BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **242,** 219–225 (1998)
ARTICLE NO. RC977941

Human Erythrocyte Glycolipids Promote HIV-1 Envelope Glycoprotein-Mediated Fusion of $CD4^+$ Cells

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Received December 1, 1997

We examined the role of target membrane glycolipids in CD4-mediated HIV-1 fusion by altering the glycolipid levels in $CD4^+$ cells. $CD4^+$ human cells exhibited 50% reduction in extent of fusion with gp120-gp41 expressing cells (TF228) when grown in the presence of a glycolipid synthesis inhibitor PPMP. We added erythrocyte glycolipids (GL) to fusion-incompetent $CD4^+$ non-human cells by influenza-hemagglutinin-mediated fusion between GL-containing liposomes and target cells. Human erythrocyte GL (HuGL)-modified $CD4^+$ non-human cells became susceptible to fusion with TF228 cells. Transfer of bovine erythrocyte glycolipids (BoGL) to $CD4^+$ non-human cells under similar conditions did not complement HIV-1 fusion. Furthermore, addition of HuGL, but not BoGL, to PPMP-inhibited cells rescued fusion to the original levels. Our observations demonstrate that human erythrocyte glycolipids promote CD4-mediated HIV-1 fusion and certain glycolipid(s) from human erythrocytes may serve as alternative and/or additional cofactors in HIV-1 entry.

Human immunodeficiency virus type-1 (HIV-1) enters permissive cells by binding to its cellular receptor CD4 (1) followed by fusion of viral and target cell membranes (2,3). HIV-1 fusion requires the participation of additional human cellular component(s) in the target membrane (4). Recent studies have shown that chemokine receptors serve as coreceptors for HIV-1 entry and fusion (5,6). Since then, several reports have indicated that alternative cofactors might also play a role in HIV-1 entry (7,8).

Lipids are integral elements in the membrane fusion process (3,9). Specific glycolipids are known to serve as receptors for influenza (10) and Sendai (11) whereas sphingomyelin and cholesterol are required for the fusion of Semliki Forest virus (12,13). HIV-1 can enter neuronal cells by a CD4-independent pathway by binding to the neutral glycolipid galactosyl ceramide (Gal Cer) and related compounds(14,15) albeit at very low efficiency. Recent studies by Delezay et al (16) suggest a role for glycolipids in CD4-mediated HIV-1 fusion based on selective interaction of a V3 loop multimeric peptide, SPC3 with target membrane glycolipids. On the other hand, contribution of lipid components in CD4-mediated HIV-1 fusion and entry has not been directly demonstrated.

Experiments from our laboratory and others have shown that the transfer of heat and protease resistant human erythrocyte (huRBC) membrane components to $CD4^+$ non-human cells (17,24) complements fusion of modified cells. In this study, we investigated the possible contribution of huRBC glycolipids (HuGL) in HIV-1 fusion. We first modified the glycolipid levels in $CD4^+$ human cells by treatment with reagents which inhibit early steps in glycolipid but not glycoprotein synthesis (18,19). We then isolated glycolipids from erythrocytes and utilized influenza hemagglutinin (HA)-mediated fusion of GL-containing liposomes to add GL to the plasma membrane of CD4-expressing mink or mouse fibroblast cells. We studied fusion of GL-modified $CD4^+$ cells using a fluorescence dye transfer assay (17). We report here that (i) Reduction in cellular glycolipids of $CD4^+$ human cells decreases their fusion efficiency which can be rescued by addition of HuGL but not

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Abbreviations used: HIV-1, Human Immunodeficiency Virus type 1; HA, influenza hemagglutinin; D10, DMEM supplemented with 10% heat inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin; R10, RPMI-1640 supplemented with 10% fetal bovine serum and 100 units/ml penicillin, 100 μg/ml streptomycin; PBS, phosphate buffered saline containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 pH 7.4; GP4F-CD4, an HA-expressing mouse fibroblast cell line transiently expressing human CD4; CMFDA, 5-chloromethylfluorescein diacetate; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine perchlorate; HuRBC, human erythrocytes, GL, glycolipid(s); HuGL, glycolipids isolated from human erythrocytes; BoGL, glycolipids isolated from bovine erythrocytes; Gal Cer, galactosyl ceramide; PPMP, 1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol; egg PC, egg L-α- phosphatidylcholine; egg PE, egg phosphatidylethanolamine; NBD-PE, N-(7-nitro-2-1,3-benzoxadiazol-4-yl)diacyl phosphatidylethanolamine; TLC, thin layer chromatography.

0006-291X/98 $25.00

BoGL; (ii) Incorporation of HuGL, but not BoGL into $CD4^+$ non-human cells renders those cells fusion competent with cells expressing HIV-1 envelope glycoprotein; and (iii) Fusion between HuGL-modified non-human cells and gp120-gp41 expressing cells occurs in a CD4-dependent manner. These observations taken together demonstrate that human erythrocyte glycolipids promote CD4-mediated HIV-1 fusion. To our knowledge this report is the first direct demonstration that human erythrocyte glycolipids play a role in HIV-1 envelope glycoprotein-mediated fusion with $CD4^+$ cells.

MATERIALS AND METHODS

Fluorescent probes were from Molecular Probes (Eugene, OR) and tissue culture media were from Gibco BRL (Life Tech, Gaithersburg, MD). Phospholipids were from Avanti Polar Lipids (Alabaster, Alabama). Other reagents were from Sigma Chemical Co. (St. Louis, MO).

Isolation of glycolipids from erythrocytes. Glycolipids (GL) were extracted using the standard protocols for glycolipid extraction by Matreya Inc. (Pleasant Gap, PA). Briefly, freeze-dried erythrocytes were solubilized in a mixture of chloroform, methanol and water (2:1:0.1 v/v). The insoluble proteins were removed by filtration and the solvents were removed under reduced pressure. The residue was treated with dilute potassium hydroxide in methanol followed by reneutralization with acetic acid. After the removal of solvents, the mixture, containing fatty acids, GL and glycerol phosphate was redissolved in minimum volume of chloroform:methanol (2:1, v/v) and was added to 40 volumes of acetone. The GL were precipitated by cooling the mixture to −20°C, and collected by filtration. Presence of GL in the extracts was detected by TLC analysis on Silica gel −60 plates (Whatman). 25-50 μg of the samples were spotted and the plates were developed in chloroform:methanol:water (65:25:4, v/v). The glycolipid spray reagent (Bials' reagent, Sigma Chemical Co.) was used to identify the GL.

Cells. Mink-CD4, GP4F and TF228 cells were obtained and grown as described (17). Human CD4 was transiently expressed on the surface of GP4F cells (GP4F-CD4) as described previously (17). HeLa-CD4 or HeLa-env cells were generated by MuLV-mediated transduction (gift of Dr. John Silver, NIAID, NIH) and were grown in D10. SupT1 cells were maintained in R10. HIV envelope proteins were transiently expressed on the surface of Hela cells using the recombinant vaccinia constructs vSC50 (HIV-2env) and vPE16 (HIV-1env) as described (20).

Treatment of $CD4^+$ human cells with PPMP. SupT1 or Hela-CD4 were grown in medium containing 10 μM PPMP (Matreya, Inc.) for at least 7 days.

Addition of glycolipids to $CD4^+$ cells. Liposomes were prepared from a mixture of egg PC: egg PE: glycolipids (3:1.5:1, w/w/w) in phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBS) and passed through a 0.2 μm filter using an extruder (Lipex Biomembranes, Inc., Vancouver, BC). HA was expressed on the surface of $CD4^+$ cells and converted to its fusogenic form by trypsin as described in (17). Liposomes (1 ml, containing 0.9 mg lipid) were bound to HA-expressing cells for 30 min at room temperature in the presence of 1 μg wheat germ agglutinin. Liposome-cell fusion was induced by brief exposure (60 sec) of the cells to pH 5.1, followed by incubation in D10 for 30 min at room temperature (17).

To quantitate liposome-mediated GL transfer to $CD4^+$ cells, a lipophilic fluorescent probe NBD-PE (ex/em 460/534 nm, 15 mol%) was also included during liposome preparation. Liposomes were fused via HA as described above, and NBD-PE fluorescence in the presence or absence of HA-mediated fusion was monitored using a cooled CCD camera (Princeton Instruments, Trenton, NJ) attached to an Olympus IX70 microscope, 40× oil immersion lens and the U-MNIBA filter cube (470-490 nm ex, 505 nm dichroic mirror, 515-550 nm em). Cell boundaries were outlined using the phase images, and the intensity of NBD signal within the boundary was calculated. After the average NBD signal intensities were obtained for the entire experiment, the data were binned into histograms, allowing comparison of the relative extent of liposome-cell fusion before and after activation of the HA.

HIV-1 envelope glycoprotein-mediated cell–cell fusion. To monitor cell-cell fusion we used the following cell pairs: SupT1/HeLa-env, HeLa-CD4/TF228, GP4F-CD4/TF228, and mink-CD4/TF228 cells. Briefly, the adherent cell lines were plated on microwells and labeled with the cytoplasmic dye CMFDA (10 μM, ex/em 492/516 nm). TF228 or SupT1 cells were labeled with the fluorescent membrane probe DiI as follows: 5×10^6 cells were washed and resuspended in 0.5 ml PBS and incubated with 5 μl DiI (10 mM,ex/em 560/590 nm) in 0.1 ml Diluent C (Sigma, cat # CGL-DIL) for 2-5 min. Unincorporated dye was removed by incubation with R10. 10^5 suspension cells were cocultured with their respective fusion partners for 2-4 hours at 37°C. For assays in which the $CD4^+$ cells were treated with PPMP, all incubation media contained the inhibitor. At the end of incubations, the phase and fluorescence images were collected as described above using the U-MNG filter cube (530-550 nm ex, 570 nm dichroic mirror, 590 nm high pass em) for DiI observation and the U-MNIBA filter cube (described above) for CMFDA. Cell-cell was fusion was determined as previously described (17).

RESULTS

Pretreatment of $CD4^+$ Human Cells with Glycolipid Synthesis Inhibitors Impairs Their Fusion Activity

In our first series of experiments, we studied the effect of inhibition of glycolipid synthesis in $CD4^+$ human cell lines upon their subsequent fusion with TF228 cells. We used PPMP to reduce the levels of cellular glycolipids. PPMP is known to block the early steps in the glycolipid but not glycoprotein biosynthetic pathway (18,19).

We cultured SupT1 and HeLa-CD4 cells with PPMP for at least 7 days and monitored the fusion of these cells with TF228 cells using the dye-transfer assay. The details of the assay are described in the Methods section and (17). The results are shown in Figure 1. As can be seen in Figure 1A, fusion between SupT1 and Hela-env cells was reduced by nearly 50%in PPMP-treated cells, as compared to fusion with uninhibited SupT1 cells. We also observed similar inhibition patterns when HeLa-CD4 cells were used as targets (Figure 1B). The inhibition of fusion was reproducible from at least three independent experiments. To rule out the possibility of an adverse effect of PPMP on the general state of HeLa-CD4 cells, we monitored fusion of PPMP-treated HeLa-CD4 cells with HIV-2 envelope expressing cells. The results are shown in Figure 1C. It is clear from the figure that treatment of HeLa-CD4 cells with PPMP did not impair their fusion potential.

Incorporation of Glycolipids into $CD4^+$ Nonhuman Cells

Glycolipids were isolated from human or bovine erythrocytes and analysed by TLC as described in the

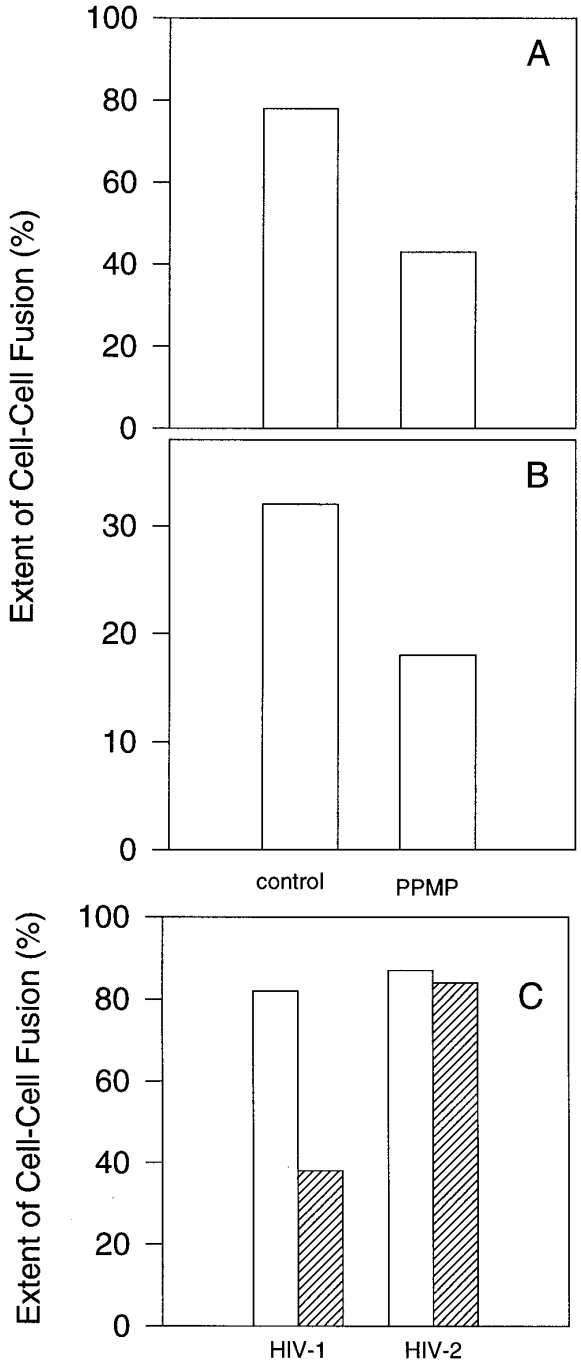

FIG. 1. Effect of glycolipid synthesis inhibition on HIV-1 fusion: Target cells were grown in the presence of 10 μM PPMP for at least 7 days. The cells were labeled with fluorescent probes as indicated and were cocultured with the effector cells for 2 hours at 37°C. After incubation, the cells were examined for the presence of both dyes in a single cell and extent of fusion was calculated as described in Methods section. A, 10^5 DiI-labeled SupT1 cells cocultured with 5×10^4 CMFDA-labeled Hela-env cells. B, 10^5 DiI-labeled TF228 cells cocultured with 5×10^4 CMFDA-labeled Hela-CD4 cells. C, Effect of glycolipid synthesis inhibition on HIV-2 fusion. 5×10^4 Hela cells were plated on microwells and infected with vPE16 (expressing HIV-1 envelope) or vSC50 (expressing HIV-2 envelope) for 14-18 hours 37°C, labeled with CMFDA and cocultured with DiI-labeled SupT1 cells for 2 hours at 37°C.

Methods section. Figure 2A shows the TLC of the glycolipids used in this study. As can be seen in the figure, human (lane 1, HuGL) and bovine (Lane 2, BoGL) glycolipids have substantially different glycolipids compositions.

To assess the role of glycolipids in HIV-1 envelope glycoprotein-mediated fusions, GL were incorporated in liposomes containing NBD-PE and transferred to mink-CD4 cells by HA-mediated fusion as described above. Images were acquired for phase and fluorescence as described. Since liposomes were approximately 0.2 μm in diameter, they were visible as faintly fluorescent punctate objects in the bound state. Upon fusion, NBD-PE fluorescence redistributed on the large surface area of the cells and could be monitored using a cooled CCD camera. Liposomal lipid transfer was quantitated as described in Methods section. Histograms of fluorescence intensity for the population of cells in a field are shown in Figure 2B. Fluorescence signal increased in the cells upon fusion with liposomes containing glycolipids (Figure 2B, a, human and b, bovine) when HAo was converted to fusion active HA with trypsin (21). On the other hand, there was very low levels of fluorescence intensity associated with the cells in the absence of HA-mediated liposome fusion (Figure 2B, c, human and d, bovine). These data show that similar amounts of human (Figure 2B, a) and bovine (Figure 2B, b) glycolipids were transferred to the target cells. This method presented here is an improved quantitative method for studying fusion between liposomes and cultured cells.

Glycolipids from Human Erythrocytes Mediate Fusion between CD4$^+$ Nonhuman and gp120-gp41-Expressing Cells

To analyze the recovery of HIV-1 fusion after the addition of glycolipids to mink-CD4 cells, the GL-modified mink-CD4 cells were labeled with a cytoplasmic dye, CMFDA and cocultured with DiI-labeled TF228 cells for 4 hours at 37°C. At the end of the incubation, cell-cell fusion was monitored by analyzing the overlap of fluorescent probes as described in the Methods section and (17). We quantitated fusion by collecting 5-7 images for each sample and counting the fraction of cells positive for both fluorescent probes. These data are shown in Figure 3. It is evident from the graph that fusion occurred only when HuGL (and not BoGL) were transferred to mink-CD4 cells. We did not observe any increase in dye transfer above background when HA was not activated (Figure 3) or when mink cells (CD4 negative) were used as targets (data not shown). Therefore incorporation of HuGL into CD4$^+$ non-human cells renders those cells susceptible to HIV-1 fusion. We obtained similar results in at least three independent experiments using glycolipids isolated from different batches of human erythrocytes. We also made

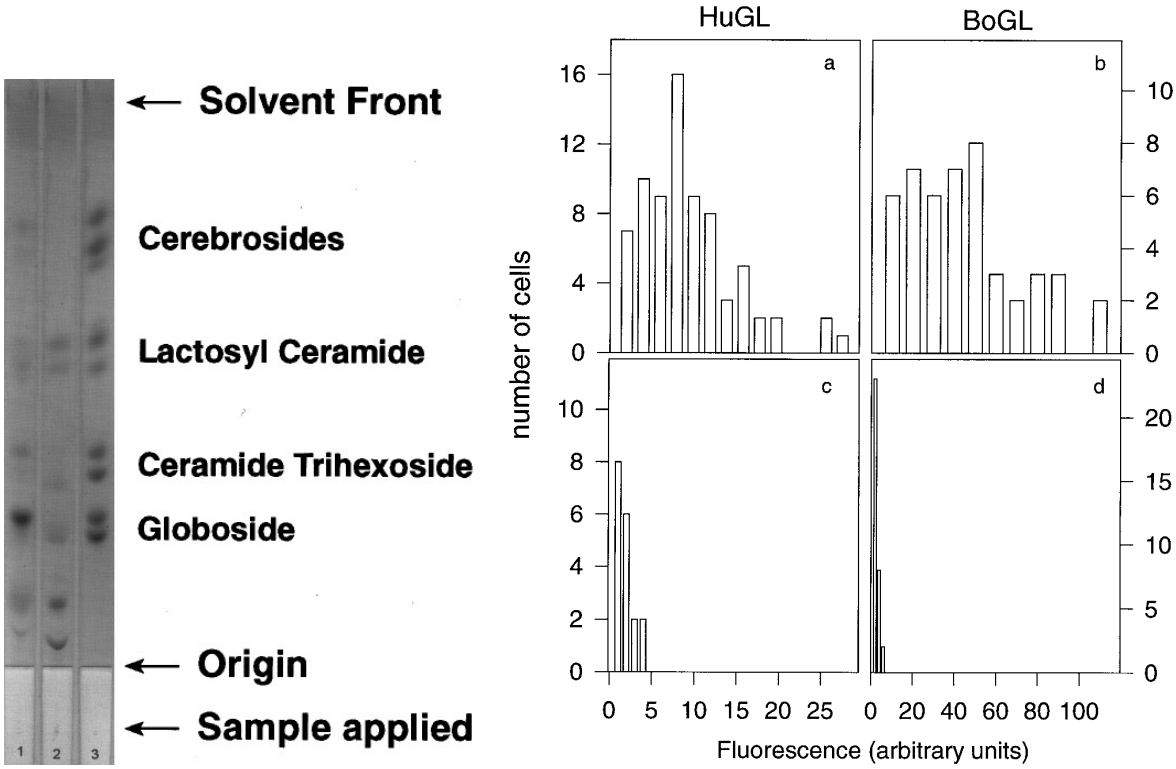

FIG. 2. TLC analysis of glycolipids used in this study: Glycolipids were extracted from erythrocytes and TLC was run as described in the Methods section The TLC plate was scanned using a Hewlett-Packard 4P scanner. 1, HuGL, 2, BoGL, 3, neutral glycolipid qualmix standard (Matreya, Inc). B. Incorporation of glycolipids to mink-CD4 cells: Mink-CD4 cells were infected with influenza virus for 4-5 hours at 37°C and cell surface-expressed HA was activated by trypsin. HAo was not activated in control samples. Binding and fusion of GL-containing liposomes was done as described in Methods section. The images were acquired and quantitation of NBD-PE fluorescence in individual cells (which reflects transfer of liposomal glycolipids) was done as described in the Methods section. a and b, fusion of GL-containing liposomes via HA; c and d, HA0 controls without fusion. a and c, HuGL; b and d, BoGL.

similar observations when GP4F-CD4 cells were used as the targets (Table 1). We did not observe any significant increase in dye-transfer above background levels when Gal Cer was incorporated into $CD4^+$ non-human cells under identical conditions (Table 1).

The Recovery of HIV-1 Fusion is CD4-Dependent

Previous reports have shown that HIV-1 can enter neuronal cells by a CD4-independent pathway by utilizing Gal Cer as alternative receptor (14,15).Therefore we wished to determine whether recovery of HIV-1 fusion by HuGL was due to the presence of Gal Cer or related compounds in the human glycolipid mixture or fusion required primary interaction of gp120-gp41 with cellular CD4. We studied the fusion of HuGL-modified cells in the presence of reagents, which are known to block CD4-dependent HIV-1 entry and fusion in vitro (22,23). The results are shown in Table 1. Fusion between HuGL-modified mink-CD4 or GP4F-CD4 cells and TF228 cells was inhibited in the presence of sCD4 or an anti-CD4 antibody OKT4A (Table 1). Furthermore, when HuGL were incorporated into CD4-negative cells we did not observe any dye-transfer above background indicating a lack of fusion recovery (unpublished observations). These results show that HuGL are required for HIV-1 fusion via the CD4-dependent pathway.

Addition of HuGL to Inhibitor-Treated Cells Rescues HIV- 1 Fusion

To further assess the specific contribution of HuGL in gp120-gp41 mediated cell-cell fusion, we tested the ability of HuGL to rescue fusion of PPMP- treated HeLa-CD4 cells. We added glycolipids to inhibitor-treated Hela-CD4 cells by HA-mediated fusion of glycolipid-containing liposomes and monitored fusion with TF228 cells as described in the Methods section. The results are shown in Figure 4. Treatment of HeLa-CD4 cells with PPMP reduced fusion to about 50% as compared to fusion of untreated cells. Addition of HuGL to the inhibitor-treated cells rescued fusion to the original levels. On the other hand, addition of BoGL under identical conditions did not result in reversal of inhibition by PPMP.

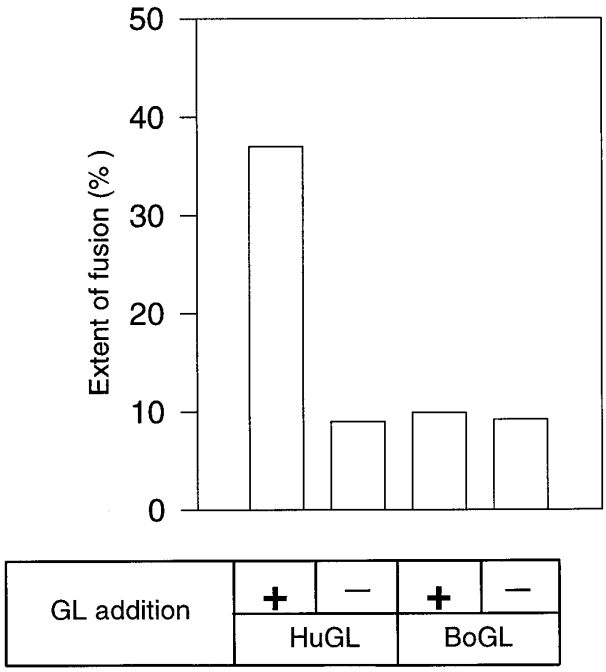

FIG. 3. Glycolipids from human erythrocytes mediate fusion between $CD4^+$ non-human and gp120-gp41 expressing cells: HA was expressed on the surface of mink-CD4 cells and activated by trypsin as described in the Methods section. Glycolipids were transferred via liposomes as described in the Methods section. GL-modified mink-CD4 cells were labeled with CMFDA and cocultivated with DiI-labeled TF228 cells for 4 hours at 37°C. To quantitate fusion, 5-7 images were collected as described above and percent fusion was calculated as described in the text.

DISCUSSION

It is well-established that distinct lipids serve as viral receptors and/or fusion prerequisites for viruses such as influenza (10), Sendai (11) and Semliki Forest virus(12,13). Gal Cer and related compounds act as receptor for the HIV-1 fusion which occurs in the absence of primary receptor CD4(14,15). To our knowledge, a requirement of specific lipids in CD4-mediated HIV-1 fusion has not been directly demonstrated. Glycolipids possess extreme structural diversity because of the nature and number of sugars, branching type and stereo specificity of chemical bonds(25). It is conceivable that specific GL(s) may play a role in HIV-1 entry (26). We undertook these studies to evaluate the participation of glycolipids in CD4-dependent HIV-1 fusion.

The obvious approach to examine the role of specific lipids in HIV-1 entry is to develop model systems for HIV-1 fusion (reconstitution of viral or target lipids and/or proteins). Unfortunately such attempts have not been successful in the study of membrane fusion mediated by CD4-gp120 interaction. Therefore, we studied the role of human erythrocyte glycolipids by manipulating the levels of glycolipids in the target cells. We reduced the glycolipid levels in fusion-competent $CD4^+$ human cells by pre-incubation with the inhibitors which are known to block early steps in glycolipid but not glycoprotein synthesis (18,19). Alternatively, we incorporated glycolipids into the plasma membrane of $CD4^+$ non-human cells which normally do not fuse with gp120-gp41 expressing cells.

Our data on the effect of glycolipid synthesis inhibitors are in agreement with previous findings (26). Although Picard et al (26) used PDMP and significantly longer incubation periods (50-100 days) to inhibit glycolipid synthesis, they observed about 46% inhibition of HIV-1 entry. We treated our cells for 7-10 days and used PPMP (instead of PDMP), which has higher specific activity than PDMP, and obtained a similar reduction in fusion activity (see Fig. 1A and B). The reduced fusion was not due to impaired fusion ability of inhibited cells, because these cells efficiently fused with HIV-2 envelope glycoprotein-expressing cells (Figure 1C).

Although techniques are available to express protein molecules of interest on the cell surface at reasonably high levels, such an approach can not be applied to modify lipid levels in the cell membranes. We initially tried direct methods to incorporate GL to the target cell membranes, but the modified cells showed very low levels of cell-cell fusion above background using the microscopic fluorescence dye-transfer assay (unpublished results). The limited fusion is probably due either to inefficient incorporation of GL or failure of the

TABLE 1

Glycolipid-Mediated Complementation of HIV-1 Fusion Occurs via CD4-Dependent Pathway

$CD4^+$ cells[a]	Glycolipid addition[b]	Inhibitor[c]	Fusion (%)[d]
Hela	none	none	46
	HuGL	none	65
Mink	none	none	05
	HuGL	none	35
	HuGL	sCD4	13
	HuGL	OKT4a	8
GP4F	none	none	4
	HuGL	none	20
	HuGL	sCD4	8
	HuGL	OKT4a	8
	Gal Cer	none	8

[a] HA was expressed on HeLa-CD4 or mink-CD4 cells as described in the Methods section. Alternatively, CD4 was expressed on the surface of GP4F cells in Methods section.

[b] Cells were modified with HuGL or Gal Cer, labeled with CMFDA and cocultured with DiI-labeled TF228 cells for 4 hours at 37°C as described in the Methods section.

[c] 0.1 mg/ml sCD4 or 5 μg/ml OKT4A were added during coculture of $CD4^+$ and TF228 cells.

[d] Similar results were obtained from two independent experiments.

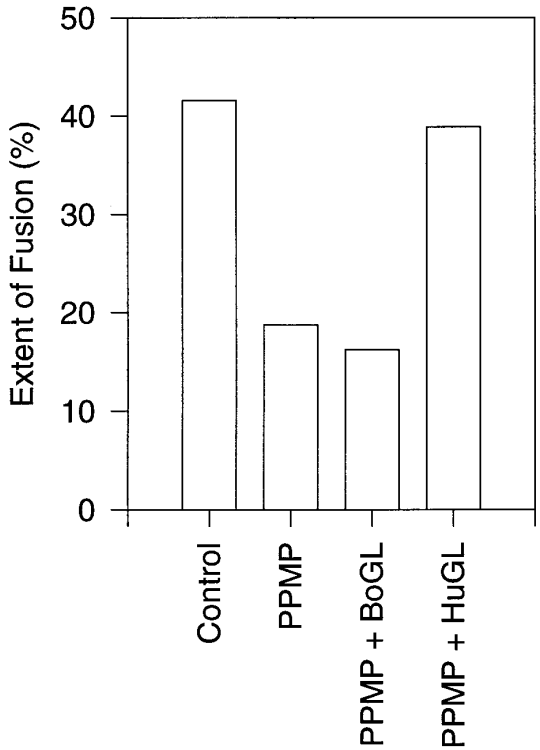

FIG. 4. Rescue of fusion by addition of human glycolipids to glycolipid-modified $CD4^+$ human cells. Hela-CD4 cells were treated with PPMP, plated on microwells, modified by the addition of GL and labeled with CMFDA as described in the Methods section. The cells were cocultured with DiI-labeled TF228 cells for 2 hrs at 37°C. Fusion was quantified as described in the Methods section.

GL to orient properly in the membrane. To obtain sufficient incorporation of GL into non-human cells, we induced fusion of GL-containing liposomes with the target cells using influenza HA (17). We quantitated the transfer of liposomal GL by observing redistribution of a fluorescent probe NBD-PE from GL-containing liposomes to the target cells (Figure 2B).

Addition of HuGL but not BoGL to mink-CD4 cells resulted in recovery of fusion of the modified cells with gp120-gp41 expressing cells (Figure 3). Fusion between HuGL-modified $CD4^+$ non-human and TF228 cells was inhibited in the presence of sCD4 and OKT4A (Table 1). Thus, human glycolipid(s) are involved in fusion mediated by CD4/gp120-gp41 interaction. Our observations support the notion that certain glycolipids might be involved in HIV-1 fusion (16). It is possible that one or more glycolipids in the HuGL mixture participate in promoting CD4-dependent HIV-1 fusion. This process is distinct from the previously reported role of Gal Cer in HIV-1 fusion because the effect we see occurs via the CD4-mediated pathway. Complementation of fusion by HuGL is much more efficient than by Gal Cer as evidenced by the lack of fusion seen in our assay after addition of Gal Cer to GP4F-CD4 cells (Table 1). Recently Feng et al (5) have reported that mink-CD4 cells expressing high levels of human CXCR4 become susceptible to HIV-1 fusion in the absence of added HuGL. To reconcile our data with those observations, we postulate that in the normal case when the cell is not forced to incorporate large amounts of CXCR4, the glycolipid might facilitate the formation of the gp120-gp41-CD4-CXCR4 complex leading to membrane fusion.

In summary, we have demonstrated that human erythrocyte glycolipids are involved in HIV-1 envelope-glycoprotein mediated cell-cell fusion. The nature of molecular species and the chemical structure of the specific glycolipid(s) which participate in the fusion process will require isolation, purification and structural analysis of individual glycolipids from the crude mixture.

ACKNOWLEDGMENTS

We thank Dr. Paul Clapham for the mink-CD4 cell line, Dr. Zdenka L. Jonak for the TF228.1.16 cell line, Dr. John Silver for HeLa cell lines and Dr. Judith White for GP4F cells. We thank Drs Edward Berger, Chris Broder, Patricia Earl, and Bernard Moss for vaccinia recombinants. SupT1 cells were obtained though NIH AIDS Research and Reference Reagent Program. This work was supported by the NIH Intramural AIDS Targeted Antiviral Program. We thank Dr. David Roberts for insightful comments on glycolipids. I. M.-B. is a postdoctoral fellow of the Direccion General de Investigacion Cientifica y Enseñanza Superior, MEC, Spain.

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