentrations of fused particles are denoted by L_f and V_f , respectively. Particle concentrations were determined from lipid concentration and the particle diameter. The average virion diameter was assumed to be 130 nm [49]. In the case of reversible binding, $L_f = L_0$ and

$$V_f = V_0(1 - \alpha)\{1 - \exp(-L_0/[V_0(1 - \alpha)])\} \quad (1)$$

In the case of irreversible binding, $L_f = (1 - \alpha)L_0$ and

$$V_f = V_0(1 - \alpha)[1 - \exp(-L_0/V_0)] \quad (2)$$

The increase in fluorescence intensity was calculated by considering it as the dilution of the probe following membrane fusion [46]. Calculations showed that there was little or no probe transfer (Table III).

Kinetics of fusion. The overall fusion process is viewed as a sequence of two kinetic steps, aggregation, which is of second order in particle concentration, followed by membrane destabilization and merging, which is a first-order process. There was no aggregation or fusion between liposomes or between virus particles.

The equations and programs used for determining rate constants have been described previously

[20,46,47]. The concentrations of liposomes were varied in certain cases by two orders of magnitude. In those ideal cases, we could first determine accurately the aggregation rate constant, C (in units of $M^{-1}s^{-1}$), from the results with relatively low liposome concentrations (where aggregation is rate limiting in the overall fusion process). In such cases, the results are relatively insensitive to the value of the fusion rate constant, f (in units of s^{-1}), provided that $f \gg C \cdot L_0$. Next, the fusion rate constant was determined from the initial time points of the concentrated suspensions, at $I \leq 10\text{--}15\%$, where I denotes the percentage fluorescence intensity increase. The rate constant of disaggregation, or dissociation, D (in units of s^{-1}), was determined from the results at later stages of the fusion reaction.

Results

HIV-1 fusion depends on target lipid composition

Fig. 1 shows the time-course of fusion (fluorescence dequenching) of R_{18} -labeled HIV-1 with liposomes of six different compositions, monitored continuously in pH 7.5 buffer at 37°C. The initial rate of HIV-1 fusion with liposomes was dependent on the lipid composition of target membranes, although the virus fused with all liposome compositions tested. HIV-1 fusion with negatively charged liposomes was significantly faster than with neutral liposomes. The sequence of fusion activity with respect to the lipid composition of the target liposome was CL \gg PI $>$ CL/DOPC (3:7), PA $>$ PS, PS/cholesterol (2:1) $>$ PS/PE (1:1), PS/PC (1:1) $>$ DOPC (Table I). Since HIV-1 fusion with PS/cholesterol (2:1) liposomes was similar to that with pure PS liposomes (Table I), the presence of cholesterol in the target membrane had only a minor effect on HIV-1 fusion.

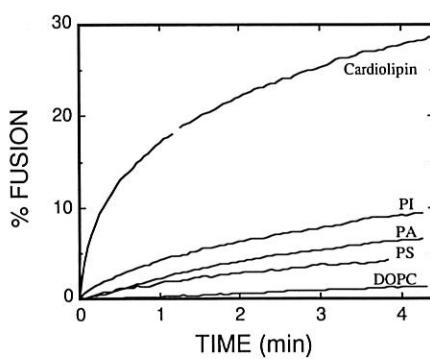


Fig. 1. Time-course of fusion between HIV-1 and liposomes of various compositions. Fusion between R_{18} -labeled HIV-1 and liposomes (50 μM lipid) was monitored continuously at pH 7.5 as the percent increase of R_{18} fluorescence (dequenching) due to lipid mixing. The kinetics of HIV-1 fusion with CL/DOPC (3:7) (data not shown) were essentially identical to those with PA.

TABLE I

Comparison of the initial rates of fusion of HIV-1 with target membranes

Target membrane	pH 7.5	pH 5.0	0.5 mM Ca^{2+}	0.5 mM Mg^{2+}
			(pH 7.5)	(pH 7.5)
CL	25	76	73	48
CL/DOPC (3:7) ^a	2.4	19	2.2	n.d. ^b
PS	1.3	16	7.8	2.1
PS/cholesterol (2:1)	1.0	12	5.1	1.5
PS/PC (1:1)	0.6	3.3	n.d.	n.d.
PS/PE (1:1)	0.8	9.3	n.d.	n.d.
PI	2.9	22	3.7	3.0
PA	2.1	27	n.a. ^c	n.a.
DOPC	0.1–1.3	0.1–1.3	1.0	n.d.
Erythrocyte ghosts ^d	0.2	0.4	2.7	n.d.

^a A different lot of HIV-1 was used for these studies. Although there were slight quantitative differences between the two lots, they behaved qualitatively the same for similar targets. Virus concentration for these experiments was 1 $\mu g/ml$.

^b n.d. refers to results not determined.

^c n.a. refers to non-applicable results. PA liposomes self-fuse above 0.2 mM calcium or 0.4 mM magnesium ion.

^d HIV-1 concentration was 2.5 $\mu g/ml$ protein. Ghost concentration was 80 $\mu g/ml$ protein.

Acidic conditions enhance HIV-1 fusion with negatively charged liposomes

We previously showed that reduced pH enhances SIV_{mac} fusion with acidic liposomes, and the fusion activity of HIV-1 with liposomes containing CL is greater at pH 5 than at pH 7.5 [17]. To study the effects of pH on HIV-1 membrane fusion more extensively, we compared the fusion activity of HIV-1 with various liposomes in the pH range 4.0 to 7.5 (Fig. 2). HIV-1 fusion with all acidic liposomes was enhanced by reduced pH, but there was essentially no effect on the initial rate of fusion with DOPC liposomes. The rate of fusion increased as the pH was lowered in this

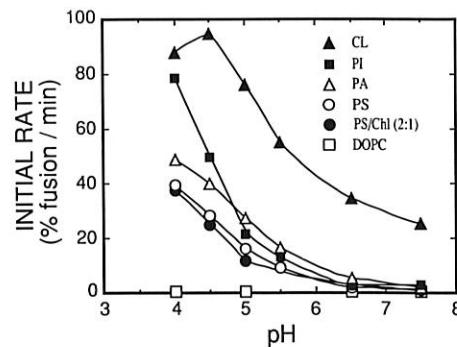


Fig. 2. Effect of pH on the fusion rate of HIV-1 with liposomes of various compositions. R_{18} -labeled HIV-1 was pre-equilibrated in fusion buffer containing 10 mM citrate at the stated pH values. (Citrate had no effect on the fusion rate as compared either with acetate (or the absence of a second buffer at pH 7.5).) Liposomes (50 μM) were then injected.

range, except that fusion with pure CL reached a maximum around pH 4.5. An analysis of the HIV-1 kinetic rate constants at different pH values is given below.

Divalent cations affect HIV-1 fusion with liposomes

Fig. 3A shows the effect of added Ca^{2+} and Mg^{2+} on HIV-1 fusion with CL vesicles. Fig. 3B shows the concentration-dependence of this effect on the rate of fusion with pure CL liposomes. Ca^{2+} or Mg^{2+} (0.5 mM) stimulated HIV-1 fusion with pure CL, pure PS and PS/cholesterol liposomes but not with DOPC liposomes (Table I). HIV-1 fusion with pure PS liposomes showed a Ca^{2+} concentration dependence similar to the HIV-CL fusion reaction (data not shown). At 0.5 mM, Ca^{2+} did not enhance HIV-1 fusion with CL/DOPC (3:7) liposomes, but did enhance fusion with other anionic liposomes (Table I). Although the initial rate of fusion with PI liposomes did not increase much at 0.5 mM Ca^{2+} , the effect on the extent of fusion at later time points was more obvious, especially at higher Ca^{2+} concentrations (1–2 mM). For all liposome compositions studied, the effect of Ca^{2+} was greater than that of Mg^{2+} .

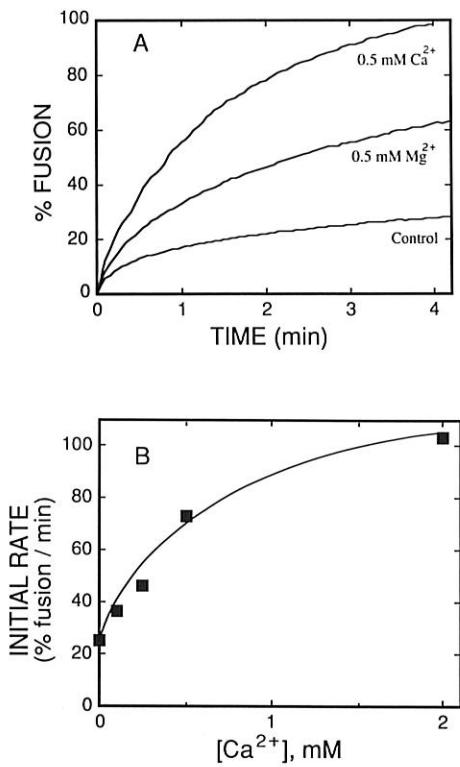


Fig. 3. Enhancement of HIV-1 fusion with CL liposomes by divalent cations at pH 7.5. (A) R_{18} -labeled HIV-1 was pre-equilibrated in the presence or absence (control) of 0.5 mM Ca^{2+} or Mg^{2+} . CL liposomes (50 μM) were then injected to initiate the fusion reaction. (B) The initial fusion rates of HIV-1 fusion with CL liposomes were plotted as a function of calcium concentration.

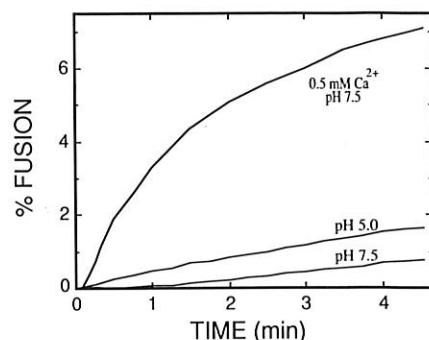


Fig. 4. Time-course of fusion between HIV-1 and erythrocyte ghost membranes. R_{18} -labeled HIV-1 (2.5 $\mu\text{g}/\text{ml}$ protein) was pre-equilibrated under several conditions. Erythrocyte ghosts (80 $\mu\text{g}/\text{ml}$ protein) were then injected.

HIV-1 fusion with erythrocyte ghost membranes

The erythrocyte ghost membrane has often been used as a standardized biological membrane target to test the fusion activity of lipid enveloped viruses [20,25,42,50,51]. Red blood cell ghosts lack the CD4 antigen, and were therefore used to study CD4-independent HIV-1 fusion activity with a biological membrane. Fig. 4 shows the fusion of HIV-1 with these membranes (see also Table I). The initial fusion rate of HIV-1 with erythrocyte ghosts, although slow, was at least as fast as with DOPC liposomes. Reduced pH had only a slight effect on fusion, whereas 0.5 mM Ca^{2+} dramatically increased HIV-1 fusion with the ghost membranes.

HIV-1 fusion with pure CL liposomes depends on intact viral envelope proteins

To ascertain the role of viral envelope proteins in fusion with CL liposomes, we demonstrated previously that HIV-1 fusion with CL liposomes can be blocked by recombinant soluble CD4 [19]. To test further the involvement of the envelope proteins in the fusogenic activity of HIV-1 toward CL liposomes, we examined whether fusion could be blocked by the removal of its envelope glycoproteins. For this purpose, the virus was trypsinized in the presence or absence of calcium (Fig. 5).

In both cases, trypsinization inhibited HIV-1 fusion with CL liposomes at pH 7.5 in the presence of 0.25 mM Ca^{2+} , but fusion was inhibited to a greater extent when the virus was trypsinized in the presence of Ca^{2+} . The initial rate of fusion was reduced by 86% and 79%, relative to the control experiment, for trypsinization in the presence or absence of Ca^{2+} , respectively. The presence of inactive trypsin (i.e., trypsin complexed with trypsin inhibitor) did not significantly inhibit fusion, e.g., by coating the virus or target.

Clearly, binding and fusion of HIV-1 with CL liposomes was not blocked completely by prior viral trypsinization. This was not unexpected. Similar results

have been obtained with influenza [52] and Sendai [48] virus fusion with liposomes. Western blot analysis showed that intact gp120 and gp41 were not present after trypsin treatment in the presence of calcium. However, a small amount of intact gp120 and gp41 were retained when HIV-1 was trypsinized in the absence of calcium (data not shown).

A kinetic model can predict the final extents of HIV-1 fusion with liposomes

Final extents of HIV-1 fusion (0.1 to 6 h) with several liposome compositions were determined at different virus:target vesicle ratios by varying the liposome concentration (at 0.75 $\mu\text{g}/\text{ml}$ HIV protein, which corresponds to 0.36 μM viral lipid) (Table II, Exptl.). For all of the liposome compositions studied, the calculated values could explain and yield predictions of final extents for a wide range of liposome concentrations

only by assuming that the fusion products consist of a single virus and any number of liposomes (calculations for other hypothetical situations are not shown).

That the fusion products consist of a single virus and any number of liposomes was confirmed for CL by another test (Fig. 6). Here, unlike the experiments described above (e.g., Figs. 1–3, where the liposomes were in excess compared to virus), liposomes and virions were incubated at a virus/lipid ratio of 1:1 at pH 7.5, until fusion was complete, i.e. until the fluorescence reached a plateau. At this stage the fluorescence was not completely quenched. When unlabeled virus was added to the system after the plateau had been reached, there was no further increase in fluorescence intensity. This indicates that virus particles cannot fuse with the fusion products. However, when more CL liposomes were added, the fluorescence showed a significant increase beyond the plateau. The initial fusion

TABLE II
Experimental and calculated values of final extents of fluorescence intensity

Target membrane	Liposomal lipid (μM)	Medium	Exptl. % max. fluorescence	Calculated % fluorescence ^a	
				irreversible binding	avg rev. & irrev. binding
CL	0.188	pH 7.5	22		(70) ^b
	0.36		28		22
	0.375		41		35
	0.75		45		36
	3.75		59		48
	18.8		69		64
CL	0.188	pH 5	23		69
	0.36		33		70
	0.75		68		70
	50		100		70
					(100) ^c
CL	0.375	0.25 mM Ca^{2+} (pH 7.5)	48		28
	50		100		45
					66
PS/PC (1:1)	0.75	pH 7.5	34		100
	3.75		44		100
PS/PE (1:1)	0.75	pH 7.5	31		(50)
	3.75		36		33
					46
DOPC	0.75	pH 7.5	31		(40)
	50		36		27
					36
DOPC	0.75	pH 5	26		(50)
	50		58		33
					50
					(60)
DOPC	0.75	pH 5	34		40
	50		64		60

^a Calculated values are based on an assumption of the percentage of virus that is active given in parentheses above each category.

^b The predicted values are different for reversible (rev.) and irreversible (irrev.) binding in this case, but the average of the two predictions most closely resembles the experimental values.

^c When all virus particles are active, Eqn. 1 reduces to Eqn. 2, and the type of binding is irrelevant for the final extents.

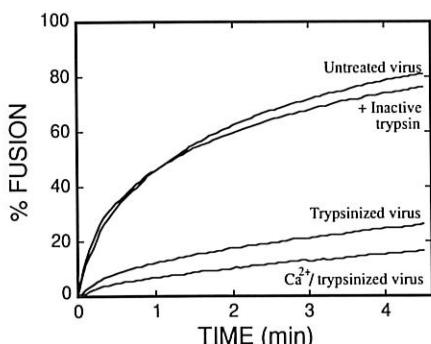


Fig. 5. Inhibition of HIV-1 fusion with CL liposomes by prior trypsinization of intact virus. Prior to fusion experiments, virus was pre-incubated at 37°C for 1 h with or without trypsin. For trypsinized virus, the protease (in fusion buffer) was added to the labeled HIV-1 in the presence or absence of 5 mM Ca^{2+} (pH 7.5; trypsin/viral protein = 1:1, w/w). Trypsin inhibitor (at an inhibitor/trypsin ratio of 1:2, twice the concentration of inhibitor required to inactivate trypsin) was then added before measuring fluorescence dequenching at pH 7.5 with 0.25 mM Ca^{2+} . A control, '+ inactive trypsin', in which inhibitor was added to the trypsin before pre-incubation with the virus, was also performed.

could have reached completion only if all active virus particles had fused to their greatest extent, or if the target population had been depleted due to multiple fusion events caused by a single virion. If each virion could only fuse once, then addition of target above the level of virus particles should not have resulted in further dequenching. When this experiment was conducted at pH 5.0, the same effects were observed (data not shown). In summary, these observations indicate that the fusion products could fuse with the additional liposomes but not with added virus.

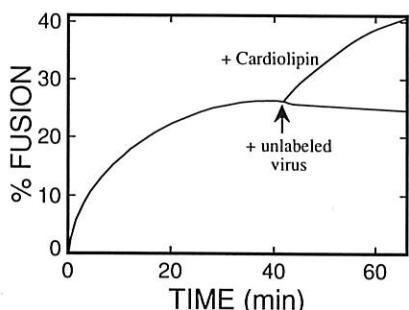


Fig. 6. HIV-1 cannot fuse with virus-liposome fusion products, but the fusion products can fuse with liposomes. R_{18} -labeled HIV-1 fusion with CL liposomes was monitored for approx. 40 min at pH 7.5. Measurement of fluorescence was continuous for the initial 6 min, followed by time-point measurements until the next injection. The virus:target lipid ratio was initially 1:1 (0.36 μM each). At the arrow, 1.1 μM of additional CL liposomes or unlabeled HIV-1 was injected into the cuvette. Fusion monitoring was continuous for the next 6 min and then measured at fixed time points. The rate of fusion after the second addition of CL liposomes was lower than after the first addition, even though three times as much target was added. In this concentration range, the rate of fusion of intact virus with liposomes was always higher for higher concentrations of target (Fig. 7). Therefore, the fusion products fused at a considerably slower rate than the intact virus.

TABLE III

Final extents of fluorescence increase (% max) for HIV-1 interacting with liposomes at pH 7.5 ^a

Target membrane	Lipid ratio liposome : virus	Exptl. value	Calculated	
			fusion	probe transfer
			best fit	equal partition
CL		$\alpha = 0.3$	$K = 0.1$	$K = 0.44$
	0.46	22	22	31
	1.85	45	48	65
	9.25	59	64	90
DOPC	139	71	70	99
		$\alpha = 0.5$	$K = 0.04$	$K = 0.44$
	2	26	33	67
	139	58	50	99

^a α represents the fraction of virions that are incapable of fusing. K is the ratio of binding constants of liposome over virus for partition of the probe. In view of the size difference between HIV and liposomes, $K = 0.44$ corresponds to the equal binding affinity, and hence to the maximal fluorescence level of the probe due to transfer.

In the case of HIV-1 fusion with CL at pH 5 or at pH 7.5 in the presence of ≥ 0.25 mM Ca^{2+} , all the virions were capable of fusing ($\alpha = 0$) (Table II). With DOPC vesicles, 60% of the virions were capable of fusing at pH 5, and 50% at pH 7.5. For DOPC, agreement between experimental and calculated values was better when irreversible binding of fusion-inactive virions to liposomes was assumed. The final extents of fusion of HIV-1 with PS/PC or PS/PE (1:1) at pH 7.5 were similar to those with DOPC liposomes. Although we do not have systematic studies of final extents of HIV-1 fusion with PA and PI liposomes, the results in Fig. 2 and data not shown indicate that all virus particles are fusion active ($\alpha = 0$) with these liposomes at or below pH 5.

Fluorescence dequenching is a measure of fusion and is not due to transfer

Significantly, no fluorescence increase was seen when the fusion products and the unlabeled virus were incubated together for more than 20 min (Fig. 6). This is another indication that R_{18} probe transfer does not occur in this system. As described in Materials and Methods, incubation of the labeled virus with an excess of unlabeled virus also did not result in any probe transfer. In the case of DOPC liposomes, where the rate of fluorescence increase was slow, and the fit of calculated to experimental fluorescence levels was only fair (Table II), we considered the possibility that probe dilution might reflect aggregation-dependent transfer of individual probe molecules to the target membrane rather than viral fusion. We employed previously determined expressions for probe transfer [53]. At equilibrium, the distribution of the probe depends on the

particle concentrations and on K , the ratio of binding constants for partition of the probe into liposomes versus into virus. In Table III, we present the experimental final extents and calculated I values assuming that probe dilution is due to either fusion or transfer (for both the case of equal partition, $K = 0.44$, as well as for the case of best fit to the data) for both DOPC and CL liposome targets. It is obvious that the equations assuming fusion better explained the final extents than those assuming probe transfer. Electron microscopy (Fig. 8) provided further evidence for HIV-liposome fusion. These results constitute proof that the increase in fluorescence intensity in HIV-liposome systems is mainly due to probe dilution into the target membrane following fusion rather than spontaneous transfer of the probe.

Kinetics of HIV-1 fusion with liposomes can be predicted by a theoretical model

Fig. 7 illustrates the effect of vesicle concentration on the overall rate of HIV-1 fusion with CL liposomes. We employed a constant concentration of virus ($0.75 \mu\text{g}/\text{ml}$ viral protein, equivalent to $0.36 \mu\text{M}$ viral lipid) and varied the liposome concentration over two orders of magnitude (from $0.188 \mu\text{M}$ to $18.8 \mu\text{M}$ lipid). An increase in liposome concentration resulted in a significant increase in the overall rate of fusion, albeit lower than proportional to the concentration change.

The rate constants are presented in Table IV, and the results of the calculated fits for CL liposomes are shown in Fig. 7. Fig. 7 also demonstrates that the model yielded good simulations and predictions for the kinetics of fluorescence increase for HIV-1 fusion with CL liposomes. This was also the case for HIV-1 fusion with the other liposome compositions analyzed (data not shown). At pH 7.5 and 37°C , the aggregation rate constant for all liposome compositions ($(4.7) \cdot 10^8 \text{ M}^{-1} \text{s}^{-1}$) is large, only one order of magnitude or less below the upper limit in diffusion-controlled processes [54]. This means that there is almost no potential energy barrier for HIV-1 adhesion to a liposome. At pH 7.5, the fusion rate constant and, to a lesser extent, the percentage of active virions have the greatest effect on the overall rate of fluorescence increase.

After fixing C and f (and, to a certain extent, D), we noted that the calculated values were overestimating the experimental values at later times for the $3.75 \mu\text{M}$ cases ($> 1 \text{ min}$), and at earlier times for the $18.8 \mu\text{M}$ case ($> 15–30 \text{ s}$, depending on pH). Accordingly, we reduced the rate constants for higher order aggregation-fusion products. This reduction was expressed by the parameters K_f and K_c , respectively, and had practically no effect on the outcome of fusion and fluorescence increase in cases of very dilute suspensions, or even for more concentrated suspensions at earlier stages ($I \leq 20\%$).

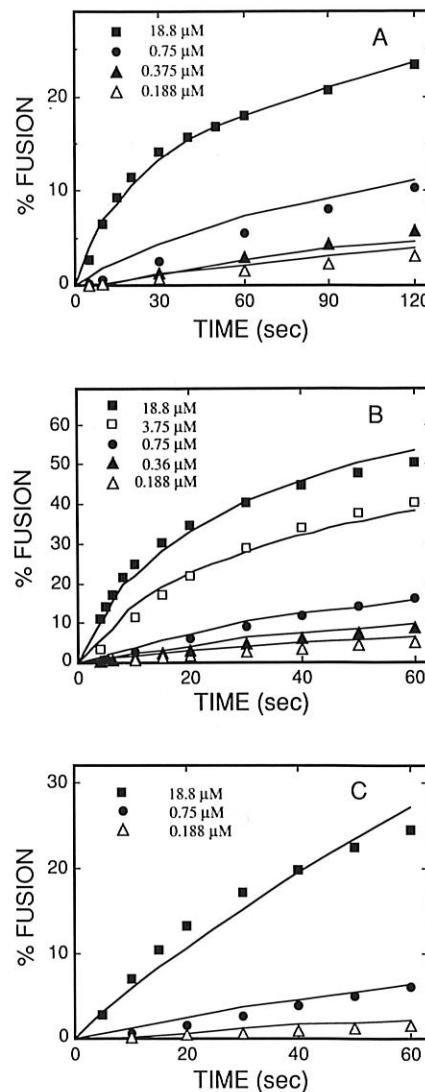


Fig. 7. Effect of liposome concentration on the kinetics of fluorescence intensity increase due to fusion between R_{18} -labeled HIV-1 and CL liposomes. Fusion experiments were performed at (A) pH 7.5, (B) pH 5.0, or (C) pH 7.5 with 0.25 mM Ca^{2+} as described in Figs. 1–3, respectively. The virus concentration was $0.36 \mu\text{M}$ viral lipid. Continuously acquired experimental values are indicated by solid lines, and calculated values (based on the rate constants presented in Table IV) are given by the symbols.

In virtually all cases, the rate constants were reduced 2- to 5-fold for liposome fusion with fusion products relative to liposome-virus fusion. The reduction factors given in Table IV merely reflect average values, since it can be expected that the rate constants may decrease with the number of liposomes in the aggregation-fusion product. The slower rate of fusion seen when unlabeled liposomes were added to a suspension of previously fused HIV-liposomes (Fig. 6) provided further evidence that the aggregation-fusion products fused slower with liposomes than did intact virions. Note that, after the second addition of target,

there was a 4-fold larger target concentration than that present initially, which, as shown in Fig. 7A, would have resulted in a much faster initial rate if the fusion products could fuse as efficiently as intact virions.

A similar pattern was seen for the much slower kinetics of HIV-1 fusion with DOPC, PS/PE (1:1) and PS/PC (1:1) liposomes (Table IV). However, there was larger uncertainty in the determination of the

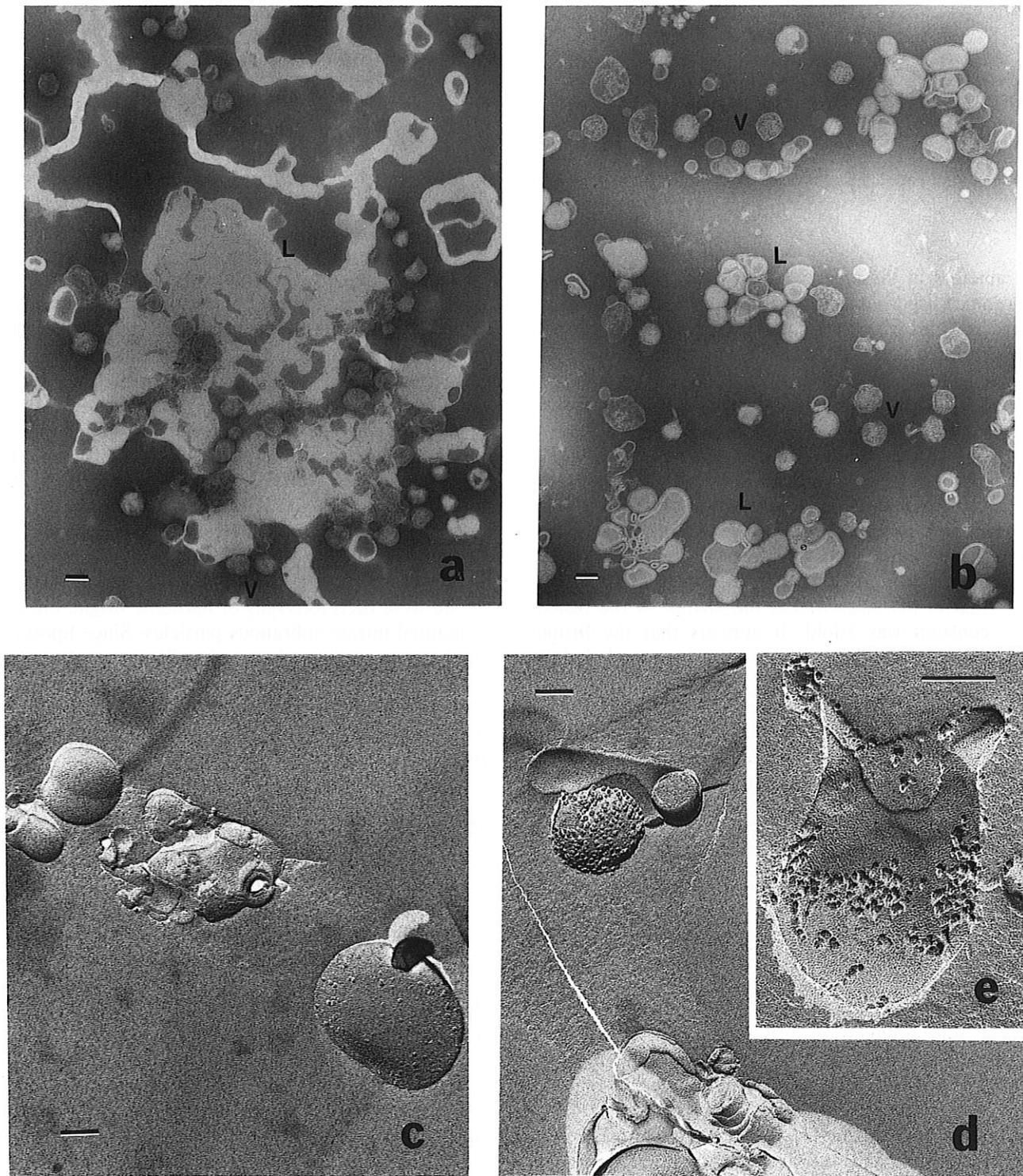


Fig. 8. Electron micrographs of HIV-1 bound to and fused with liposomes at pH 7.5. (a,b) CL (a) or DOPC (b) liposomes were mixed with unlabeled HIV-1 at 37°C for 5 min and then fixed prior to performance of negative stain EM (bar = 100 nm). (c,d) CL liposomes were mixed with unlabeled virus at 37°C for 5 min and fixed prior to freeze-fracture EM (bar = 100 nm). (e) DOPC liposomes were mixed with virus at 37°C for 4 h prior to freeze-fracture EM (bar = 100 nm).

TABLE IV

Percent of HIV-1 fusion activity, rate constants of aggregation (C), fusion (f) and dissociation (D), and rate constant reduction factors (K_f and K_c)^a

Target membrane	Medium	%Active virus	C ($M^{-1} s^{-1}$)	f (s^{-1})	D (s^{-1})	K_f	K_c
CL	pH 7.5	70	7.00 E08	0.1	0.03	0.2	0.2
CL	pH 5	100	1.35 E09	0.35	0.05	0.3	0.3
CL	pH 7.5/0.25 mM Ca ²⁺	100	7.00 E08	0.07	0.05	0.25	0.25
PS/PC (1:1)	pH 7.5	50	5.00 E08	0.0007	0.016	0.65	1
PS/PE (1:1)	pH 7.5	40	5.00 E08	0.001	0.014	0.65	1
DOPC	pH 7.5	50	4.00 E08	0.03	0.03	0.33	0.33
PI	pH 4.5	100	8.00 E07	0.3	0.05	0.25	0.25
PA	pH 4.5	100	5.00 E07	0.35	0.05	0.2	0.2

^a The percent uncertainty of the constants α , C, f, D, K_f and K_c were 10%, 20%, 30%, 50%, 30% and 30%, respectively.

parameters in these cases because, in the most dilute liposome suspension, the condition $f \gg C \cdot L_0$ was not satisfied. This means that the rate-limiting step was not the aggregation of the liposomes to the virus.

Effect of environmental parameters on HIV-1 fusion extents and kinetics

At pH 5, all HIV-1 particles could fuse with the acidic liposomes CL, PA or PI (Table IV; although the data for PA and PI are only shown for pH 4.5, the same is true for pH 5.0). At pH 7.5, only 70% of the virions could fuse with CL liposomes. At pH 5, the rate constant of aggregation, C, was about 2-fold larger than that at pH 7.5, whereas the change in the fusion rate constant was 3-fold. It appears that the fusion capacity (final extent) of HIV-1 also increased slightly toward pure DOPC liposomes at lower pH, but the effect was less than in the case of acidic liposomes (Table II). As shown in Table IV, the kinetic rate constants of aggregation and fusion for HIV-1 fusion with CL liposomes were essentially unchanged by the presence of Ca²⁺ ion at pH 7.5, but the percentage of virions capable of fusing increased from 70% to 100% in the presence of 0.25 mM Ca²⁺. Therefore, while the calcium ion concentration primarily affects the overall rate of fusion by determining the percentage of active virus, pH also alters the aggregation and fusion kinetics of HIV-1.

EM confirms HIV-1 fusion with liposomes

The end products of fusion between HIV-1 and liposomes were visually examined by two electron microscopic methods. Figs. 8a and 8b show negative stain electron micrographs of HIV-1 with CL and DOPC liposomes, respectively. The liposome membranes could be distinguished from the virus due to the oligolamellar structure and large size of the liposomes we used for the EM studies. Extensive aggregation among virus particles and liposomes was seen with CL, but less was seen with DOPC.

To obtain clearer evidence, we used freeze-fracture preparation to study the interaction of HIV-1 virions with CL and DOPC liposomes. Figs. 8c–8e show freeze-fracture micrographs of HIV-1 with liposomes. Large aggregated structures and enlarged membranes were observed when the virus was incubated with CL liposomes, and the liposome membranes contained intramembranous particles (Figs. 8c and 8d). DOPC, when incubated with HIV-1 for 4 h, also showed intramembranous particles (Fig. 8e). Virus alone has the tendency to cross-fracture (Ref. 55, and Friend, D.S., personal communication), and will not fracture along the envelope bilayer. The fractured membranes in Figs. 8c–8e were therefore only of liposome origin, but they contained intramembranous particles. Since liposomes of the type used in this study will not produce such structures on their own, the intramembranous particles must be from the viral envelope. These intramembranous particles must therefore be located within fusion products.

Discussion

Model membranes allow rigorous kinetic characterization of the fusion process [43,47], enable the target composition to be manipulated to identify the dependence of viral fusion on target membrane lipids and incorporated receptors [48,52,56–58], and allow for the direct quantitative comparison of the fusion characteristics of different viruses. For example, liposomes have been used to show that the fusion of Semliki Forest virus requires cholesterol in the target membrane [57], and to demonstrate the role of conformational changes of influenza hemagglutinin in membrane fusion [56]. Unlike previously studied lipid enveloped viruses that can fuse with a large variety of mammalian cells [25], HIV-1 seems to have a more restricted host cell range: it preferentially infects CD4-expressing peripheral blood cells [1]. On the other

hand, HIV-1 can infect certain CD4-negative neural cells [7,10–12] and many other CD4-negative cell types [12], suggesting that the virus may use more than one entry mechanism. Alternative receptor mechanisms have been demonstrated [11,16], but there is no single identified alternative high-affinity binding moiety common to most susceptible CD4-negative cells.

We demonstrated here the use of a quantitative lipid-mixing assay to determine which target membrane structures and environmental conditions promote HIV-1 fusion in a well-defined model system. We showed that HIV-1 can fuse with liposomes of several lipid compositions and with human erythrocyte ghost membranes. Since none of the membranes used here contained CD4, its presence in a target membrane is clearly not required for HIV-1 fusion. It is likely that the presence of CD4 in a target membrane serves to enhance the overall rate of fusion. HIV-1 preferentially fuses with negatively-charged liposomes, and the presence of cholesterol in the target membrane has no effect on HIV-1 fusogenic capacity. The preferential fusion of HIV-1 with negatively-charged liposomes is qualitatively similar to the fusion of other lipid enveloped viruses, including Sendai virus [46,48], influenza virus [52] and SIV_{mac} [17]. HIV-1 fusion activity is dependent upon target lipid composition and the pH of the reaction mixture, and fusion is often enhanced by physiological levels of Ca²⁺. Electron microscopy studies and a mathematical analysis of the final extents of fluorescence dequenching at different virus:liposome ratios confirmed that the fluorescence assay used here was a direct measure of HIV-1 fusion.

Our results may seem to differ with two reports that studied HIV fusion using liposomes or red blood cells as delivery vehicles or targets, respectively [59,60]. The first study used fluorescence and EM to deduce that CD4-containing liposomes interact with HIV-infected cell lines, but that liposomes lacking CD4 do not interact significantly with HIV-infected cells [59]. However, the study utilized a lipid composition that would be expected to show relatively slow fusion kinetics in our system. The second report used the R₁₈ lipid-mixing assay to monitor HIV-1 fusion with red blood cells and red blood cells containing electroinserted CD4 [60]. Although this study indicated an absence of HIV-1 fusion with native red blood cells, it is possible that the presence of hemoglobin in this system masks the relatively low level of fluorescence dequenching (fusion) we describe here for erythrocyte ghosts. Furthermore, we have optimized the percent of HIV particles undergoing fusion by using a small number of virions per cell, about an order of magnitude less than in Zeira et al. [60]. We must emphasize that we do not dispute the findings that the presence of CD4 in a target membrane may increase the efficiency of HIV-1 binding and fusion.

Electron microscopy provides visual evidence that HIV-1 fusion does not require CD4 in the target liposome. The negative staining method we used for EM was adopted from earlier studies that examined the fusion products between liposomes and Sendai or influenza virus [45]. This technique demonstrated here that the interaction of HIV-1 with liposomes results in post-fusion structures similar to those observed with other viruses. Additional EM using freeze-fracture showed that the HIV-1 envelope proteins dilute into the liposome bilayer after fusion. Thus, EM confirmed that HIV-1 is capable of fusing with CL or DOPC membranes lacking any protein receptor.

Although the kinetics of HIV-1 fusion were highly dependent on target lipid composition, it is significant that HIV-1 fused with all liposome compositions tested. Galactosyl ceramide has been suggested as a receptor for HIV-1 in certain CD4-negative cell lines derived from brain tissue [11] and colon cells [16]. Since fusion is preceded by binding of the virus to its target, our results indicate that intact HIV-1 can bind to many types of membranes, both net neutral and negatively charged. We do not question the possibility that galactosyl ceramide is an alternative receptor to CD4 for HIV-1 entry in some cells. It may, however, not be the only such alternative. It is possible that the virus might fuse exceptionally efficiently with galactosyl ceramide-containing liposomes; these experiments are in progress.

Whether endocytosis of HIV-1 precedes viral-cell membrane fusion is controversial [27,61–63]. The physiological significance of our results which show enhanced HIV-1 fusogenic activity toward liposomes at lowered pH is unknown at this time. Most reports suggest that HIV-1 entry occurs at the plasma membrane [26,27] and is not dependent on acidification of endocytic vesicles [6,27,61]. Although HIV-1 exhibits enhanced fusogenic activity at lower pH, with liposomes as target membranes, reduced pH may not be required for viral entry into cells. Furthermore, this enhanced fusion is only observed with acidic liposomes, and reduced pH has little effect on HIV-1 fusion with erythrocyte ghost membranes. We should note, however, that the acidic phospholipid PS is generally accepted as the cellular receptor for at least one virus, vesicular stomatitis virus [64]. Thus acidic lipids should not be dismissed completely as potentially relevant cellular targets.

The presence of ≥ 0.1 mM Ca²⁺ enhances HIV-1 fusion activity and extent toward acidic liposomes, whereas similar levels of Ca²⁺ have no effect on the fusion capacity of influenza [52] or Sendai virus (Ref. 65 and Hoekstra, D., personal communication) toward liposomes. Low levels of Mg²⁺ also enhance HIV-1 fusion with several acidic liposomes. In addition, HIV-1 fusion with erythrocyte ghost membranes is signifi-

cantly enhanced by the presence of physiological levels of Ca^{2+} , which is unlike even SIV_{mac} [17]. Thus, a striking difference exists in the effect of divalent cations on HIV-1 fusion with acidic liposomes and erythrocyte ghost membranes as compared with influenza or Sendai virus fusion. Ca^{2+} does not enhance HIV-1 fusion with neutral liposomes. The physical explanation for these results is unclear at this time.

The results show that HIV-1 fusion with cardiolipin liposomes at pH 7.5 and 0.25 mM Ca^{2+} is significantly inhibited by prior trypsinization of intact virus. We previously showed that recombinant soluble CD4 inhibits HIV-1 fusion with CL liposomes [19], and recent results show that soluble CD4 and glutaraldehyde pre-treatment also inhibit dequenching of R_{18} labeled HIV-1 with both CD4^+ and CD4^- cells (Stamatatos, L., Nir, S. and Düzungüneş, N., unpublished data). While our results do not prove that the HIV-1 envelope glycoproteins are acting in a physiologically relevant manner, the results do show that the envelope glycoproteins are required for at least most of the fluorescence dequenching in this system, which is a quantitative assay of virion fusion. To our knowledge, antibodies against gp120/gp41 that neutralize HIV-1 infectivity toward CD4-negative cells have not been identified. Once such antibodies are available, they will be useful in determining whether HIV-1 fusion with particular model membranes is related to the fusion required for viral entry into the relevant cell types. Neutralizing antibodies will also help clarify whether the same epitopes on gp120/gp41 are involved in both CD4-dependent and -independent fusion.

Trypsinization may block the binding and/or fusion roles of gp120/gp41. Although trypsinization did not completely block the fusion reaction, this was not unexpected. A similar effect was seen for both influenza [52] and Sendai [48] virus fusion with CL liposomes. For Sendai virus, fusion is inhibited by 47% and 82% at pH 5 and 7.4, respectively.

The lipid-mixing fusion assay used here was originally developed for use with Sendai and influenza viruses to measure virus fusion quantitatively [42], and has been used previously to study phenomenologically the fusion of HIV-1 with cells [6,60,66] and the fusion of SIV_{mac} and HIV-1 with liposomes [17]. The kinetic analysis of virus-liposome fusion presented here, however, allows quantitative comparison of HIV-1 fusion with the extensively studied fusion characteristics of influenza and Sendai viruses.

As with influenza and Sendai viruses, the fusion activity of HIV-1 toward neutral liposomes or mixed neutral-acidic liposomes is lower, compared to pure acidic liposomes. When CL is replaced by PS/PC (1:1) or PS/PE (1:1) as the target membrane, the rate constant of aggregation, C , is reduced by 30% and the rate constant of fusion, f , is reduced by two orders of

magnitude, at neutral pH. We also demonstrated that, like influenza and Sendai viruses, an HIV-1 virion cannot fuse with a fusion product, and therefore the HIV-liposome fusion products consist of a single virion and several liposomes. This limitation may be due to a saturation in the capacity of the liposomal membranes for the viral glycoproteins, and perhaps due to a negative cooperativity (i.e., the presence of viral glycoproteins in the fusion product membrane is inhibitory to the close approach and fusion of a second virion) [46,47].

An important characteristic of HIV-liposome fusion is a significant reduction in the fusion capacity of the fusion products relative to that of the virus toward liposomes. In contrast, influenza and Sendai viruses do not exhibit this property. The rate constant of fusion is reduced 2- to 5-fold for the HIV-1 fusion products, and the aggregation rate constant is usually affected to the same extent. This correlates well with the fact that HIV-liposome fusion products exhibit reduced infectivity toward CD4-positive cells as compared with intact virus [19,35,36]. The initial organization of viral glycoproteins within the viral envelope may be more conducive to fusion than that attained after the initial round of fusion. Determination of whether the key alteration in glycoprotein organization in the fusion product is a change in gp120/gp41 surface density, a change in multimer/monomer ratios, an increased proportion of conformationally rearranged fusion-inactive spikes or a combination of these factors will help clarify the molecular mechanism of the HIV-1 fusion process.

The effect of pH exhibits the same trend for influenza and Sendai viruses and HIV-1. The rates and final extents of viral fusion with liposomes increase when the pH is lowered from 7.5 to 5. All three viruses show complete fusion activity ($\alpha = 0$) toward CL liposomes at pH 5. For HIV-1, we have shown that the same holds true for its fusion with PA and PI liposomes at or below pH 5. The fact that both the aggregation and fusion rate constants for HIV-1 fusion with CL liposomes are increased at pH 5 versus pH 7.5 suggests that reduced pH enhances both the binding capacity and the fusogenicity of the viral membrane proteins towards negatively-charged membranes. Viral glycoprotein protonation is most likely responsible for the low pH enhancement of HIV-1 fusion activity: the surface charge of liposomes containing these lipids would not change sufficiently to explain the observed enhancement in the fusion rate, at least down to pH 5 [17].

The present study does not attempt to prove that the erythrocyte ghosts or lipid compositions of some of the model membranes investigated here represent a physiological model for typical CD4-independent HIV-1 fusion with most cell membranes. In fact, the wide

variety of CD4-negative cells that are susceptible to HIV-1 infection suggests that there may not be a broadly representative model membrane target for CD4-independent fusion. However, it is clear that a specific membrane lipid or protein in the target membrane is not required for immunodeficiency virus fusion activity. Comparison of the present results with the kinetics of fusion of HIV-1 with liposomes containing reconstituted CD4 and/or galactosyl ceramide will prove useful in determining whether specific receptors significantly affect the rate of HIV-1 fusion. Finally, screening of neutralizing antibodies (for HIV-1 infection of both CD4-positive and -negative cells) for inhibition of HIV-1 fusion with targets of a 'representative' lipid composition may help identify the required epitopes for fusion induced by gp120/gp41.

It is ultimately necessary to compare the fusion activity toward liposomes with the activity toward living human cells, and to compare the fusion activities of various HIV strains toward a variety of membrane targets. These studies are in progress in our laboratory. Liposomes may also prove useful as carriers of antiviral or cytotoxic compounds that could be directed preferentially toward cell-free virus or infected cells expressing viral membrane glycoproteins on their surface. The potential of inhibiting cell-free virus infectivity using liposomes, even without incorporated CD4, is demonstrated by our recent observations that pre-incubation of HIV-1 with CL liposomes, or the presence of such liposomes during the initial interaction of the virus with A3.01 and H9 cells, inhibits the production of viral antigen by these cells [35,36].

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