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Galactosyl Ceramide (or a Closely Related Molecule) Is the Receptor for Human Immunodeficiency Virus Type 1 on Human Colon Epithelial HT29 Cells

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The gastrointestinal tract is considered to be a major route of infection for human immunodeficiency virus (HIV). Infection of human colon epithelial cells by HIV is not blocked by anti-CD4 antibodies known to block infection of lymphoid cells (J. Fantini, N. Yahi, and J. C. Chermann, Proc. Natl. Acad. Sci. USA 88:9297–9301, 1991), suggesting the presence of an alternate receptor for HIV on these cells. In this report, we show that (i) a monoclonal antibody specifically directed against galactosyl ceramide inhibited the infection of HT29 cells by two markedly different strains of HIV-1, as assessed by polymerase chain reaction amplification and reverse transcriptase assay; (ii) this antibody strongly labeled the surface of HT29 cells by immunofluorescence and electron microscopic immunolocalization; (iii) the labeling was preferentially but not totally restricted to the basolateral membrane domain of differentiated colonic cells, in agreement with the ability of HIV to infect both the apical and basolateral surfaces of these epithelial cells; and (iv) in thin-layer chromatography-immunostaining experiments with neutral glycolipids prepared from HT29 cells, the antibody specifically reacted with a ceramide monoglycoside fraction corresponding to galactosyl ceramide. We did not detect this glycolipid in lymphoid cells, and anti-galactosyl ceramide antibodies consistently failed to inhibit HIV infection of these cells. These data suggest that galactosyl ceramide (or a derivative) is an essential component of the receptor for HIV on the surface of HT29 cells.

It is now well established that CD4, a 55-kDa glycoprotein expressed by lymphocytes, monocytes, and macrophages, is the main receptor for human immunodeficiency virus (HIV) (21). However, HIV infection in CD4-negative cells such as fibroblasts (26) or brain-derived cells (13) has been reported, suggesting the existence of one or more alternate receptors for HIV. Recently, Harouse et al. have screened antibodies against neural cell surface components for their ability to block HIV infection of neural cell lines (13). This approach led to the identification of a sphingolipid, galactosyl ceramide (GalC), as a putative cellular receptor for HIV in neural cells on the basis of the following data (2, 13): (i) antibodies against GalC inhibited entry of HIV into neural cells, and (ii) recombinant gp120 specifically bound to GalC and its derivative, galactosyl sulfatide. These results prompted us to test anti-GalC antibodies for inhibition of HIV infection in human colon epithelial cells of the HT29 cell line. Indeed, HIV infection of HT29 cells was not inhibited by anti-CD4 antibodies known to block infection of lymphoid cells, despite the fact that these cells expressed an immunologically related CD4 antigen whose function remains unclear (10, 28). Since the colorectal mucosal surface epithelium represents a major route of HIV infection, identification of the epithelial receptor for HIV is of fundamental importance. In this report, we present evidence that GalC or a closely related molecule is an essential component of this receptor.

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MATERIALS AND METHODS

Antibodies. The monoclonal antibody (MAb) anti-GalC is a mouse immunoglobulin G3 (IgG3) purchased from Boehringer Mannheim. In preliminary experiments, this antibody was kindly provided by Barbara Ranscht (19). Anti-GalC is highly specific for GalC and, to a lesser extent, for its sulfated derivative, galactosyl sulfatide, but it does not cross-react with sphingosine, ceramide, mixed ganglioside, or glucosyl ceramide (19). OKT4A (Ortho Diagnostics) and Leu3A (Becton-Dickinson) are two anti-CD4 MAbs directed against the binding site for gp120 (20). IOT-2 (Immunotech) is a mouse MAb directed against HLA class I antigens.

Cell culture. The human colon adenocarcinoma cell lines HT29 (ATCC HTB38) and T-84 (a generous gift from Bernard Verrier) and the clonal subpopulation HT29-D4 (7) were routinely grown in Dulbecco's modified Eagle's medium (DMEM)-Ham's F12 medium (1:1 [vol/vol]) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.4). Differentiated HT29-D4 cells were cultured in glucose-free DMEM supplemented with 5 mM galactose and 10% FCS (5). When indicated, HT29-D4 cells were grown on Nuclepore filters (pore size, 3 μm) in two-compartment Transwell cell culture chambers (Costar) as previously described (8). Human T-lymphoblastoid CEM-CL5 cells (27) were grown in RPMI 1640 supplemented with 10% FCS. Human peripheral blood lymphocytes (PBL) obtained from normal seronegative donors were separated by Ficoll-Hypaque gradient centrifugation, cultured in RPMI 1640 supplemented with 10% FCS, and stimulated with phytohemagglutinin 3 days before infection.

Virus stocks. The viruses used were the prototype HIV-1

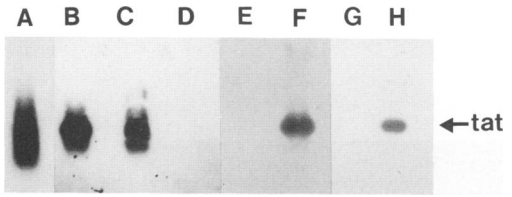

FIG. 1. Detection of HIV-1 DNA sequences by PCR and Southern blot hybridization. HT29 cells were incubated with either a anti-GalC MAb (lanes E and G) or anti-CD4 MAb OKT4A (lanes F and H) for 2 h at 4°C. The cells were then exposed to HIV-1 LAV (lanes G and H) or HIV-1 NDK (lanes E and F) for 16 h at 37°C (0.05 $TCID_{50}$ per cell for both strains) in the continuous presence of antibodies, washed extensively, and treated with trypsin to remove excess inoculum. After three subcultures obtained by trypsinization (12 days postinfection), total cellular DNA was extracted and subjected to HIV-specific amplification with nucleotide primers from *gag* and *tat* genes. The amplified products were analyzed on a 1.5% agarose gel, transferred to a nylon membrane, and hybridized with radiolabeled probes specific for *gag* and *tat* sequences (the autoradiogram shows the results obtained with the *tat* probe). Positive and negative controls for PCR were CEM cells chronically infected with HIV-1 LAV, used as a positive control (A), HT29 cells infected with HIV-1 NDK (0.05 $TCID_{50}$ per cell), 3 months postinfection (B), HT29 cells infected with HIV-1 LAV (0.05 $TCID_{50}$ per cell), 3 months postinfection (C), and T-84 cells 3 months after exposure to HIV-1 LAV (0.05 $TCID_{50}$ per cell) (D) (this isolate did not infect T-84 cells [10] and consistently gave no PCR signal).

TABLE 1. RT activity in HT29 cells or PBL infected with two strains of HIV-1 in the presence of MAbs[a]

Cell type	Expt	RT activity (cpm/ml)	
		HIV-1 LAV	HIV-1 NDK
HT29 cells[b]	Negative control	196, 348	362, 142
	No antibody	658,396, 496,128	432,764, 584,772
	OKT4A	692,126, 712,226	474,656, 373,680
	Anti-GalC	512, 1,078	170, 364
PBL[c]	Negative control	402, 292	674, 346
	No antibody	33,780, 36,712	99,552, 140,336
	Leu3A	666, 306	98,848, 117,658
	Anti-GalC	37,012, 24,038	51,768, 80,894

[a] Cells were pretreated with MAb anti-GalC, OKT4A, or Leu3A (10 μg/ml) for 2 h at 4°C and exposed to HIV-1 for 1 h at 37°C in the continuous presence of MAbs. In the negative control experiment, cells were first incubated at 4°C and then at 37°C with PBS alone. Control of infection was assessed by incubating the cells at 4°C in the absence of antibodies and then exposing the cells to HIV-1 at 37°C.

[b] After the indicated sequence of incubations, HT29 cells were washed and subcultured by trypsinization as indicated in Materials and Methods. At the third and fourth subcultures (days 12 and 20 postinfection), HT29 cells were cocultivated with indicator CEM-CL5 cells. After 24 h, CEM-CL5 cells were harvested, cultured in separate flasks, and tested for RT activity. The results shown are from two separate experiments performed on two successive passages of HT29 cells.

[c] PBL were washed by dilution in culture medium and assayed for RT activity. RT determinations were performed at days 16 (first entry in each column) and 20 (second entry in each column) postinfection.

strain LAV (1) and the Zairian strain NDK, which is highly cytopathic for T lymphocytes (4). Culture supernatants from PBL infected with HIV-1 LAV or HIV-1 NDK were passed through 0.22-μm-pore-size membrane filters (Millipore) and stored aliquoted at −80°C until use. Values of 50% tissue culture infectious doses ($TCID_{50}$) per milliliter of these viral stocks were 5×10^4 and 5×10^5 for HIV-1 LAV and HIV-1 NDK, respectively.

HIV-1 infection of cells. Exponentially growing HT29 cells were exposed at 37°C for 16 h to HIV-1 at a multiplicity of infection of 0.05 $TCID_{50}$ per cell. After extensive washing, cells were treated with trypsin to remove excess inoculum (25), harvested, and subcultured several times before analysis at day 12, 20, or 90 postinfection. PBL were washed in culture medium and then exposed to viral inocula (0.05 $TCID_{50}$ per cell) for 1 h at 37°C. Infected cells were washed and resuspended in complete medium supplemented with interleukin-2 (30 IU/ml; Sanofi), Polybrene (2 μg/ml), and anti-alpha interferon serum (1).

Inhibition of infection by anti-GalC MAbs. HT29 cells were treated at 4°C for 1 h with 10 μg of either anti-GalC or OKT4A and subsequently exposed to HIV-1 (0.05 $TCID_{50}$ per cell) for 16 h at 37°C in the continuous presence of MAbs. After extensive washing, the cells were trypsinized and subcultured three times before analysis.

RT assay. Levels of reverse transcriptase (RT) activity in culture supernatants were determined as previously described (10).

PCR. After extensive washing in cold sterile phosphate-buffered saline (PBS), HIV-infected cells were lysed in situ in 10 mM Tris-HCl (pH 8.3)–50 mM KCl–2.5 mM $MgCl_2$–0.01% gelatin–0.45% Tween 20–0.45% Nonidet P-40–200 μg of proteinase K per ml and digested overnight at 56°C. The reaction was stopped by incubation at 95°C for 10 min. Samples (25 μl of lysate corresponding approximately to 1.25×10^5 cells) were submitted to HIV-specific DNA amplification with nucleotide primers homologous to the HIV-1 LAV sequence ranging from 949 to 976 and 1177 to 1204 (*gag*-specific primers) or from 5419 to 5446 and 5563 to 5590 (*tat*-specific primers) and 2 U of *Taq* polymerase (Cetus) per ml. The following sequence of amplification was used: 5 min at 94°C for initial denaturation and then 30 cycles (1 min at 94°C, 45 s at 55°C, and 2 min at 72°C), followed by 5 min at 72°C before cooling at 4°C. The polymerase chain reaction (PCR) products were loaded on a 1.5% agarose gel, electrophoresed, and transferred to a Hybond N+ nylon membrane (Amersham), using the Southern blot technique. The probes for detection of PCR products after *gag*- and *tat*-specific amplification were oligonucleotides spanning positions 1061 to 1065 and 5463 to 5472, respectively, of the HIV-1 LAV genome. All primers and probes used were kindly provided by CIS Bio International, Grenoble, France.

Immunofluorescence labeling. Indirect immunofluorescence was performed on paraformaldehyde-fixed HT29 cells as described previously (7). Anti-GalC was used at a concentration of 10 μg/ml, and the labeling was revealed with a tetramethylrhodamine isothiocyanate (TRITC)–anti-mouse goat conjugate (dilution of 1/50) purchased from Sigma. Immunofluorescent staining of CEM-CL5 cells was performed as previously described (27) with anti-CD4 MAb OKT4A (10 μg/ml), anti-HLA class I MAb IOT-2 (2 μg/ml), or anti-GalC (10 μg/ml).

Electron microscopic immunolocalization. HT29 cells were washed in PBS and incubated for 2 h at 4°C with the anti-GalC MAb at a concentration of 10 μg/ml. Cells were washed three times with ice-cold PBS and further incubated with a 1/25 dilution of gold-conjugated protein A (Sigma). After three washes in PBS, cells were fixed with glutaraldehyde and processed for electron microscopy as previously described (7).

TLC-immunostaining. Folch lower-phase lipids (11) extracted from HT29 cells were chromatographed on Silica Gel 60 plates (Merck, Darmstadt, Germany), using chloroform-methanol-water (60:35:8, vol/vol/vol) as the solvent. Neutral

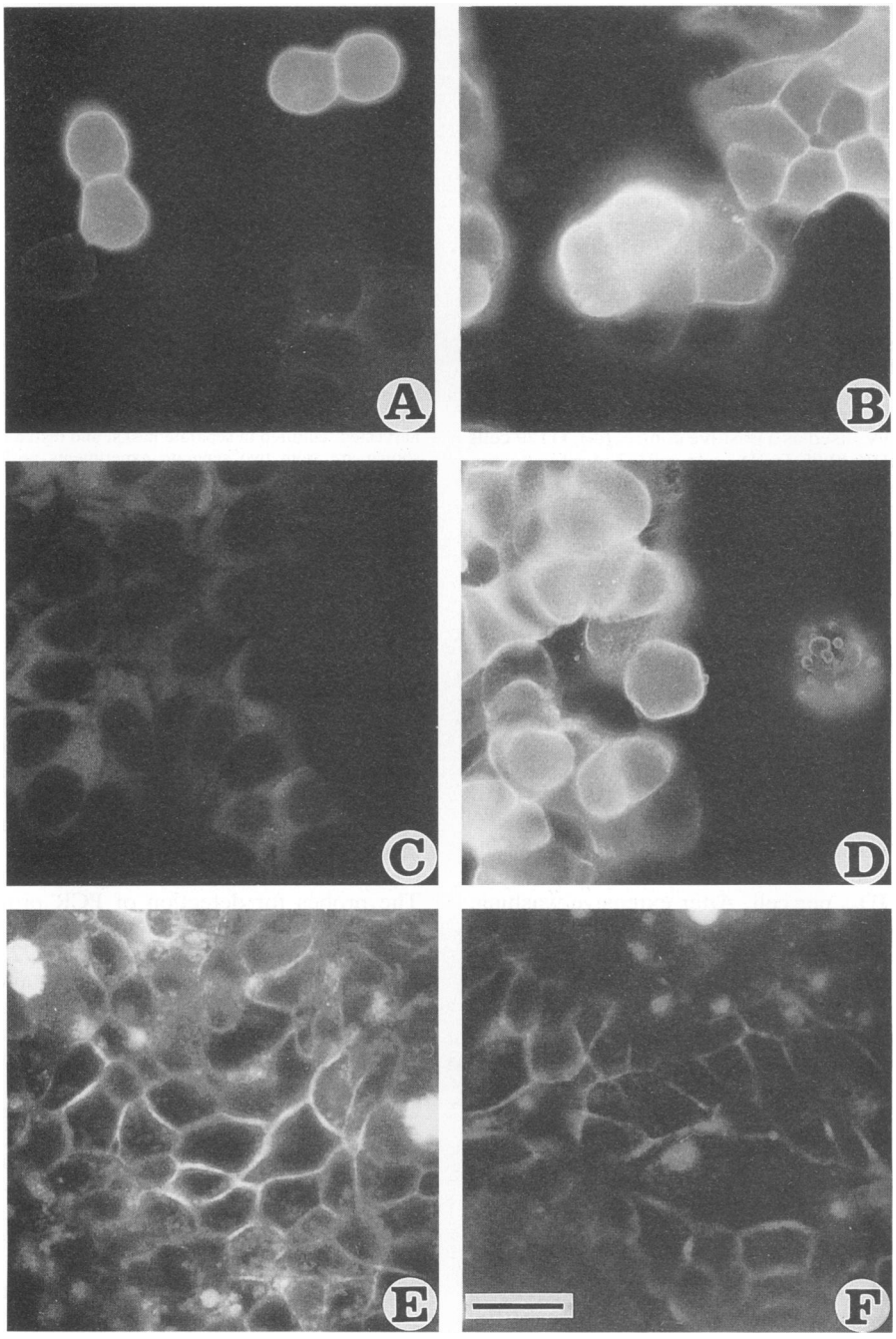

FIG. 2. Immunofluorescence labeling of HT29 cells with an anti-GalC MAb. Noninfected HT29 cells in the active phase of growth were labeled with an anti-GalC MAb (2 [A] or 4 [B] days after seeding) or an irrelevant IgG3 mouse antibody (C). GalC was also detected on the surface of HT29 cells chronically infected with HIV-1 NDK (D). Differentiated HT29-D4 cells cultured in two-compartment cell culture chambers were used for basolateral and apical detection of GalC. In this case, the labeling was mainly basolateral (E), but a nonnegligible fluorescence was observed on the apical membrane (F). Bar, 20 μm.

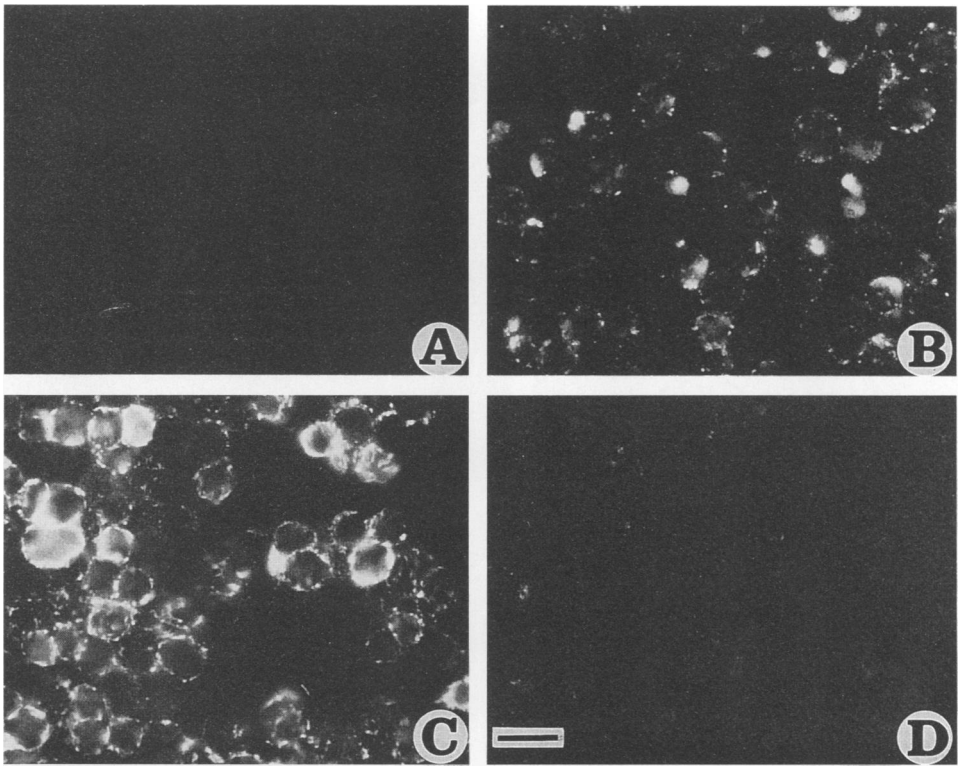

FIG. 3. Lack of detection of GalC in lymphoid cells. The expression of GalC on lymphoid cells was studied by indirect immunofluorescence, using anti-CD4 and anti-HLA MAbs as positive controls. CEM-CL5 cells showed strong labeling with anti-CD4 MAb Leu3A (B) and anti-HLA class I MAb IOT-2 (C). In contrast, no significant labeling was observed with an anti-GalC MAb (D). Nonspecific labeling was determined with an irrelevant IgG3 antibody (A). Bar, 20 μm.

glycolipids were visualized by spraying with orcinol-sulfuric acid, followed by heating at 110°C for 10 min. For immunostaining, the thin-layer chromatography (TLC) plates were first mechanically stabilized by a 2-min bath in 0.1% polyisobutyl metacrylate in *n*-hexane. The dried TLC plates were then immersed in 5% nonfat milk in PBS (milk buffer) and allowed to stand for 3 h at 4°C to block the nonspecific reaction of antibodies. The plates were further incubated overnight at 4°C with the murine anti-GalC MAb (10 μg/ml). After the plates were washed four times with ice-cold PBS, ^{125}I-protein A (10^6 cpm/ml) was added and reacted for 2 h at room temperature in milk buffer. The plates were washed four times in ice-cold PBS, dried, and exposed to Fuji X-ray film for autoradiography.

RESULTS AND DISCUSSION

Treatment of HT29 cells with anti-GalC MAb blocks HIV-1 infection. HT29 cells can be infected with several HIV isolates, including HIV-1 LAV and HIV-1 NDK (9). HIV-1 NDK was able to perform its complete replication cycle in HT29 cells. In contrast, infection with HIV-1 LAV could be detected only by cocultivation with CD4-positive lymphoid cells or by PCR amplification of viral DNA (6, 9, 10). Both methods have been carefully validated by us in a recent report (10). In particular, HIV-1 could be rescued from latently infected HT29 cells as late as 1 year after infection, demonstrating the persistence of HIV infection in these cells. Therefore, one can rule out the possibility that both the transmission of infection to indicator T cells and the presence of a positive signal in PCR experiments are due to carryover virus from the inoculum.

In the first set of experiments, we examined the effect of the anti-GalC MAb on infection of HT29 cells by both strains of HIV-1. The results of a PCR analysis with *tat* primers (Fig. 1) showed that treatment of HT29 cells with 10 μg of anti-GalC MAb per ml resulted in the absence of the PCR *tat* signal in cells exposed to HIV-1 LAV and HIV-1 NDK (lanes E and G), whereas treatment with anti-CD4 MAb OKT4A did not (lanes F and H). The specificity of the PCR signals was further assessed with a positive control cell line (CEM cells chronically infected with HIV-1 LAV [lane A]) and a negative cell line (T-84 intestinal cells [lane D]). In addition, total DNA extracted from HT29 cells 3 months after exposure to HIV-1 LAV (lane C) or HIV-1 NDK (lane B) gave strongly positive PCR signals, in agreement with our previous data (10). Similar results were obtained with *gag* primers (not shown). Taken together, these data demonstrated the reliability of PCR to assess HIV infection in HT29 cells.

Productive infection of HT29 cells with HIV-1 NDK was further demonstrated by the observation of HIV particles budding from HT29 cells in electron microscope studies (6, 9), the detection of HIV-1 mRNA by in situ hybridization (not shown), and the presence of RT activity in the cell-free culture supernatant (around 10,000 cpm/ml 12 days postinfection). In contrast, RT activity was not detectable for cells treated with the anti-GalC MAb. Since viral production was greatly enhanced after cocultivation in the presence of CD4-positive cells, we used this more sensitive procedure to

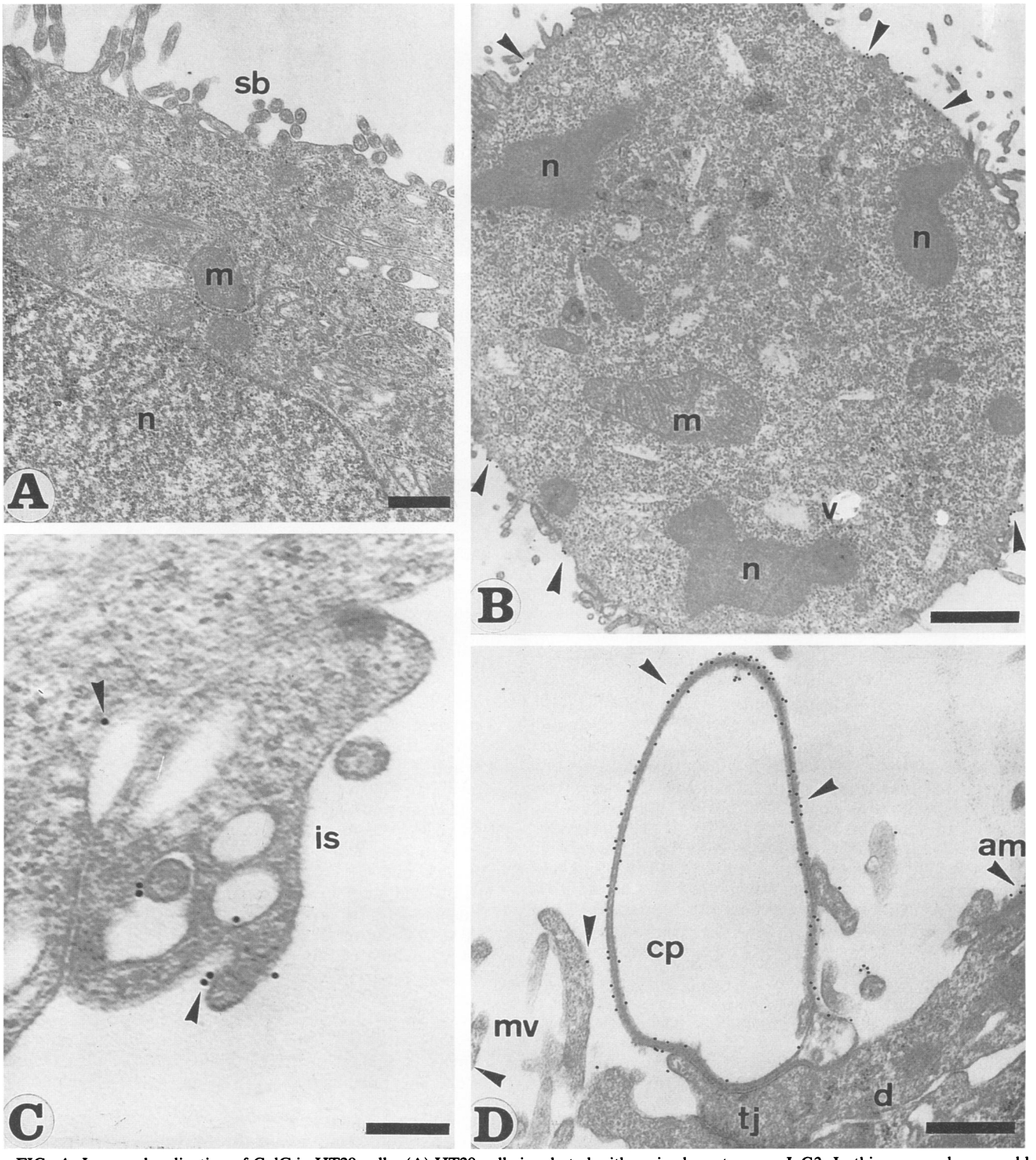

FIG. 4. Immunolocalization of GalC in HT29 cells. (A) HT29 cells incubated with an irrelevant mouse IgG3. In this case, only one gold particle was detected per 100 cells (bar, 0.5 μm). m, mitochondria; n, nucleus; sb, striated border (apical plasma membrane). (B) Undifferentiated HT29 cells labeled with an anti-GalC MAb (bar, 1 μm). Arrowheads indicate gold particles associated with protein A. m, mitochondria; n, part of the nucleus; v, vacuole. (C) Basolateral labeling of GalC (arrowheads) in differentiated HT29-D4 cells (bar, 0.2 μm). is, intercellular space. (D) Apical labeling in differentiated HT29-D4 cells incubated with an anti-GalC MAb (bar, 0.5 μm). The labeling was detected on a cytoplasmic process (cp) in the vicinity of a tight junction (tj), on the apical plasma membrane (am), and on microvilli (mv). The basolateral plasma membrane displays a desmosome (d).

measure inhibition of infection by anti-GalC antibodies. The results shown in Table 1 demonstrated that anti-GalC but not anti-CD4 antibodies were able to block the infection of HT29 cells. In contrast, the anti-GalC MAb did not block CD4-dependent (with HIV-1 LAV) or CD4-independent (with HIV-1 NDK) infection of PBL. Taken together, these findings strongly suggested the specific involvement of GalC in the infection process of HT29 cells.

Detection of GalC in HT29 cells. The most likely explanation for these results would be the presence of the sphingolipid on the surface of HT29 cells. GalC is a marker for oligodendrocytes and Schwann cells in the nervous system (19) but is also one of the major glycolipids detected in the plasma membrane from normal and tumoral human colon epithelial cells (15, 22, 23). Additionally, it has been shown recently that after incubation with a fluorescent ceramide analog, HT29 cells were able to synthesize several labeled sphingolipids, including GalC (17). Whether these cells could synthesize GalC de novo and express it in the outer leaflet of the membrane bilayer was still unknown. To answer this question, we used immunofluorescence microscopy. As shown in Fig. 2A and B, the anti-GalC MAb strongly illuminated the surface of HT29 cells. The labeling was heterogeneous (around 50% of positive cells), in agreement with the percentage of HIV-infected cells (9). Interestingly, chronic infection with HIV-1 NDK did not lead to a significant decrease of expression of GalC (Fig. 2D). Next, we analyzed the distribution of GalC in differentiated epithelial cells. As with other polarized epithelial cells, the columnar cell of the bowel mucosal epithelium is characterized by the presence of two distinct plasma membrane domains, the apical (facing the lumen) and the basolateral (facing the internal milieu). These two domains are separated by tight junctions which maintain the unique protein-lipid composition of each domain. When cultured in two-compartment cell culture chambers on a polycarbonate filter, cloned HT29-D4 cells formed a polarized monolayer which represents a valuable model for the intestinal epithelium (8, 10). In a recent study, we demonstrated that differentiated HT29-D4 cells could be infected by HIV-1 after either an apical or a basolateral incubation of the virus (10). In perfect agreement with these data, we found that GalC was expressed on the apical and basolateral membranes of filter-grown HT29-D4 cells (Fig. 2E and F). One should note that the fluorescence was more intense on the basolateral side, suggesting a preferential basolateral sorting of the sphingolipid.

By the pre-embedding immunoelectron microscopy technique, we visualized the presence of GalC on the surface of HT29 cells (Fig. 3). In undifferentiated HT29 cells, the labeling was homogeneously distributed all around the cells (Fig. 3B), in agreement with the immunofluorescence data (for comparison, see Fig. 2A). In polarized HT29-D4 cells, gold particles were clearly seen (i) on the apical plasma membrane in the vicinity of tight junctions and on brush border microvilli (Fig. 3D) and (ii) on the basolateral plasma membrane (Fig. 3C). The specificity of the immunolocalization was assessed by incubating the cells with an irrelevant MAb of the same subclass (IgG3) as the anti-GalC MAb. In this case, about one gold particle was seen for every 100 cells (Fig. 3A), illustrating the specificity of the immunolabeling procedure. These data demonstrated that GalC (or a closely related molecule) was an accessible component of the surface of both undifferentiated and differentiated HT29 cells. In contrast, we failed to detect the glycolipid on the surface of lymphoid cells, including CEM-CL5 cells (Fig. 4) and PBL (not shown). This result is in agreement with the lack of effect of anti-GalC antibodies on lymphocyte infection (Table 1).

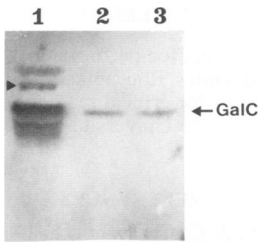

FIG. 5. TLC-immunostaining of the neutral glycolipids of HT29 cells. Lane 1, anti-GalC MAb immunostaining of glycosyl ceramides (50 μg) purified from bovine brain. The major labeled component corresponds to GalC, and the minor one corresponds to galactosyl sulfatide (arrowhead). Lanes 2 and 3, duplicate experiments of anti-GalC MAb immunostaining of neutral glycolipids extracted from 10^6 HT29 cells. A single band corresponding to GalC is detected. The specificity of the immunostaining was assessed by incubating the plate with an irrelevant IgG3 and ^{125}I-protein A. In this case, the GalC band was not detected (not shown). Neutral glycolipids were developed with chloroform-methanol-water (60:35:8).

The nature of the glycolipid recognized by the anti-GalC MAb in HT29 cells was studied in TLC-immunostaining experiments. Neutral glycolipids were extracted from 10^6 HT29 cells, separated on silica gel plates, and detected with orcinol. The major neutral glycolipids had the mobility of ceramide monohexosides (not shown). As shown in Fig. 5 (lanes 2 and 3), the anti-GalC antibody specifically reacted with a unique species of ceramide monohexosides. This sphingoglycolipid had the same chromatographic mobility as did the major ceramide monohexoside extracted from bovine brain (lane 1), i.e., GalC. The other component recognized by the antibody in lane 1 corresponds to galactosyl sulfatide, a GalC derivative known to react with the antibody (19). These data strongly suggested that GalC (or a close derivative such as fucosyl ceramide, a glycolipid found in some colon tumor cells [12]) is indeed expressed by HT29 cells and specifically recognized by a neutralizing MAb. Since HIV-1 gp120 specifically binds to GalC (2), it is likely that HIV may use this glycolipid as a receptor to enter colon epithelial cells. This finding is not surprising, since several glycosphingolipids or gangliosides are used as epithelial receptors for various microorganisms, including simian rotavirus (24) and *Escherichia coli* (16), and for bacterial toxins such as cholera toxin (3).

Conclusions. From a fundamental point of view, it is now clear that the cellular tropism of HIV is not exclusively dependent on the expression of CD4 on target cells, since CD4-negative neural and epithelial cells that express GalC on their surfaces are infectible. From a clinical point of view, the productive infection of epithelial intestinal cells by HIV induces a defect of brush border assembly (9), which could explain the syndrome of malabsorption observed in some patients with AIDS (18). The identification of an HIV receptor on mucosal cells may give rise to new therapeutic concepts for the prevention of HIV transmission in the case of nonprotected sexual intercourse and for the treatment of HIV-induced gastrointestinal disorders. Additionally, this finding raises the interesting possibility that HIV infections in the nervous system and in the gastrointestinal tract have similar features, consequent to a common mechanism of virus entry.

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