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Lipid composition and fluidity of the human immunodeficiency virus envelope and host cell plasma membranes

(H9 plasma membranes)

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ABSTRACT **Previous studies have indicated that human immunodeficiency virus (HIV) is enclosed with a lipid envelope similar in composition to cell plasma membranes and to other viruses. Further, the fluidity, as measured by spin resonance spectroscopy, is low and the viral envelope is among the most highly ordered membranes analyzed. However, the relationship between viral envelope lipids and those of the host cell is not known. Here we demonstrate that the phospholipids within the envelopes of HIV-1_{RF} and HIV-2-L are similar to each other but significantly different from their respective host cell surface membranes. Further, we demonstrate that the cholesterol-to-phospholipid molar ratio of the viral envelope is approximately 2.5 times that of the host cell surface membranes. Consistent with the elevated cholesterol-to-phospholipid molar ratio, the viral envelopes of HIV-1_{RF} and HIV-2-L were shown to be 7.5% and 10.5% more ordered than the plasma membranes of their respective host cells. These data demonstrate that HIV-1 and HIV-2-L select specific lipid domains within the surface membrane of their host cells through which to emerge during viral maturation.**

Electron microscopic studies (1–3) have demonstrated that the human immunodeficiency virus (HIV) is surrounded by a lipid envelope derived from the host cell plasma membrane. The initial report of the lipid composition of the HIV demonstrated that the envelope phospholipids were similar to those of other enveloped viruses and the erythrocyte plasma membrane (4, 5). The demonstration of high levels of phospholipids normally found in cell surface membranes [sphingomyelin (Sph), phosphatidylethanolamine (PE), phosphatidylcholine (PC), and phosphatidylserine (PS)] supported the electron microscopic observations depicting the enclosure of the viral capsid with a lipid envelope derived from the host cell plasma membrane during the budding process (4, 5). Further, these studies (4, 5) and others (6) indicated that HIV had a low lipid-to-protein (weight) ratio and a high cholesterol-to-phospholipid molar ratio (C/P ratio).

Electron spin resonance (ESR) analyses of HIV have indicated that the flexibility of an incorporated spin-labeled probe molecule (a 5-nitroxide derivative of stearic acid; 5-NS) is restricted and that the viral envelope is among the most "ordered" membranes analyzed (5, 7, 8). Order parameters, which were calculated to be >0.7 (when spectral recordings were made at 37°C), were comparable to those of other enveloped viral systems exhibiting high order parameters (>0.7) and C/P ratios >1.0 (5, 9–11).

Although all HIV isolates examined to date exhibit high C/P ratios and elevated order parameters, differences in phospholipid classes have been reported. For example, in HIV isolates LK013, F7529, and 4105 (5) and HZ321 (9) Sph is 25–28 mol %, whereas in isolates HTLV-III_B and HTLV-III_{RF}, Sph is 11–13 mol % (6). It is presently unclear what physical mechanism(s) account for these lipid variations. They could reflect variations in cell lines and growth conditions or genetic differences in HIV isolates, in which envelope or other proteins select different lipids from the bulk lipid pool within the cell surface membrane during the budding process (5). Lipid envelopes of other viruses possess a lipid profile different from their host surface membranes, as has been shown for Semliki Forest virus (12), simian virus 5 (13, 14), respiratory syncytial virus (15), Sindbis virus (16), and vesicular stomatitis virus (17). Viral budding through specific regions of the cell membrane has been suggested for some of these enveloped viruses (see refs. 9, 18, and 44 for reviews).

Here we present evidence that HIV types 1 and 2 (HIV-1_{RF} and HIV-2-L) exhibit a phospholipid profile and fluidity significantly different from those of the plasma membranes of the host cells in which they are grown. Such differences are strongly suggestive of a selective sequestration of lipids occurring during the budding process, in which the viral proteins select specific domains within the host cell membrane through which to emerge during maturation. Further, we provide data on the C/P ratios of six additional laboratory and clinical HIV and simian immunodeficiency virus (SIV) isolates.

MATERIALS AND METHODS

Materials. Spin probes were purchased from Molecular Probes and Aldrich and checked with two-dimensional chromatography for purity (19). All solvents were HPLC grade from Fisher.

HIV Isolates. HIV-1_{RF} was purchased from John Dahlberg (formerly at Universal Biotechnology; Rockville, MD) and HIV-2-L was from James Whitman, Jr. (Advanced Biotechnologies; Columbia, MD), along with H9 cells, from which the viral isolates were grown. Essentially 15 liters of infected H9 cells (1×10^6 per ml) were clarified with a JCF-Z (Beckman) rotor in continuous flow centrifugation. The cell pack was washed and banded between 1.12 and 1.18 g/ml on a 5–45% sucrose density gradient, washed again with phosphate-buffered saline (PBS), and resuspended in 1.5 ml for analysis. Purity was checked by viral particle count, PAGE/Western blot analysis, and p24 and protein concentration, and it was determined to be greater than 92.5%. Viral isolates were stored frozen at −70°C and shipped on dry ice. Infected

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Abbreviations: HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; 5-NS, the *N*-oxy-4′,4′-dimethyloxazolidine derivative of 5-ketostearate; C/P ratio, cholesterol-to-phospholipid molar ratio; Sph, sphingomyelin; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid.

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and uninfected H9 cells were grown under identical conditions and washed three times in PBS before being frozen as a pellet and shipped on dry ice.

Plasma Membrane Isolation. All manipulations of cells during plasma membrane isolation were performed within a sterile hood of a biosafety level 3 (P3) facility. H9 cells were homogenized by using a Kirkland Emulsiflex device (Avestin, Ottawa), which is completely enclosed to permit the isolation of membrane fractions with a minimum of aerosol formation. Twenty milliliters of a homogenizing buffer (10 mM Hepes/5 mM $MgCl_2$/1 mM EDTA/0.1 mM phenylmethylsufonyl fluoride/1 mM dithiothreitol/10 mM KCl, pH 7.6) was added to a packed pellet of 2–5 × 10^9 frozen cells and the mixture was allowed to thaw briefly at 37°C. The cells were disrupted by the piston action (5-cm stroke distance; 1 cycle per sec) of the Emulsiflex device, forcing the suspension through a 5-mm orifice apposed by a plunger of slightly smaller diameter.

After cell disruption, plasma membranes were isolated by a modified sucrose gradient procedure (20–23). The cell homogenate was transferred from the syringe receptacle of the Emulsiflex to a 50-ml tube, placed in a JA-20 rotor within a sterile hood of the P3 facility, and spun at 400 × *g* (Beckman JA21 centrifuge). The supernatant was decanted into a 28-ml tube, which was bottom loaded with 22.5%, 32%, 37%, and 45% sucrose and centrifuged for 3 hr at 100,000 × *g* with a Beckman L4 ultracentrifuge (within the P3 facility). The plasma membrane fraction (22.5%/32% interface) was diluted with resuspension buffer (50 mM Hepes/100 mM NaCl/20 mM KCl, pH 7.6) and washed twice in a Beckman Ti-60 rotor at 36,000 × *g* for 30 min. Mitochondria were removed from the 37%/45% interface with a Pasteur pipette, washed twice at 12,000 × *g*, and resuspended in the same buffer.

The uninfected H9 preparations were analyzed by a plasma membrane marker enzyme [Na^+,K^+-ATPase; EC 3.6.1.3 (24)], and a mitochondrial-specific enzyme [succinate dehydrogenase; EC 1.3.99.1 (25)]. These analyses indicated a 12- to 20-fold enrichment in [Na^+,K^+-ATPase and less than 10% mitochondrial contamination in the H9 plasma membrane fraction.

Lipid Extraction and Analysis. Viral lipids and plasma membranes of cells were extracted by a modification of previously published procedures (5, 26, 27) using an Omni-mixer (South Shore Scientific, Santa Ana, CA), in a completely enclosed glass vessel sealed with a Teflon washer and a stainless steel cap. The extracting solvent was chloroform/methanol (2:1, vol/vol), 5% (vol/vol) freshly prepared saturated (28% wt/wt) ammonia, and 0.05% butylated hydroxytoluene (5). A rotor blade projecting through the cap of the Omni-mixer homogenized the samples without aerosol dispersion in an atmosphere flushed with argon gas. The lipid extracts were removed from the P3 facility, concentrated by low-pressure, low-temperature, rotary evaporation, and purified of nonlipid contaminants on a Sephadex G-25 column in a solvent of chloroform/methanol (19:1, vol/vol) saturated with water (5, 26, 27). The column eluate was concentrated and chromatographed, and lipid spots were identified from standards run under identical conditions (see Fig. 1 for solvent composition). Lipid phosphorus was quantified as described previously (5, 27, 28). Cholesterol was assayed with the Boehringer Mannheim Diagnostics High Performance-K kit (catalog no. 692905) (27). Protein was determined by the Bradford assay (29) according to Bio-Rad.

HIV Spin-Labeling and Spectral Recording. HIV-1_{RF} and HIV-2-L (7–9 mg/ml) and plasma membranes (14–16 mg/ml) were spin-labeled as described (5, 7, 8) within the P3 facility. Basically, 20 μl of sample was added to the 5-NS spin label (*N*-oxy-4',4'-dimethyloxazolidine derivative of 5-ketostearate) previously dried at the bottom of a microcentrifuge tube, and after mixing, the sample was drawn into a 50-μl capillary and the ends were heat sealed. The capillary was taken from the P3 facility, placed in a Kornberg holder, and introduced into the variable temperature cavity of an ESR spectrometer equipped with a trap to prevent accidental release of "live" HIV aerosols. The Varian E-109 ESR spectrometer was fitted with a Deltron (Sydney, Australia) DCM 20 temperature-control accessory and coupled to a DOS-based computer with a program for capture and integration of spectra. Probe-to-lipid molar ratio (1:240) was determined by double integration of ESR spectra, comparing strong pitch with a known number of spins to the recorded membrane and HIV spectra (5, 7, 8). To evaluate the mobility of the 5-NS probe executing rapid, anisotropic motion, a polarity-uncorrected order parameter $S(T_\parallel) = \frac{1}{2}[3(T_\parallel - T_{xx})/(T_{zz} - T_{xx}) - 1]$, was calculated as shown in Aloia *et al.* (5). Values range from 0 to 1, with the extremes indicating, respectively, fluid and immobilized environments.

Table 1. C/P ratios of viral isolates

Name	C/P ratio		Supplier	Description
	Group 1	Group 2		
HIV-LK013	0.84		Fred C. Jensen	Isolated from the peripheral blood lymphocytes of a male homosexual who subsequently died in December 1985 (1)
HIV-F7529	0.91		Fred C. Jensen	Isolated from the lymph nodes of a female sex partner of an intravenous drug abuser with AIDS-related complex (1)
HIV-4105	0.92		Fred C. Jensen	Isolated from the peripheral blood cells of a male homosexual (1)
HIV-HZ321		1.23	ImmuneResponse	Isolated at the Centers for Disease Control from the serum of a Zairian female by J. P. Getchell (30) and previously analyzed (9)
$HTLV\text{-}III_B$		1.2	—	Laboratory isolate (8)
$HTLV\text{-}III_{RF}$		1.2	—	Laboratory isolate (8)
$HTLV\text{-}III_{MN}$		1.6	—	Laboratory isolate (8)
HIV-HZ321		1.24	ImmuneResponse	HIV immunogen purified from Hut-78 cells October 1990
HIV-HZ321		1.27	ImmuneResponse	HIV immunogen purified from Hut-78 cells November 1990
HIV-BK		1.25	John Dahlberg	Clinical isolate passaged three times before being grown on CEM-SS cells for isolation and purification by Universal Biotechnology (Rockville, MD)
HIV-OT	0.77		John Dahlberg	Clinical isolate passaged three times before being grown on CEM-SS cells for isolation and purification by Universal Biotechnology
HIV-1_{RF}	0.96		John Dahlberg	Laboratory isolate from Universal Biotechnology
HIV-2-L	0.88		James Whitman	Laboratory isolate from Advanced Biotechnologies (Columbia, MD)
SIV-B670	0.79		ImmuneResponse	SIV originally isolated by M. Murphey-Corb, Delta Regional Primate Center, Tulane Univ. (59)

RESULTS AND DISCUSSION

Table 1 gives the C/P ratios of 14 different isolates of HIV-1, HIV-2, and SIV, the first 7 of which have been previously published but are included here for comparison. These data show that the C/P ratios of both laboratory and clinical HIV isolates fall into two distinct groups, those between 0.8 and approximately 1.0 and those >1.2. The reason for this difference is unknown. However, all C/P ratios are comparable to those of other viral isolates (5, 10, 11).

Fig. 1 depicts the two-dimensional thin-layer chromatograms for HIV-1_{RF} and HIV-2 and the plasma membranes of the H9 cells from which they were grown. All lipid spots are clearly resolved and differences between plasma membrane lipids and HIV lipids are apparent. Table 2 illustrates the phospholipid class ratios for HIV-1_{RF} and HIV-2-L, showing the differences between viral and host cell plasma membranes. For example, the levels of PC and PI in the viral envelope are reduced by 50% and 80%, respectively, whereas Sph is enriched 3-fold in both viral isolates compared with their respective host cell plasma membranes. PS is also elevated by 40% in HIV-1_{RF} and by 140% in HIV-2-L, compared with the lipids of the host cell. Further, the C/P ratio of the virus is seen to be approximately 2.5 times that found in the plasma membranes from infected cells.

Differences between the phospholipid composition of uninfected and infected H9 cells were also observed. PC increased by approximately 6% and PS decreased 38% in plasma membranes from both infected cell lines compared with the membranes from uninfected cells, while Sph was lower by 14% and 25% in membranes from cells infected with HIV-2-L and HIV-1_{RF}, respectively. The C/P ratio was also found to be lower in plasma membranes isolated from infected cells compared with uninfected cells, consistent with reports of Sindbis virus-infected chick embryo and baby hamster kidney cells (31).

Table 2. Membrane lipid composition of infected plasma membranes and HIV-1_{RF} and HIV-2-L isolates

	Lipid composition, mol %				
	H9 plasma membranes*			Virus†	
Lipid	Uninfected	Infected with HIV-1_{RF}	Infected with HIV-2-L	HIV-1_{RF}	HIV-2-L
PC	47.55	50.51	50.48	29.85	27.57
PE	23.34	24.87	24.45	24.58	27.26
Sph	9.64	7.14	8.26	24.06	22.97
PI	4.87	4.72	5.50	0.42	1.14
PS	10.26	6.36	6.34	9.02	15.50
PA	0.59	0.33	0.47	1.21	1.21
Other‡	3.44	6.07	4.49	8.25	4.81
Recovery§	97.70	100.17	99.69	97.40	96.13
C/P ratio (n = 2)	0.476	0.371	0.343	0.958 (×2.58)	0.876 (×2.55)

*Plasma membranes isolated from uninfected H9 cells and H9 cells infected with HIV-1_{RF} and HIV-2-L (see Table 1 for source).
†Lipids extracted from HIV-1_{RF} and HIV-2-L virus grown in H9 cells (see Table 1 for source).
‡Other = minor, unidentified phosphorus-containing spots, lysophospholipids, and material remaining at the origin (see Fig. 1).
§Percent recovery is determined by dividing the amount of phosphorus recovered from the chromatography plate by the phosphorus content of an aliquot of lipid sample not chromatographed.

These data strongly imply that HIV-1_{RF} and HIV-2-L are selective in the regions of the host cell membrane through which they emerge during maturation. Further, the data are similar to reports of six other enveloped viruses which exhibit a lipid composition and C/P ratio significantly different from those of their host cell surface membranes (see table 2 of ref. 9). A mechanism to account for the HIV envelope lipid profile

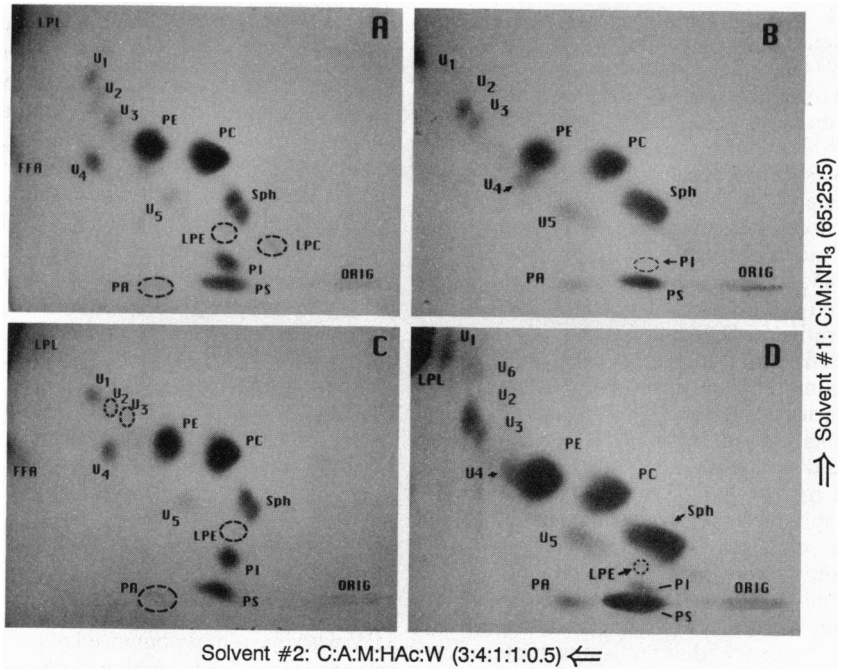

FIG. 1. Two-dimensional thin-layer chromatograms of lipids extracted from plasma membranes of H9 cells infected with HIV-1_{RF} (*A*) or HIV-2-L (*C*) and from intact HIV-1_{RF} (*B*) or HIV-2-L (*D*). Solvent 1, C:M:NH_3 [chloroform/methanol/ammonia (28%)]; and solvent 2, C:A:M:HAc:W (chloroform/acetone/methanol/acetic acid/water) (28). Lipid spots were identified by comparison with standards run under identical conditions. PI, phosphatidylinositol; PA, phosphatidic acid; Orig, origin; LPE, lysophosphatidylethanolamine; LPC, lysophosphatidylcholine; LPL, less polar lipid; FFA, free fatty acid; U_1–U_5, unknown phosphorus-containing spots. U_4 in the viral isolates (*B* and *D*) was thought to be an unknown spot which had migrated close to PE. However, phosphorus analysis revealed the spot to contain <0.1% of the total phosphorus, and so it was included in the percent composition of PE. All values in Table 1 were derived from the lipids in these chromatograms and are expressed as molar percent.

is thought to be a selection process by the envelope or other viral proteins for a domain of lipids which envelop the virus capsid during the budding process. The reduced C/P ratio found within the infected H9 cell membranes vs. the uninfected cells reinforces the concept of cholesterol domains through which HIV evaginates. Such domains were predicted to be either passive (preexisting within the lateral plane of the host cell surface membrane) or dynamic (being induced by insertion of viral peptides—e.g., myristoylated p55 gag protein—into the inner monolayer of the host cell plasma membrane during viral budding) (5). However, regardless of the mechanism, it seems that both HIV-1 and HIV-2-L emulate the same process, since the lipid profiles of the two virions are similar but significantly different from those of their respective host cells.

Sterol within host cell plasma membranes has been shown to be a specific requirement for infection and/or fusion with many viral isolates (31–37). Further, a high C/P ratio within viral envelopes has also been shown to be required for infectivity of many enveloped viruses. For example, removal of about 50% of the sterol from vesicular stomatitis virus (VSV) resulted in a >85% decrease in the infection of BHK cells (38, 39). Incubation of HIV-1 with a phospholipid liposome, A1-721 [7 parts neutral lipid/2 parts PC/1 part PE (40)] removed approximately 50% of the cholesterol (6) and correlated with previous studies showing that a similar treatment of HIV-1 reduced infectivity by 50% (41). These reports are consistent with studies in which lymphocytes and erythrocytes were incubated with A1-721, resulting in cholesterol removal and a consequent increase in membrane fluidity measured by fluorescence spectroscopy (42). In view of the C/P ratio of HIV-1_{RF} and HIV-2-L being significantly greater than that found in infected cell plasma membranes, a vital role of cholesterol in the infectivity process is considered a distinct possibility. Similar elevations in C/P ratios in other envelope viruses over their respective host cell surface membranes, by 1- to 5-fold, have been demonstrated for bovine leukemia virus (BLV), equine infectious anemia virus (EIAV), Friend murine leukemia virus (FMLV), and avian myoblastosis virus (AMV) (11). ESR analyses of these viral envelopes indicated them to be highly ordered (11). Similarly, for VSV and influenza virus grown in Madin–Darby bovine kidney cells, calculated order parameters were 5–10% greater than those of host cell plasma membranes (43, 44). Confirmatory results were obtained from fluorescent spectroscopic analysis of enveloped viruses, indicating that the viral envelopes were significantly more ordered than their host cell surface membranes (39, 45, 46).

Previously we attributed the highly ordered HIV-1 envelope to the elevated C/P ratio (5). Spin resonance analysis of HIV and intact, uninfected HUT-78 cells revealed that the viral envelope was approximately 11% more ordered than the lymphocyte (5). Since earlier studies of 5-NS-labeled lymphocytes indicated that this probe most likely resided in the plasma membrane and did not penetrate into intracellular membranes (47, 48), we interpreted our analysis of intact cells to indicate that the spin probe was reporting the milieu of the surface membrane only. However, spectral contributions of the probe residing within intracellular membranes (i.e., a composite spectrum) could not be ruled out, since purified plasma membranes were not previously analyzed (5).

Here we also present evidence that indicates that the fluidity of the 5-NS spin-labeled HIV-1_{RF} and HIV-2-L envelope is less than that of isolated plasma membranes from the host cells (Fig. 2). These spectra were derived from isolated plasma membranes of infected cells (spectra A-1 and B-1 in Fig. 2) and from HIV envelopes (spectra A-2 and B-2 in Fig. 2). They clearly show the outer hyperfine splittings ($2T_{\parallel}$) to be greater for the viral envelope than for the plasma membrane of the infected host cells. The polarity-

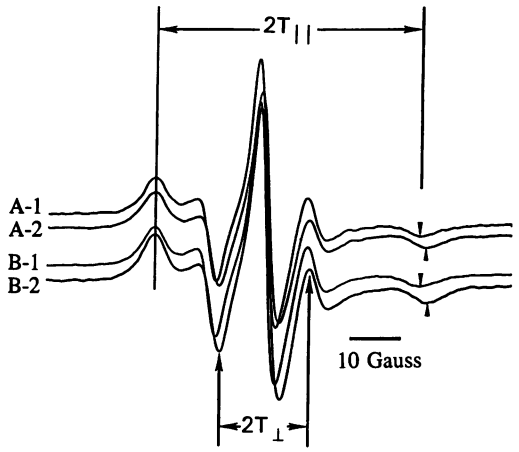

FIG. 2. ESR spectra of plasma membranes isolated from H9 cells infected with HIV-1_{RF} (A-1) and HIV-2-L (B-1) and from the intact HIV-1_{RF} (A-2) and HIV-2-L (B-2). All membranes and virus were spin labeled with 5-NS in a sterile hood within a biosafety level 3 facility and subsequently transferred to a Varian E-109 electron paramagnetic resonance spectrometer (1–3). Spectra were recorded at 38°C. Instrument conditions were 8-min scan time, 3.2-G modulation amplitude, 10-mW microwave power, and 1-sec time constant. The horizontal axis represents varying magnetic field, while the vertical axis reflects microwave absorption. Outer and inner hyperfine splittings, $2T_{\parallel}$ and $2T_{\perp}$, were measured as shown, with the latter being corrected by the addition of 1.6 G. The low-field peaks of the spectra are aligned and the high-field troughs of the outer hyperfine splittings are indicated by arrowheads.

uncorrected order parameters [$S(T_{\parallel})$ for these spectra recorded at 38°C were 0.637 and 0.641 (plasma membranes from H9 cells infected with HIV-1_{RF} and HIV-2-L, respectively) and 0.685 and 0.707 (for HIV-1_{RF} and HIV-2-L, respectively). The order parameters for the viral isolates indicate that HIV-1_{RF} and HIV-2-L are, respectively, 7.5% and 10.5% more ordered (less fluid) than the plasma membranes of the host cells from which they are derived. These results remove previous doubts that our earlier analyses were reporting composite spectra (contributions from both surface and intracellular membranes). The HIV order parameters calculated here are similar to those reported previously for three HIV isolates (5) and for other enveloped viruses (bovine leukemia virus, equine infectious anemia virus, Friend murine leukemia virus, and avian myeloblastosis virus) (11). These data demonstrate that HIV most probably emerges through specific domains within the host cell surface membrane during budding.

At the present time the importance of the viral lipid composition in the infectivity process has only begun to be understood. For example, elevated temperatures, known to alter the fluidity of the HIV envelope (5), also reduce infectivity (by three orders of magnitude) in a time- and temperature-dependent manner (49). Treatment of HIV and other enveloped viruses with various lipophilic drugs, such as the spermicide nonoxynol-9 (50, 51), oleic acid (52), AL-721 (6, 49), and butylated hydroxytoluene (BHT) (5, 53–55), have been shown to significantly block infectivity. Pretreatment of HIV with liposomes composed of cardiolipin, one of the major mitochondrial lipids, inhibits subsequent infection of A3.01 and H9 cells (56), and the presence of such liposomes during infection leads to a dose-dependent reduction of subsequent p24 production (56–58). Preincubation of HIV-1_{LAV} with a synthetic lipid, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA), has been shown to enhance infection of A3.01 and H9 cells by more than 30-fold (58). As such, the alteration in HIV lipid composition by fusion

with these liposomes caused major alterations in the capacity of the viral isolate to infect host cells.

The lipid profiles of HIV-1_{RF} and HIV-2-L are similar to each other except for the higher content of PI (0.42 vs. 1.14 mol %) and PS (9.02 vs. 15.50 mol %) in HIV-2 (Table 2). At this time the significance of the differences in phospholipid classes between HIV-1 and HIV-2-L isolates is unknown, since the range of values of all phospholipids is within those previously reported (5, 6, 9, 10). However, the evidence presented here lends support to the critical nature of the lipid composition of the HIV envelope derived during emergence by means of a sequestration of specific lipids from the host cell surface membrane lipid repertoire. It would thus be of considerable interest to modify the plasma membrane lipids of the host cells and to examine the lipid composition of the virions emerging from these cells, as well as the capacity of these virions to infect various host cells.

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