Original Paper

Journal of Biomedical Science

J Biomed Sci 2003;10:156–166
DOI: 10.1159/000068089

Received: June 27, 2002
Accepted: August 30, 2002

The Virotoxin Model of HIV-1 Enteropathy: Involvement of GPR15/Bob and Galactosylceramide in the Cytopathic Effects Induced by HIV-1 gp120 in the HT-29-D4 Intestinal Cell Line

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Key Words
HIV-1 enteropathy · Virotoxin · Calcium signaling · Protein kinase C · Intestinal cell line · GPR15/Bob coreceptor · Glycolipid · Lipid raft

Abstract
Background: Malabsorption and diarrhea are common, serious problems in AIDS patients, and are in part due to the incompletely understood entity HIV enteropathy. Our prior in vitro work has shown that increased transepithelial permeability and glucose malabsorption, similar to HIV enteropathy, are caused by HIV surface protein gp120, although the mechanism remains unclear. ***Results:*** We studied the effects of HIV surface protein gp120 on the differentiated intestinal cell line HT-29-D4, specifically the effects on microtubules, transepithelial resistance, and sodium glucose cotransport. gp120 induced extensive microtubule depolymerization, an 80% decrease in transepithelial resistance, and a 70% decrease in sodium-dependent glucose transport, changes closely paralleling those of HIV enteropathy. The effects on transepithelial resistance were used to study potential inhibitors. Neutralizing antibodies to GPR15/Bob but not to CXCR4 (the coreceptor allowing infection with these HIV strains) inhibited these effects. Antibodies to galactosylceramide (GalCer) and a synthetic analog of GalCer also inhibited the gp120-induced changes, suggesting the involvement of GalCer-enriched lipid rafts in gp120 binding to intestinal epithelial cells. ***Conclusion:*** We conclude that direct HIV infection and gp120-induced cytopathic effects are distinct phenomena. While in vivo confirmation is needed to prove this, gp120 could be a virotoxin significantly contributing to HIV enteropathy.

Gastrointestinal symptoms, such as chronic diarrhea, dehydration and malabsorption, have been described in many patients infected with HIV-1 [22, 25]. Although an infectious etiology can often be detected, extensive diagnostic evaluation may fail to explain the intestinal symptoms in at least 25–30% of cases [41]. The term 'HIV enteropathy' was first used by Kotler et al. [25] to describe those patients who did not have an identifiable enteric pathogen after a thorough evaluation. HIV-associated his-

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topathologic and functional abnormalities in the small intestine include villus atrophy and maturational defects in enterocytes [25, 39] resulting in carbohydrate and lipid malabsorption [14, 29]. Detection of HIV nucleic acids in the intestinal mucosa [31] reinforced the view that HIV itself may be an enteropathogen. Indeed, diarrhea may occur very early in infection and has been shown to increase in severity as disease progresses [41]. Moreover, zidovudine treatment of infected patients improved enterocyte maturation [40]. Since the enteropathy resolves with short-term antiretroviral treatment, these data strongly support the hypothesis that HIV-1 is a direct cause of the enteropathy [26].

As HIV enteropathy canot be studied in an in vivo system, several attempts have been made to develop an in vitro model to study HIV-induced malabsorption and diarrhea. Adachi et al. [1] were the first to report in vitro infection of intestinal cell lines with HIV-1. This pioneer study was confirmed by several investigators using the cell line most permissive to HIV-1 and HIV-2 infection: the HT-29 human colon adenocarcinoma cell line [4, 15, 32]. The demonstration that productive HIV-1 infection of HT-29 cells resulted in impaired enterocyte differentiation provided, for the first time, a potential mechanism for HIV-induced enteropathy [16]. The high sensitivity of HT-29 cells to HIV infection allowed rapid characterization of the glycolipid galactosylceramide (GalCer) as a receptor for HIV-1 in these CD4-negative cells [43]. HT-29 cells also express the HIV-1 coreceptor CXCR4 that cooperates with GalCer during the fusion process [9, 18, 37, 44]. However, elucidation of the mechanism of entry of HIV-1 into HT-29 cells did not explain how HIV-1 causes diarrhea and malabsorption. Although HIV-1 nucleic acids have been detected in the intestinal epithelium of infected patients [31], the role of direct HIV-1 infection of intestinal epithelial cells in this enteropathy is still a point of controversy [41]. Differentiated enterocytes have a very short life (1–2 days), which may not be sufficient to allow a full cycle of productive HIV infection. Also, in situ hybridization suggests that most infected cells are in the lamina propria rather than the epithelium, and are probably macrophages [20]. Thus, it is reasonable to suspect that HIV could perturb enterocytic functions without necessarily infecting intestinal epithelial cells.

The recent demonstration that the surface envelope glycoprotein of simian immunodeficiency virus (SIV) induces an experimental enteropathy in an animal model strongly supports this view [35]. Recombinant gp120 causes calcium signaling and microtubule disruption in vitro [8, 10]. Moreover, microtubule depolymerization was evidenced in intestinal biopsies from HIV-infected patients, suggesting that it could be an important mechanism causing the physiological changes of HIV enteropathy [5, 6]. Namely, we showed that picomolar concentrations of recombinant gp120 activate GPR15/Bob, a G-protein-coupled receptor abundantly expressed on the basolateral surface of intestinal epithelium [6].

The aims of the present study were: (1) to finalize the virotoxin concept of HIV enteropathy and assess whether it applies to authentic (i.e. nonrecombinant) gp120 purified from laboratory and primary HIV-1 isolates, (2) to determine the role of GalCer in GPR15/Bob-gp120 interactions, (3) to assess whether chloride secretion is affected by gp120 virotoxins, and (4) to define original strategies for protecting intestinal cells from gp120-induced cytopathic effects.

Methods

Materials

Six peptides corresponding to the deduced amino acid sequence of human GPR15/Bob [11] were synthesized and purified by high performance liquid chromatography : HAEDFARRRKRSVSL (P1), DKEASLGLWRTGSFLCK (P2), MDPEETSVYLDYYYATS (P3), SGLRQEHYLPSAILQ (P4), RELTLIDDKPYCAEKKAT (P5), and KNYDFGSSTETSDSHLTK (P6). Rabbits were injected with either the first three (P1–P3) or the following three (P4–P6) peptides, and immune sera were affinity purified with the corresponding antigen peptides [6]. The antibodies were named Bob37 and Bob39, respectively. This work was performed at Research Genetics, (Huntsville, Ala., USA). The anti-CXCR4 mouse monoclonal antibody (clone 12G5) was purchased from R&D. Rabbit anti-GalCer antibodies were from Chemicon. The anti-α-tubulin antibody (clone B-5-1-2) and rhodamine (TRITC)-labeled phalloidin were from Sigma. The synthetic soluble analog of GalCer CA52 [17] was designed and synthesized in the laboratory of I. Rico-Lattes (Toulouse, France).

Cell Culture

HT-29-D4 cells were routinely grown in 75-cm^2 flasks (Costar) in DMEM/F12 medium (Biowhittaker) supplemented with 10% fetal calf serum (Dutscher). To induce differentiation, half-confluent HT-29-D4 cells were grown in glucose-free DMEM (Sigma) supplemented with 5 m*M* galactose and 10% dialyzed fetal calf serum, as previously reported [15].

Virus Production

HIV-1 viruses were produced in peripheral blood mononuclear cells (PBMC). The isolates used in this study were the laboratory strain HIV-1(IIIB) [33] and two primary isolates, HIV-1(89.6) [7] and HIV-1(SEN), obtained in our laboratory from a lymph node biopsy [23]. Viruses were titered in PBMC obtained from a healthy donor. Viral titer is expressed as the dilution of virus stock required for infecting 50% of cultured PBMC ($TCID_{50}$). Surface envelope glycoproteins were purified by lectin affinity chromatography as previously reported [23].

Immunocytochemistry

HT-29-D4 cells grown on glass coverslips were fixed with 4% paraformaldehyde (w/v) in 0.1 *M* phosphate buffer, pH 7.4, for 30 min at room temperature. The cells were then treated with 50 m*M* NaCl (15 min), rinsed with phosphate buffer containing 1% bovine serum albumin (BSA), and permeabilized with 0.2% Triton X-100 in phosphate buffer containing 5% BSA (30 min). For α-tubulin staining, coverslips were incubated in a humid atmosphere at 4°C for 4 h with the primary antibody (5 μg/ml) in phosphate buffer containing 1% BSA. After washing, the cells were incubated with fluorescein-conjugated secondary antibodies for 90 min at 4°C. The coverslips were mounted in a Mowiol solution and analyzed by confocal microscopy (Leica TCS inverted laser scanning microscope) as previously reported [10]. For actin staining, rhodamine-phalloidin was added to the secondary antibody solution. To determine the saturating concentrations of anti-GPR15/Bob, anti-CXCR4, and anti-GalCer antibodies, HT-29-D4 cells were incubated with various dilutions of each antibody and revealed with appropriate peroxidase-coupled secondary antibodies [9].

Electrophysiological Measurements

HT-29-D4 cells were cultured in two-compartment cell culture chambers on a polycarbonate filter (Transwell-clear, Catalog No. 3450, Costar) and analyzed for electrical parameters in a modified Ussing chamber as previously reported [10]. The chamber was maintained at 37°C on a hot plate. Apical and basal compartments were filled with electrophysiological medium: 137 m*M* NaCl, 5.36 m*M* KCl, 0.4 m*M* Na_2HPO_4, 0.8 m*M* $MgCl_2$, 1.8 m*M* $CaCl_2$, 20 m*M* N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), pH 7.4. Transepithelial potential difference was measured with Ag electrodes and continuously recorded using a voltage clamp unit (Physiologic Instrument). Bipolar current pulses (20 μA for 2 s, every 30 s) were passed through Ag electrodes to measure transepithelial electrical resistance (TEER), which was determined according to Ohm's law [10]. The sodium-dependent electrogenic glucose transport activity was measured as an increase in short-circuit current (ΔIsc) following apical incubation of HT-29-D4 cells with the nonmetabolizable analog of glucose α-methylglucose [19]. Except when indicated, purified gp120s were incubated in the apical compartment of the cell culture chamber (total volume 1.5 ml instead of 10 ml for the basal compartment of the Ussing chamber device unit).

Surface Pressure Measurements

GalCer with an α-hydroxylated fatty acid (referred to as GalCer throughout this study) was either prepared from bovine brain (Sigma) or purified from HT-29-D4 membrane rafts [19]. The surface pressure was measured with a microtensiometer (Kibron Inc., Finland) specially designed for studying protein-lipid interactions [19]. The experiments were performed at 25°C in a laboratory equipped with a filtered air supply. A solution of GalCer was prepared just before use in hexane:chloroform:methanol, 11:5:4 (v:v:v) at a concentration of 1 mg/ml. The lipid was carefully spread onto the meniscus of a pure water subphase (800 μl). HIV-1 gp120 was injected into the subphase with a 10 μl Hamilton syringe. The surface pressure was continuously measured as a function of time until reaching equilibrium corresponding to the maximal surface pressure increase (Δπmax) expressed in mN/m.

Table 1. HIV-1 gp120-induced impairment of intestinal barrier and absorption functions

Treatment	TEER, $\Omega \cdot cm^2$	ΔIsc after α-methylglucose treatment, $\mu A \cdot cm^{-2}$
Control (16 h)	1,569 ± 225	1.18 ± 0.13
+ gp120 (16 h)	300 ± 108	0.35 ± 0.07
Control (48 h)	1,228 ± 384	1.44 ± 0.25
+ gp120 (48 h)	320 ± 267	0.29 ± 0.11

Differentiated HT-29-D4 cells cultured in Transwell chambers were either not treated (control) or treated with 50 n*M* purified gp120 (IIIB isolate) for the time indicated in serum-containing culture medium. TEER values and ΔIsc after incubation with 3 m*M* α-methylglucose (sodium-dependent glucose transport activity) were measured as described in Materials and Methods. The results are expressed as the mean ± SD of 4 separate experiments.

Results

Effect of HIV-1 gp120 on Cytoskeletal Organization in a Model Intestinal Epithelium

α-Tubulin and actin staining in HT-29-D4 cells is shown in figure 1a–c. The α-tubulin labeling was particularly intense in the central part of the cells where it formed a complex network of microtubules surrounding the nucleus (fig. 1a, b). Actin filaments were observed in most confocal planes, especially in the basal part of the cells (fig. 1c). After 1 h of incubation with gp120 purified from HIV-1(IIIB) virions (50 n*M*), a dramatic decrease of α-tubulin staining was observed in most cells, whereas the pattern of actin labeling was nearly unchanged (fig. 1d–f).

Effect of Purified HIV-1 gp120 on Intestinal Barrier and Absorption Functions

Differentiated HT-29-D4 cells cultured on permeable filters were incubated with 50 n*M* purified gp120 (IIIB isolate) in complete, serum-containing medium. In these experiments, gp120 was added to both compartments of the culture chamber (table 1). After 16 h of incubation, TEER was decreased by 81%. At the same time, the electrogenic activity (ΔIsc) of the apical sodium/glucose cotransporter (SGLT1) was reduced by 70%. After 48 h of treatment with the purified gp120, TEER and SGLT1 activity were decreased by 74 and 80%, respectively. Similar data were obtained when gp120 was added only in the apical compartment of the cell culture chamber.

To study the kinetics of gp120-induced defects in barrier and absorption functions, the electrophysiological

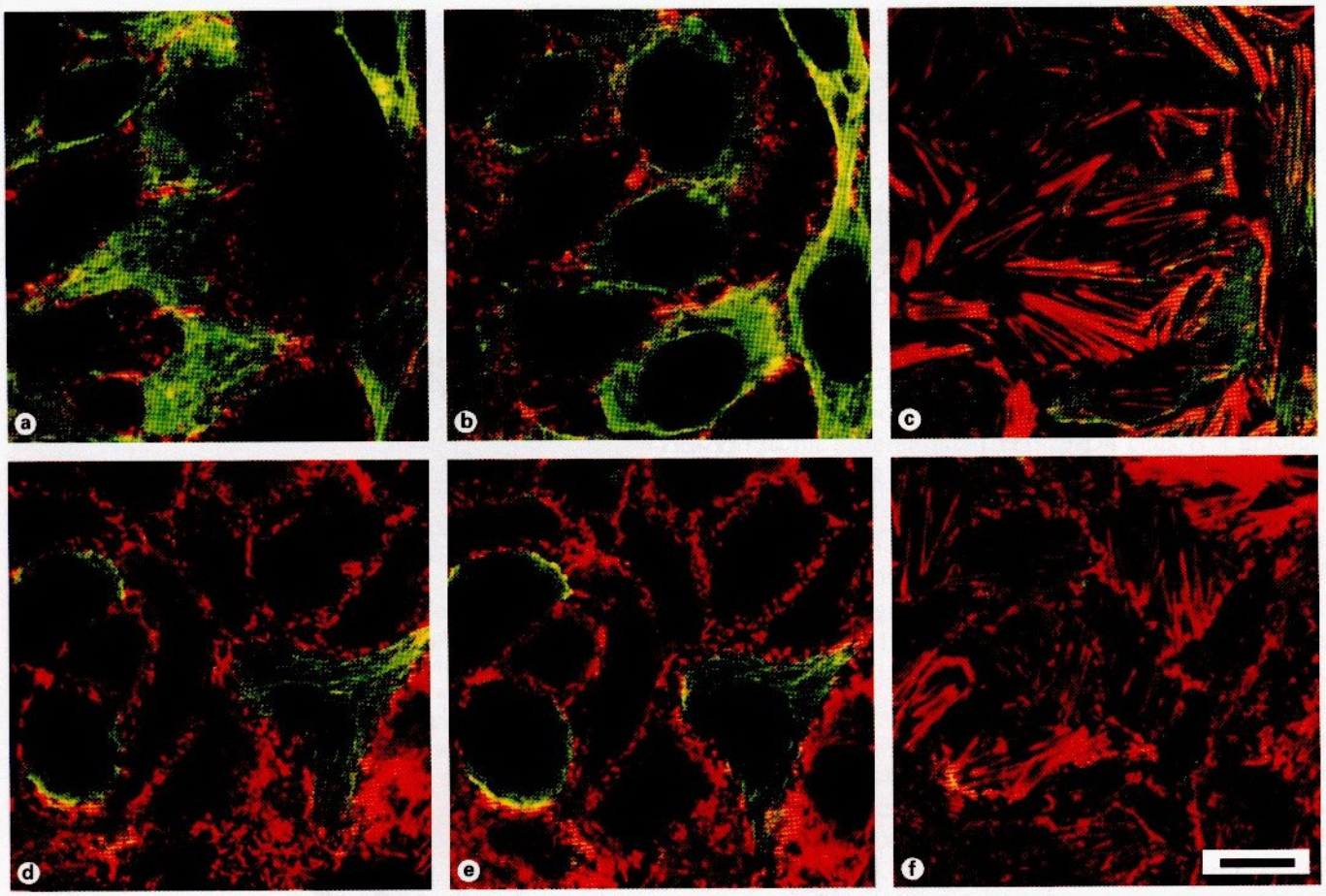

Fig. 1. Microtubule depolymerization induced by purified gp120 (IIIB isolate). Differentiated HT-29-D4 cells were either not exposed (**a–c**) or exposed (**d–f**) to 50 n*M* purified gp120 (IIIB isolate) for 1 h. Microtubules were stained with an α-tubulin antibody followed by fluorescein-conjugated anti-mouse IgG. Actin filaments were stained with rhodamine-phalloidin. Confocal images were acquired with a Leica laser scanning microscope. A series of three confocal planes corresponding to the apical, mid and basal regions of the cells (from left to right, respectively) is presented. Scale bar: 20 μm.

activity of HT-29-D4 cells was continuously recorded over a period of 14 h (fig. 2a). In these experiments, the cells could not be bathed in complete medium, which interferes with real-time electrode recordings. Instead, we used a buffer specifically designed for electrophysiological studies [10]. Under these conditions, the TEER of control cells was slightly decreased after 4 h of analysis, but it remained stable for the next 10 h. When the cells were incubated apically with 0.45 nmol gp120 (IIIB isolate), the TEER was dramatically increased during the first hour, then it gradually decreased to null values after 12 h. Similar variations of TEER were observed during the incubation with 0.45 nmol gp120 purified from a primary isolate, HIV-1(SEN) that does not infect HT-29 cells [23]. In this case however, TEER was found to increase again after 10 h of incubation with the gp120, so that it returned to a value identical to control cells after 14 h (fig. 2a). The transient drop below baseline which occurred upon gp120(SEN) incubation was reproducibly observed in three separate experiments. The initial increase in TEER induced by gp120(IIIB) and gp120(SEN) was a saturable, dose-dependent process (fig. 2b). 20 min after the addition of gp120, TEER was enhanced by 2.52 ± 0.19 and 1.60 ± 0.17 (n = 3) for gp120(SEN) and gp120(IIIB), respectively. The effect was strain specific since gp120 from 89.6, another primary isolate that does not infect HT-29 cells [23, 44], was totally inactive (fig. 2c). A similar increase in TEER was measured upon treatment of the cells with neurotensin, a potent activator of inositol triphosphate-dependent calcium signaling in intestinal cells

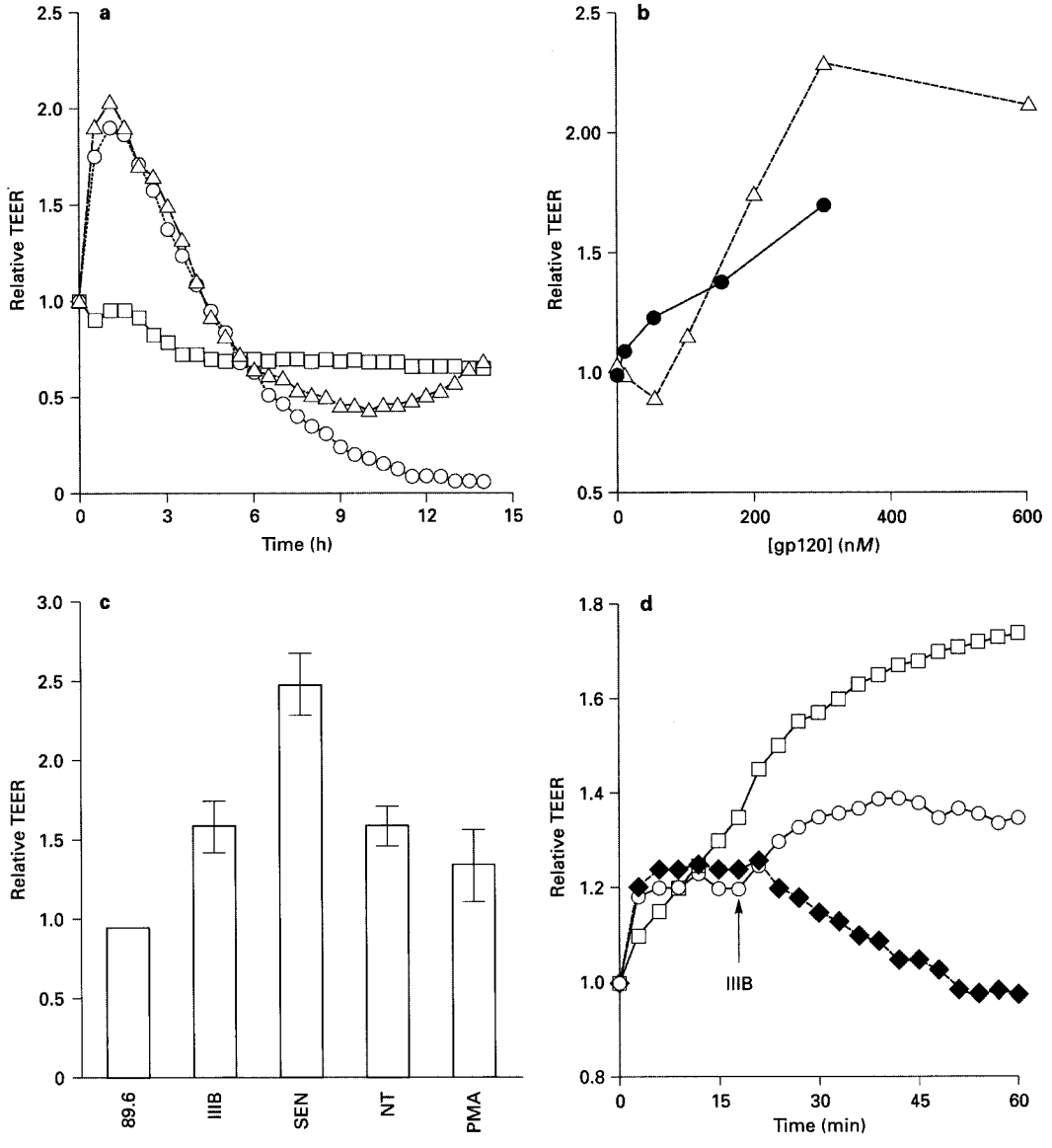

Fig. 2. Strain-specific effects of gp120 on the intestinal barrier function. **a** Differentiated HT-29-D4 cells cultured in Transwell chambers were either not exposed (squares) or exposed to gp120 (300 n*M*) purified from HIV-1(IIIB) (circles) or HIV-1(SEN) (triangles). Electrophysiological parameters were continuously recorded for 14 h in electrophysiological medium and the relative value of TEER (corresponding to the TEER value at a given time divided by TEER at time 0) is shown. **b** Dose-dependent effects of gp120 purified from HIV-1(IIIB) (circles) or HIV-1(SEN) (triangles) on TEER after 20 min of incubation. **c** Initial increase in TEER expressed as relative TEER after 20 min of treatment with various gp120 (300 n*M*), with neurotensin (5 n*M*), or with PMA (2 μ*M*). **d** Differentiated HT-29-D4 cells were treated with 2 μ*M* PMA (diamonds), 300 n*M* gp120(IIIB) (squares), or sequentially with PMA (2 μ*M*) and gp120 (300 n*M*) as indicated by the arrow (circles). The TEER was recorded continuously during the experiments.

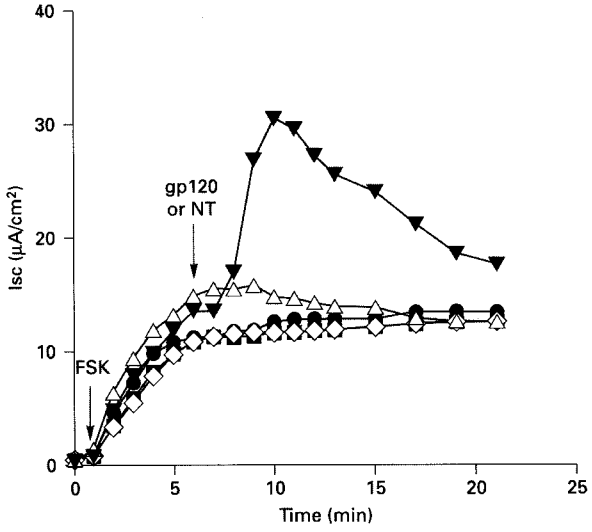

Fig. 3. Lack of effect of purified gp120 on chloride secretion. Differentiated HT-29-D4 cells cultured in Transwell chambers were first treated with forskolin and then with either neurotensin (full triangles) or gp120 purified from HIV-1(IIIB) (full circles), 89.6 (open circles), or SEN (open triangles). The electrophysiological recording of control, mock-treated cells is shown with full squares.

[8], and with the protein kinase C activator phorbol myristate acetate (PMA) (fig. 2c). When the cells were first treated with PMA, the subsequent response to gp120(IIIB) was considerably decreased (fig. 2d), suggesting that PMA and gp120 activated a common protein kinase C (PKC)-dependent signal transduction pathway.

Lack of Effect of Purified HIV-1 gp120 on Chloride Secretion

Since abnormal stimulation of intestinal chloride secretion through the cAMP-regulated channel CFTR induces watery diarrhea [27], we studied whether gp120 stimulated cAMP-dependent chloride secretion. As shown in figure 3, the cAMP activator forskolin (10 μ*M*) induced a dramatic increase in the short-circuit current (ΔIsc of 13.5 ± 2.3 $\mu A \cdot cm^{-2}$, n = 8), corresponding to a net apical chloride secretion through CFTR. None of the gp120s tested (up to 300 n*M*) induced such an effect in HT-29-D4 cells (not shown). The cAMP-dependent chloride secretion could be potentiated by calcium agonists. Indeed, addition of neurotensin (5 n*M*) after forskolin (10 μ*M*) induced an additive variation of Isc (ΔIsc of 17.5 ± 3.0 $\mu A \cdot cm^{-2}$, n = 3) (fig. 3). Since gp120 virotoxins activate calcium-signaling pathways in HT-29-D4 cells [8], it was postulated that HIV-associated diarrhea could, at least in part, result from calcium-dependent potentiation of chloride secretion [19]. For this reason, we tested the effect of various gp120s (300 n*M*) after forskolin activation of CFTR. Under these conditions, we did not detect any additive effect of Isc (fig. 3). Taken together, these data show that gp120 virotoxins do not activate or potentiate chloride secretion in resting or stimulated HT-29-D4 cells.

Inhibition of the Cytopathic Effects Induced by gp120 in HT-29-D4 Cells

Overall, the most dramatic electrophysiological effect of gp120 on differentiated HT-29-D4 cells is a gradual decrease in TEER systematically preceded by a transient increase which is characteristic of activators of the phosphatidylinositol-4,5-bisphosphate (PIP2) pathway. This early TEER response is almost complete after 20 min of incubation with gp120(IIIB) or gp120(SEN), and is not observed with gp120(89.6). It is therefore indicative of a strain-specific enterotoxic effect of gp120. We used this electrophysiological signature of gp120 virotoxins to develop a reliable assay for screening potential protective agents. In this assay, HT-29-D4 cells were incubated with a saturating concentration of the most potent gp120, i.e. 300 n*M* of gp120(SEN), in competition with the tested agent. As shown in figure 4, neutralizing anti-CXCR4 antibodies did not affect the gp120-induced TEER response. In contrast, significant protection was achieved by anti-GR15/Bob antibodies, anti-GalCer antibodies, and a synthetic soluble analog of GalCer. These data suggested that gp120 virotoxins could interact with both GalCer and GPR15/Bob on the surface of HT-29-D4 cells.

Interaction between GalCer and Purified gp120s

In a recent study, we showed that the interaction between GalCer and gp120 purified from HIV-1(IIIB) was very efficient, whereas gp120s from 89.6 and SEN isolates interacted very weakly [23]. The kinetics of this interaction are an important physicochemical parameter allowing the direct comparison of GalCer recognition by various gp120s. In the experiment shown in figure 5, gp120 purified from IIIB, SEN, or 89.6 was injected underneath a monomolecular film of GalCer at the air-water interface. Immediately after the addition of gp120(IIIB) in the aqueous subphase, an exponential increase in the surface pressure of the film was measured, and equilibrium was reached after 45 min of incubation. The surface increase indicates a condensing effect of the

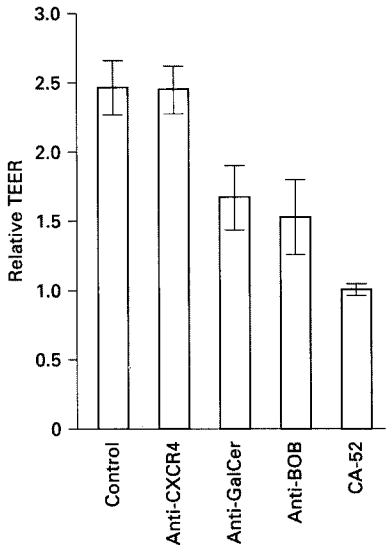

Fig. 4. Partial protection of intestinal cells from gp120-induced toxicity. Differentiated HT-29-D4 cells cultured in Transwell chambers were exposed to 300 n*M* of gp120 purified from HIV-1(SEN) in the presence of either the indicated antibody or the synthetic soluble analog of GalCer CA52 (100 μg/ml). Anti-CXCR4, anti-GalCer and anti-GPR15/Bob (Bob37 + Bob39) antibodies were used at 20 μg/ml, 1:400, and 1:100 dilutions, respectively. These concentrations corresponded to saturation binding of each antibody on HT-29-D4 cells as determined by quantitative cellular enzyme-linked assay. The results show the initial increase in TEER expressed as relative TEER after 20 min of treatment.

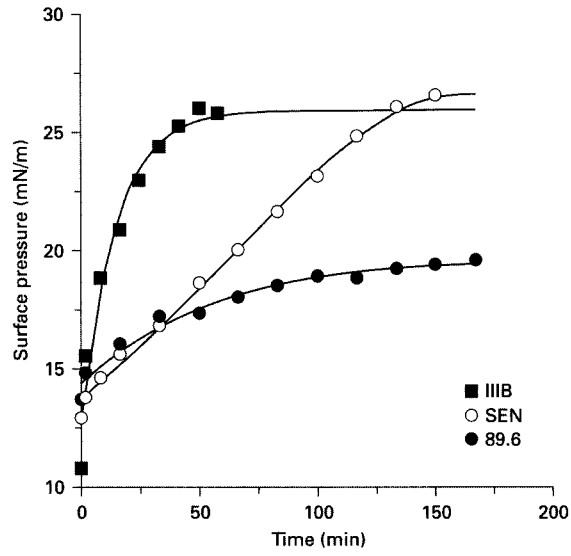

Fig. 5. GalCer-gp120 interactions measured at the air-water interface. A monomolecular film of GalCer was prepared at the air-water interface and gp120 purified from either IIIB (full squares), SEN (open circles) or 89.6 (full circles) isolates was added in the aqueous subphase at a concentration of 50 n*M*. The kinetics of the surface pressure increase induced by the viral glycoproteins are shown.

gp120 on the lipid film due to its interaction with GalCer molecules [23]. In contrast, the interaction with gp120(SEN) was very slow, linear rather than exponential, and the equilibrium was not reached even after 150 min of incubation. At this time, however, the level of interaction indicated by the absolute increase in surface pressure was identical to that of gp120(IIIB). Finally, gp120(89.6) showed very little association with GalCer with a maximal increase in surface pressure of less than 50% of that measured for IIIB and SEN. Taken together, these data confirmed the lack of a significant interaction between GalCer and gp120(89.6) and the high affinity of gp120(IIIB) for the lipid. The GalCer-binding capacity of gp120(SEN) was reevaluated and shown to occur much more slowly, but not less efficiently, than that of gp120(IIIB).

Discussion

The main outcome of the present study is that gp120 purified from selected HIV-1 strains can induce specific morphological and functional alterations in a model intestinal epithelium. These changes include microtubule disruption, perturbation of TEER and decrease in sodium-dependent glucose absorption. In contrast, gp120 did not affect chloride secretion. This is in agreement with the data obtained by Stockmann et al. [34] with duodenal biopsies of HIV-infected patients suffering from diarrhea. Overall, the perturbations induced by gp120 in HT-29-D4 cells are consistent with the symptoms of HIV-associated enteropathy [5, 22, 25, 29]. The gp120s used were purified from laboratory and primary HIV-1 isolates produced in human PBMC. Indeed, to our knowledge, this is the first report that a native (i.e. nonrecombinant) gp120 from a primary HIV-1 isolate can induce specific cytopathic effects in cultured intestinal epithelium. This is a significant increment to the body of data obtained with

HT-29 cells either productively infected or stimulated with a recombinant gp120 [2, 8, 16].

The concentrations of gp120 required for cytopathic effects of gp120 virotoxins in differentiated HT-29 cells are in the range of 50–300 n*M*. Similar concentrations of SIV surface envelope glycoprotein (in the range of 75–300 n*M*) are needed to induce calcium or inositol triphosphate signals in HT-29 cells [35]. Swaggerty et al. [35] observed that SIV surface envelope glycoprotein (0.02–0.45 nmol) induced a dose-dependent diarrhea in a mouse pup model. Overall, the concentrations of gp120 used in the present study are very similar to those previously used to demonstrate the enterotoxic properties of SIV surface envelope glycoprotein [35]. On the other hand, we observed that the sensitivity to gp120 varies markedly, depending on whether the HT-29 cells are differentiated. Indeed, calcium signals were induced by as little as 1 pmol of gp120 in undifferentiated HT-29 cells [6, 8]. However, it should be emphasized that all the experiments described in the present study have been performed on differentiated HT-29-D4 cells, since undifferentiated HT-29-D4 cells do not display the biochemical and morphological features of human enterocytes. When grown in standard glucose-containing medium, HT-29-D4 cells do express GalCer as well as GPR15/Bob, but have no detectable SGLT1 activity [6, 10]. Therefore, studies with undifferentiated HT-29-D4 cells were restricted to gp120-induced calcium fluxes and microtubule depolymerization [8, 10].

The intestinal tropism of HIV-1 has been previously established on the basis of infection of intestinal epithelial cell lines, chiefly HT-29 [37, 44]. These studies concluded that those isolates that are able to infect intestinal cells in vitro recognized both GalCer and CXCR4 [9]. HIV-1(IIIB), which interacts strongly with GalCer and uses CXCR4 as coreceptor to gain entry into target cells, can infect HT-29 cells. In contrast, HIV-1(89.6), an R5X4 isolate that can use CXCR4 but does not interact with GalCer, does not infect HT-29 cells [23]. HIV-1(SEN) is an R5 primary isolate that interacts weakly with GalCer and does not infect HT-29 cells [23]. In the present study, we showed that the gp120s purified from IIIB and SEN, but not from 89.6, induced marked alterations of TEER. Therefore, the enterotoxin activity of gp120 is not correlated with the ability of the corresponding virus to infect HT-29 cells. Instead, these data strongly support the concept that HIV-associated gastrointestinal symptoms are due to a toxin-like effect of gp120 from specific isolates (e.g. IIIB and SEN) on intestinal epithelial cells.

Since the TEER changes induced by these gp120s could be inhibited by anti-GPR15/Bob, it is likely that this receptor is involved in the pathogenesis of HIV enteropathy, as recently suggested [6]. In this respect, it should be emphasized that anti-CXCR4 antibodies that block HIV-1 infection of HT-29 cells [9, 37] do not protect the cells from the toxic effects of gp120. Moreover, both anti-GalCer antibodies (which bind to the cell surface of intestinal cells) and a synthetic soluble analog of GalCer (which neutralizes soluble gp120), inhibited gp120-induced TEER changes. Finally, quantitative determinations by cellular ELISA [9] showed that GalCer and GPR15/Bob were expressed in both plasma membrane domains (i.e. apical and basolateral) of differentiated HT-29-D4 cells (not shown). Correspondingly, cytopathic effects could be induced after either apical or bipolar addition of gp120. In the intestine of infected individuals, the co-expression of GalCer and GPR15/Bob is on both the apical and the basolateral membrane [6]. In particular, GPR15/Bob is abundantly expressed on the basolateral surface of intestinal epithelium whereas the other coreceptors stimulated by gp120 (CCR5 and CXCR4) are mostly on the apical side in vivo [13]. Given that HIV exposure is likely on the basal surface from subepithelial macrophages [20], one could reasonably conclude that gp120-induced GPR15/Bob activation is a plausible cause of HIV enteropathy [6]. Finally, incubation of HT-29-D4 cells with ultracentrifuged virus particles (IIIB isolate) resulted in a transient increase in TEER after a lag ranging from of 10 to 20 min (data not shown). This preliminary experiment suggested that viral-associated trimeric gp120 could, like monomeric purified gp120, induce an enterotoxic effect, although with slightly different kinetics.

Based on these data and on previous reports [in particular 6, 8, 10, 35], we propose the following hypothesis for explaining the role of gp120 in the pathogenesis of HIV-1 enteropathy.

(1) Virus-associated gp120 or free gp120 released from infected immune cells of the lamina propria binds to GPR15/Bob, presumably in a GalCer-rich subdomain (raft) of the basolateral plasma membrane of an enterocyte. In this respect, there is a striking analogy between HIV-1 gp120 and bacterial neurotoxins. Tetanus and botulinum toxins bind to presynaptic membranes through low affinity, high concentrations of ganglioside receptors (GD1a, GD1b and GT1b), and then move laterally to bind to a high-affinity protein receptor [36]. Since the final binding affinity is the product of these two binding constants, a very high affinity is achieved. This concept of

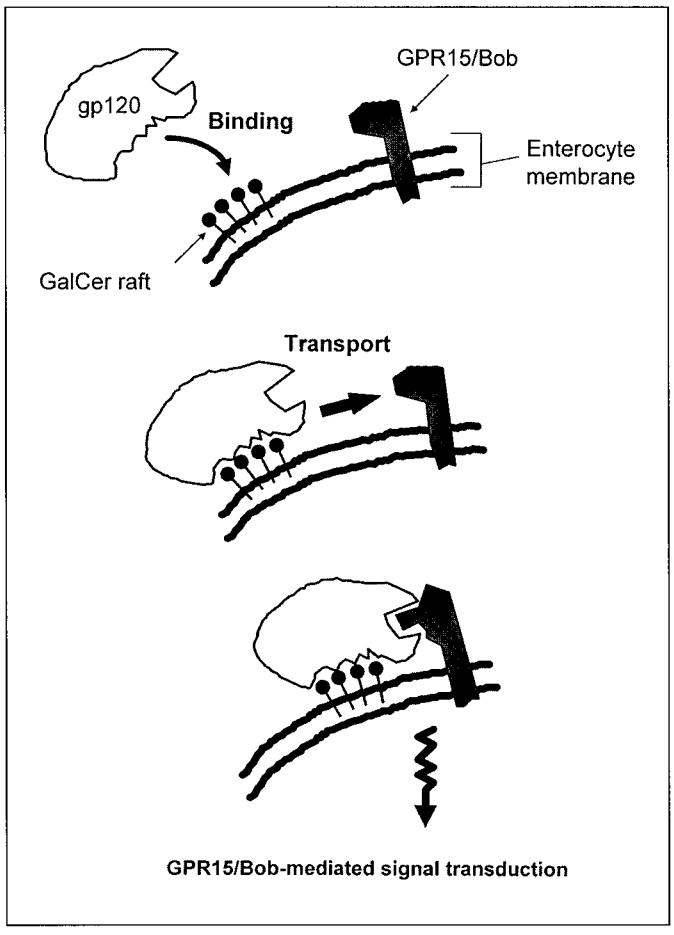

Fig. 6. The 'double receptor' model of gp120 binding to intestinal epithelial cells.

a 'double receptor,' originally developed by Montecucco [30], may explain why the cytopathic effects of gp120 are inhibited with both anti-GalCer and anti-GPR15/Bob antibodies (fig. 6). On the surface of intestinal cells, lipid rafts enriched in GalCer may provide abundant binding sites that could (a) stabilize the virotoxin on the cell surface, and (b) transport it to GPR15/Bob. Under these conditions, even if the signal transduction cascade is primarily activated following gp120 binding to GPR15/Bob, it could be inhibited by both anti-GalCer and anti-GPR15/Bob antibodies. Based on this model, one can predict an effective blocking of gp120 toxicity with a combination of anti-GalCer and anti-GPR15/Bob antibodies. Experiments are currently in progress to validate this point.

(2) Gp120-induced activation of GPR15/Bob results in the initiation of a calcium-signaling pathway involving a pertussis-toxin-sensitive G protein, inositol triphosphate synthesis, calcium release from endoplasmic reticulum stores, and activation of PKC [6, 8]. Consistent with this hypothesis, GalCer and transducer G proteins were co-localized in detergent-insoluble membranes (rafts) purified from differentiated HT-29-D4 cells [19]. Moreover, SIV envelope glycoprotein induced a transient increase in intracellular calcium levels and increased inositol-1,45-triphosphate levels in HT-29 cells [35].

(3) The gp120-induced short-term increase in TEER is probably due to activation of PKC which may stabilize junctional complexes by phosphorylation of tight-junction proteins such as ZO-1, ZO-2, and p130 [12]. Moreover, PKC has been shown to decrease phosphorylation of the regulatory light chain of myosin II (MLC), resulting in a short-term TEER increase [38]. Consistently, a transient increase in TEER was observed upon treatment of HT-29-D4 cells with the PKC activator PMA (fig. 2c). Moreover, pretreatment of the cells with PMA resulted in a decreased response to gp120 (fig. 2d). Overall, these data strongly suggest that the effects of gp120 involve a calcium-dependent activation of PKC.

(4) The long-term decrease in TEER may also involve PKC, as previously demonstrated [38]. Alternatively, one cannot exclude the activation of RhoA due to microtubule disruption [42]. Indeed, RhoA activation increases actin-myosin contraction and dramatically affects the equilibrium of tight junctions [24]. In support of this hypothesis, a decrease in TEER has been observed in intestinal Caco-2 cells exposed to a bacterial activator of RhoA [21].

(5) The microtubule defect impairs apical plasma membrane renewal which involves the transport of brush border components into vesicles originating from the supranuclear Golgi apparatus. This may lead to specific missorting of apical membrane transporters, such as the sodium-dependent glucose transporter SGLT1 [19]. Since SGLT1 is responsible for the intestinal absorption of at least 5 liters of water per day in a healthy subject [28], a defect in SGLT1 could be responsible for the chronic diarrhea observed in HIV-infected patients in the absence of identifiable pathogens.

In conclusion, although the etiology of HIV enteropathy is likely multifactorial, these data suggest that gp120 from selected HIV-1 strains could directly affect the intestinal epithelium. We are aware that the enterotoxic properties of gp120 could be demonstrated only for two HIV-1 strains, including only one primary isolate. The finding that the toxicity of gp120 is strictly isolate dependent is certainly intriguing and the characterization of more enterotoxic gp120 will be required to validate our model. Nevertheless, gp120 from HIV-1(IIIB) and HIV-1(SEN) may

be considered as new members of the recently characterized family of viral enterotoxins (virotoxins) which comprises rotavirus NSP4 [3] and SIV surface envelope glycoprotein [35]. It will be of great interest to study whether a correlation can be found between the viral isolate circulating in a patient and enteropathy, and between the interaction of the specific gp120 with intestinal receptors and cytopathic effects in HT-29-D4 cells.

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