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Direct Effect of Type 1 Human Immunodeficiency Virus (HIV-1) on Intestinal Epithelial Cell Differentiation: Relationship to HIV-1 Enteropathy

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Human immunodeficiency virus (HIV)-infected patients display severe impairments of gastrointestinal functions, including diarrhea and malabsorption, even in the absence of opportunistic infections. Since HIV-1 proteins and nucleic acids have been detected in several cell types of the intestinal mucosa, it has been postulated that HIV-1 itself could alter enterocytic functions. In the present study, we analyzed the effect of HIV-1 on the differentiation process of the epithelial intestinal cell clone HT-29-D4, which mimics the maturation of enterocytes along the crypt–villus axis of the small intestine. We found that HIV-1 infection impairs cellular differentiation (i) by affecting the barrier function of the epithelium, as evidenced by a decrease in the transepithelial electrical resistance, and (ii) by inhibiting the activity of one major glucose absorption function, i.e., sodium/glucose cotransport. At the morphological level, HIV-1 infection of HT-29-D4 cells was associated with the formation of lumina, which are representative of a defect in cellular organization. These morphofunctional perturbations induced by HIV-1 could be mimicked by nocodazole, a microtubule-disrupting agent. Correspondingly, HIV-1 exposure of HT-29-D4 cells evoked a massive disruption of microtubules, as revealed by α-tubulin indirect immunofluorescence staining. A similar effect was observed after incubation of the cells with either recombinant gp120 or a monoclonal antibody against galactosylceramide (GalCer), the intestinal receptor for HIV-1 gp120, suggesting that the effect of HIV-1 was mediated by the binding of gp120 to GalCer. Based on these data, we propose that HIV-1 may selectively alter enterocytic functions through a direct effect on the intracellular architecture of the cells. In contrast with previous theories for HIV-1 enteropathy, our data support the concept that HIV-1 may perturb intestinal functions without necessarily infecting intestinal epithelial cells. © 1997 Academic Press

INTRODUCTION

Gastrointestinal disorders, including diarrhea, malabsorption, and weight loss, are frequently reported during the course of human immunodeficiency virus type 1 (HIV-1) infection (Kotler *et al.*, 1984; Ullrich *et al.*, 1989). The origin of these symptoms is still uncertain, since in as many as 30–40% of cases, there is no identifiable enteric pathogen (Dworkin, 1992). In the absence of opportunistic infection, it has been proposed that HIV infection could be directly responsible for these symptoms (Ullrich *et al.*, 1989, 1992a,b). The concept of an "HIV enteropathy" is supported by the detection of HIV-1 proteins and/or nucleic acids in various intestinal cell types: lymphocytes, macrophages, epithelial cells (Bigornia *et al.*, 1992; Heise *et al.*, 1991; Kotler *et al.*, 1991; Levy *et al.*, 1989; Mathijs *et al.*, 1988; Nelson *et al.*, 1988). In particular, the infection of intestinal epithelial cells by HIV-1, which was confirmed by *in vitro* studies (Adachi *et al.*, 1987; Omary *et al.*, 1991), has been proposed as a direct cause of enteropathy. However, it should be noted that the detection of HIV-1 in the intestinal epithelium from AIDS patients has been inconsistently reported (Fleming *et al.*, 1992; Winter *et al.*, 1992), leading to a controversy as to the direct role of HIV-1 in the enteropathy (Sharpstone and Gazzard, 1996).

In vitro, intestinal epithelial cells have been infected by various laboratory and primary isolates of HIV-1 (Asmuth *et al.*, 1994; Barnett *et al.*, 1991; Fantini *et al.*, 1992; Omary *et al.*, 1991; Phillips and Bourinbaiar, 1992). The infection of epithelial intestinal cell lines by HIV-1 correlates with cell surface expression of the glycosphingolipid galactosylceramide (GalCer), an alternative receptor allowing HIV-1 entry into some CD4-negative cells (Harouse *et al.*, 1991; Yahi *et al.*, 1992). Since GalCer is one of the most abundant glycosphingolipids in normal human epithelial cells from the small and large intestines (Yahi *et al.*, 1994), these data may have an important physiological significance. In this respect, we have recently reported that the binding of HIV-1 surface envelope glycoprotein gp120 to the GalCer receptor of intestinal HT-29-D4 cells generated an important increase in intracellular calcium concentration (Dayanithi *et al.*, 1995, 1996). Inter-

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estingly, pertubations of calcium metabolism have been observed in HIV-1-infected HT-29 cells displaying functional alterations of their absorptive capacities, e.g., a transient decrease in the activity of several brush-border enzymes (Asmuth *et al.,* 1994). Since similar perturbations occur in the intestinal epithelium from HIV-1-infected patients, the HT-29 cell line is a relevant model for studying the effect of HIV-1 infection on intestinal epithelial cell physiology (Fantini *et al.,* 1994). The differentiation of enterocytes, which occurs during the migration of intestinal stem cells along the crypt–villus axis, can be mimicked *in vitro* by the intestinal cell clone HT-29-D4 (Fantini, 1992). These cells ressemble undifferentiated crypt cells and can be induced to differentiate into a polarized monolayer of mature enterocytes with morphological and biochemical features of normal intestinal absorptive cells (Fantini *et al.,* 1986, 1989).

Despite the considerable interest in elucidating the mechanisms of enteropathic dysfunctions associated with HIV-1 infection, very few studies have focused on the development of major absorptive functions of the enterocyte in the presence of HIV-1. In particular, since sugar absorption defect is one of the main complications in the late phases of HIV-1 infection (Ehrenpreis *et al.,* 1992), it is of great interest to study the effect of HIV-1 on sugar transport capacity of differentiating enterocytes. The sodium/glucose cotransporter, which appears gradually on the apical membrane during the differentiation of enterocytes (Hwang *et al.,* 1991), plays a major role in intestinal sugar absorption. In the present study, we have analyzed the effect of HIV-1 on the morphological and functional differentiation of the HT-29-D4 cell clone.

MATERIALS AND METHODS

Materials

Cell culture media and fetal calf serum were from Biowhittaker (Les Ulis, France). α-Methyl-D-glucopyranoside (AMG), 2-deoxyglucose (DOG), cytochalasin B, and nocodazole were purchased from Sigma (Les Ulis, France). Phlorizin was obtained from Aldrich Chimie (Les Ulis, France) and prepared as a 100 mM stock solution in Me_2SO. α-[U-^{14}C]Methyl]-D-glucopyranoside (NEC-659, 282 mCi/mmol) and 2-[^{3}H]deoxyglucose glucopyranoside (NET-328, 8.2 Ci/mmol) were obtained from Dupont (Les Ulis, France). Iodine-125 (100 mCi/mmol) was from Amersham. All reagents were of the highest purity available. The monoclonal anti-α-tubulin antibody (clone B-5-1-2), rhodamine (TRITC)-labeled phalloidin, and control mouse IgG1 were purchased from Sigma. Fluorescein-conjugated F(ab′)$_2$ fragment goat anti-mouse and anti-human IgGs were from Immunotech (Marseille, France). The anti-GalCer R-mAb was generously provided by F. Gonzalez-Scarano (University of Pennsylvania). Recombinant gp120 was from Intracel Corporation (Cambridge, MA).

Cell culture

HT-29-D4 cells were routinely grown in 75-cm^2 flasks (Costar) in DMEM/F-12 medium supplemented with 10% fetal calf serum, penicillin (40 units/ml), and streptomycin (40 μg/ml). To induce differentiation, half-confluent HT-29-D4 cells were grown in glucose-free Dulbecco's modified Eagle medium (DMEM) supplemented with 5 mM galactose and 10% dialyzed fetal calf serum, as previously reported (Fantini *et al.,* 1986). Undifferentiated cells will be referred to as HT-29-D4-UD cells, whereas cells cultured in the galactose-containing differentiating medium (i.e., differentiated cells) will be referred to as HT-29-D4-D cells.

Electrophysiological measurements

HT-29-D4-D 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, custommade Ussing chamber as previously reported (Fantini *et al.,* 1989). The chamber was maintained at 37° on a hot plate. Apical and basal compartments were filled with B medium [137 mM NaCl, 5.36 mM KCl, 0.4 mM Na_2HPO_4, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)] adjusted to pH 7.4 with NaOH. Under these conditions, the monolayer remained stable for 3–4 h. Transepithelial potential difference (TPD) was measured with electrodes and continuously recorded using a voltage clamp unit (Physiologic Instrument, Dutscher, Strasbourg, France). Bipolar current pulses (20 μA for 2 s, every 30 s) were passed through electrodes to measure the transepithelial electrical resistance, which was determined according to the Ohm's law (Fantini *et al.,* 1989).

Iodide efflux experiments

Fully differentiated HT-29-D4 cells cultured on 24-well plastic supports were washed twice with B medium and then loaded with 20 μCi/ml ^{125}I for 40 min in B medium containing 5 mM KI. After three washings, the cells were incubated with 0.5 ml of either B medium alone, B medium containing 10 μM forskolin, or B medium containing 10 μM calcium ionophore A23187. After 6 min, the supernatant was removed and the cells were disrupted in 0.1 N NaOH. The radioactivity in the supernatants and in the cell lysate was counted in a Beckman gamma counter. The results are expressed as the percentage of intracellular remaining iodide (±SD).

Sugar uptake measurements

HT-29-D4-UD or -D cells, cultured in 24-well plates, were washed with B medium and then incubated for the time indicated in the same medium containing the nonmetabolizable radiolabeled glucose analog ([^{14}C]-

AMG or [^{3}H]DOG) at the concentration of 0.1 mM. For uptake experiments performed in sodium-free medium, the cells were washed with sodium-free B medium (137 mM choline chloride instead of NaCl and 0.4 mM K_2HPO_4 instead of Na_2HPO_4 adjusted to pH 7.4 with KOH). At the end of the incubation, the medium was removed and the cells were washed three times with 1 ml of either B medium or sodium-free B medium. The cells were then disrupted with 0.1 N NaOH, and radioactivity was measured in a Beckman beta counter. The results are expressed as nanomoles of AMG (or DOG) per milligram of proteins. The protein content was evaluated with a Pierce kit using bovine serum albumin as standard.

HIV-1 infection of HT-29-D4 cells

HIV-1(LAI) was produced in human T-lymphoblastoid CEM cells and harvested from the culture supernatants during the peak of viral replication as previously described (Fantini *et al.,* 1992). Viral stocks were filtered through 0.22-μm filters and stored at $-80°$ in 1-ml aliquots. The viral titer was 10^4 tissue culture infectious dose 50% ($TCID_{50}$) per milliliter as determined in CEM cells. The infection of HT-29-D4 cells by HIV-1 was performed as described (Fantini *et al.,* 1991), with slight modifications. Exponentially growing cells were exposed to HIV-1(LAI) at a m.o.i. of 0.1 TCID50 per cell for the time indicated in each experiment. The cells were then switched to the differentiation medium. The level of infection was assessed by a quantitative PCR assay using the Amplicor kit (Roche) with 8E5 cells (one HIV-1 copy per cell) for standardization, and by p24 antigen determinations in cell-free supernatants using the p24 capture assay from Dupont. Under these conditions, a peak of p24 production was usually observed between Days 8 and 16 postinfection. At this time, immunofluorescent studies with an anti-HIV-1 human serum revealed a yield of infection of 20–30% of cells in the monolayer. From Day 18, viral production was below detectable levels (10 pg of p24/ml) but the cells remained infected since a vigorous infection could be transmitted to indicator T cells.

Immunocytochemistry

The cells 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 mM NaCl (15 min), rinsed with phosphate buffer containing 1% bovine serum albumin (BSA) (15 min), and permeabilized with 0.2% Triton X-100 in phosphate buffer containing 5% BSA (30 min). For antibody staining, coverslips were incubated in a humid chamber at 4° for 4 h with primary antibodies at appropriate dilutions in phosphate buffer containing 1% BSA. After washing, the cells were incubated with fluorescein-conjugated secondary antibodies for 90 min at 4°. The coverslips were mounted in a Mowiol solution and stored in the dark at 4° until analyzed by confocal microscopy. For actin staining, rhodamine–phalloidin was added to the secondary antibody solution.

Confocal microscopy

For confocal microscopy, a Leica TCS inverted laser scanning microscope equipped with a krypton–argon mixed gas laser was used. Images were acquired with a ×40 or a ×100 oil immersion objective. Additional magnification was achieved using the zoom feature. The dual-wavelength mode was used for two-color analyses, allowing fluorescence from both dyes to be detected simultaneously with two different photomultiplier tubes. In this configuration, contamination (cross-talk) of the green signal (fluorescein) by the red signal (rhodamine) is impossible. Nevertheless, absence of a green signal under rhodamine-selective excitation was controlled. All images were acquired with the laser power excitation set to values less than 40 mW, and stored in separate (green and red) image channels.

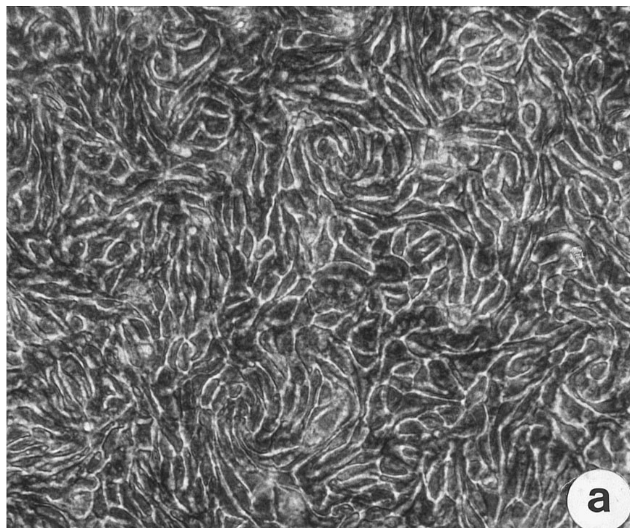
RESULTS

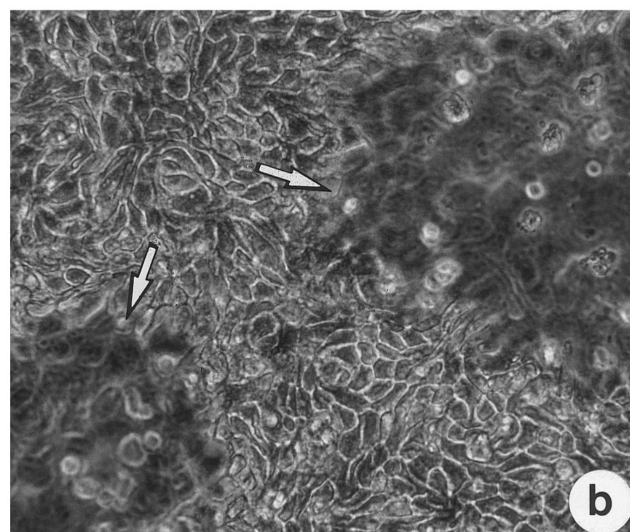
Effect of HIV-1 on the organization of intestinal cells

First, we tested the effect of HIV-1 infection on the overall morphology of HT-29-D4 cells during the differentiation process. In these experiments, undifferentiated HT-29-D4 cells (HT-29-D4-UD) were exposed to HIV-1(LAI) for 3 days and then switched to the differentiation medium as described under Materials and Methods. PCR analysis of HIV-1 DNA showed that a mean number of 2 HIV-1 DNA copies were integrated per cell. After 12 days of culture in the differentiation medium, noninfected control cells formed a regular monolayer of tightly bound cells (Fig. 1a). As previously reported (Fantini *et al.,* 1986), domes corresponding to local detachments of the cell monolayer could appear in the cultures, generally 1 week later, as the result of vectorial liquid secretion entrapment between the basis of the cells and the plastic wall of the culture flasks (not shown). In contrast, domes always occurred earlier in HT-29-D4 cells infected with HIV-1(LAI) (Fig. 1b), which suggests a significant activation of transepithelial ionic transport in infected cells (Lever, 1982). The main other cytopathic effect associated with HIV-1 infection was the presence of numerous cysts (Fig. 1c), which were characterized by electron microscopy as both inter- and intracellular lumina (not shown). Taken together, these data show that HIV-1 infection induced important morphological and functional perturbations during the course of HT-29-D4 differentiation.

Effect of HIV-1 on the electrical properties of intestinal cells

Since chloride channels are involved in the control of fluid secretion in the intestinal tract (Field and Semrad,





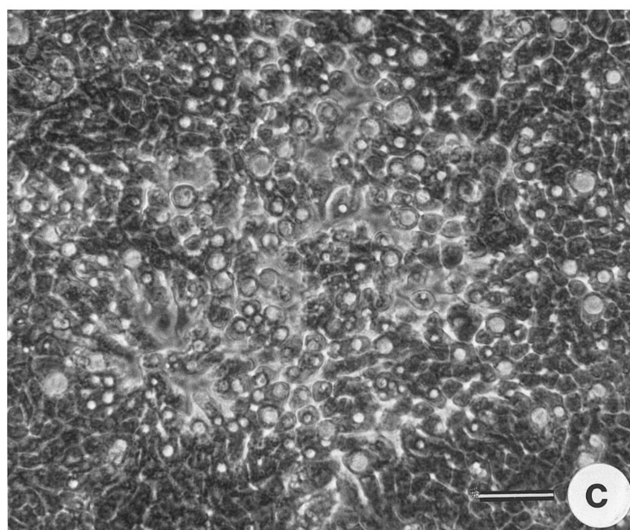


FIG. 1. Morphological effects induced by HIV-1 in HT-29-D4 cells. HT-29-D4-UD cells were either exposed (b, c) or not exposed (a) to HIV-1 and then induced to differentiate as described under Materials and Methods. After 12 days of culture in the differentiation medium, noninfected control cells form a regular monolayer of polarized cells (a). Infected cells display several domes (b, arrows) as well as numerous lumina (c). Scale bar = 25 μm.

TABLE 1

Effect of Forskolin and Calcium Ionophore A23187 on Iodide Efflux

Effector	Intracellular remaining iodide (%)
None	34.64 ± 2.9
Forskolin	16.05 ± 2.0
A23187	18.62 ± 3.0
Forskolin + A23187	11.32 ± 1.9

Note. HT-29-D4-D cells were loaded with ^{125}I, washed, and incubated with 10 μM forskolin, 10 μM A23187, or 10 μM concentrations of each as described under Materials and Methods. The radioactivity remaining inside the cells after 6 min of incubation was measured in a gamma-counter. Results are expressed as the means of three independent experiments ± SD.

1993), we studied the effect of HIV-1 infection on Cl^- secretion processes. Two chloride channels have been previously characterized on uncloned HT-29 cells (Morris *et al.,* 1992): the cystic fibrosis transmembrane conductance regulator (CFTR), which is regulated by cAMP, and a calcium-activated chloride channel. The presence of these channels has been studied in differentiated HT-29-D4 cells (HT-29-D4-D) by iodide efflux. As shown in Table 1, drugs increasing the intracellular concentrations of either cAMP or Ca^{2+} (respectively forskolin and calcium ionophore A23187) activated iodide efflux. Moreover, when the cells were incubated with both effectors, a cumulative response was obtained. Thus, HT-29-D4-D cells express both cAMP and Ca^{2+}-activated chloride channels.

In a second series of experiments, the activity of Cl^- secretion by HT-29-D4-D cells was evaluated by electrophysiological techniques. To this end, the cells were cultured on permeable supports in two-compartment cell chambers where they formed a leakproof and electrically active epithelial monolayer (Fantini *et al.,* 1989). The transepithelial resistance of the cells, measured according to Ohm's law, was in the range 700–1500 ohms·cm^2, indicating that tight junctions were functional and homogeneously distributed over the cell monolayer. The addition of forskolin in the apical compartment induced a rapid increase in the transepithelial potential difference (−19.17 mV), confirming the presence of an electrogenic cAMP-activated chloride channel, i.e., the CFTR (Fig. 2A). Moreover, the effect of forskolin was completly reversed by bumetanide (an inhibitor of the $Na^+K^+Cl^-$ cotransporter) delivered in the basal compartment. These data showed that forskolin generated an electrogenic secretion of chloride, in full agreement with

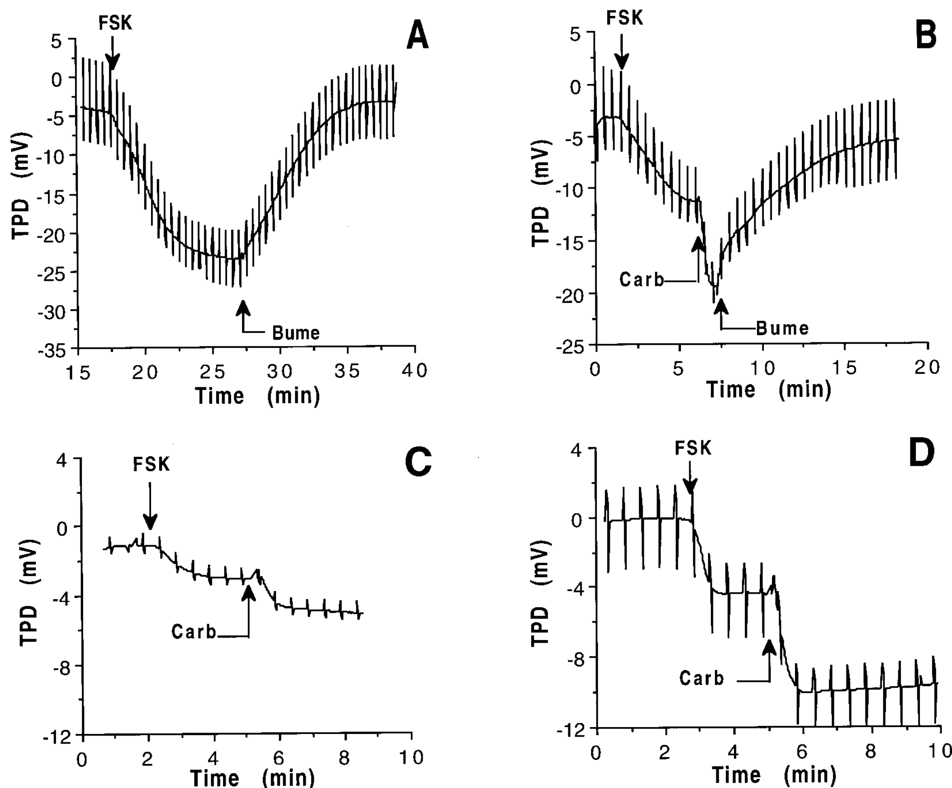


FIG. 2. Effect of HIV-1 on electrical properties of HT-29-D4-D cells. Electrophysiological parameters of HT-29-D4-D cells grown on filters were analyzed as described under Materials and Methods using a modified Ussing chamber. (A) addition of 10 μM forskolin (FSK) in the apical compartment evoked an important increase in the transepithelial potential difference (TPD, expressed in mV), which returned to the basal level after addition of 250 μM bumetanide (Bume) in the basal compartment. (B) Addition of 200 μM carbachol (Carb) in the basal compartment after forskolin treatment generated a further decrease in the transepithelial potential difference. Addition of 250 μM bumetanide then inhibited the effect. (C) HT-29-D4-D cells were infected at low density with HIV-1 and cultured for 13 days before electrophysiological determinations. These cells displayed weak electrical activity compared with noninfected control cells cultured for the same time and analyzed on the same day (D), but could respond to forskolin and carbachol.

iodide efflux experiments. Similarly, addition of carbachol (a Ca^{2+}-mobilizing agonist) in the basal compartment during the course of forskolin response, further increased the transepithelial potential difference (Fig. 2B). Interestingly, when carbachol was added first, it did not modify the transepithelial potential difference (data not shown). Taken together, our data are consistent with the presence of at least two different electrogenic chloride channels acting synergetically on HT-29-D4-D cells, in agreement with previous studies (Morris *et al.,* 1992).

Based on this characterization, we analyzed the effect of HIV-1 infection on electrogenic chloride secretion. In this experiment, HT-29-D4-D cells were seeded at low density on permeable supports, infected with HIV-1(LAI), and periodically tested for their electrophysiological activity. Noninfected cells, cultured under the same conditions, were analyzed in parallel for comparison. After 13 days, these control cells displayed a transepithelial resistance of 618 ± 42 ohms·cm^2 ($n = 3$) and responded to forskolin (−4.28 mV) and carbachol (−5.64 mV) (Fig. 2D). The main feature of infected cells was a dramatic decrease in the transepithelial resistance (218 ± 86 ohms·cm^2, $n = 3$), indicating that the maturation of junctional complexes was affected by HIV-1 (Fig. 2C). However, these cells were responsive to forskolin and carbachol (−1.9 and −2.17 mV, respectively), showing that the chloride channels were present and functional on infected cells. After 16 days, the transepithelial resistance of infected cells increased, but was still lower than that of control cells (721 ± 245 ohms·cm^2 vs 1640 ± 212 ohms·cm^2 for infected and noninfected cells, respectively). After 20 days, the infected cells displayed the same transepithelial resistance as control cells, indicating that the main effect of HIV-1 was to delay the maturation of the epithelial monolayer (Table 2).

Effect of HIV-1 on glucose transport

Since HIV-1 infection was associated with a delay in the differentiation process of HT-29-D4 cells, we studied the effect of HIV-1 on the development of glucose transport during enterocyte maturation. The activity of glucose transporters on HT-29-D4 cells was analyzed by uptake measurements using nonmetabolizable radiolabeled glu-

TABLE 2

Effect of HIV-1 Infection on Electrophysiological Parameters of HT-29-D4 Cells during the Course of Cell Differentiation

Time (days)	Uninfected cells		HIV-1-infected cells	
	TR (ohms·cm^2)	FSK (mV)	TR (ohms·cm^2)	FSK (mV)
13	618 ± 42	4.16 ± 0.16	218 ± 86	2.57 ± 0.95
16	1640 ± 212	7.52 ± 0.80	721 ± 245	4.40 ± 0.14
20	1188 ± 166	8.90 ± 1.30	1117 ± 109	7.23 ± 0.33

Note. HT-29-D4 cells cultured at low density on permeable support were either noninfected or infected with HIV-1 (LAI), and then cultured in the differentiating medium for the time indicated. Development of the electrophysiological properties of the cell monolayer was followed as a function of time in culture. Results are expressed as the means of three independent experiments ± SD. TR, transepithelial electrical resistance; FSK, 10 μM forskolin added in the apical compartment. In response to the activation of cAMP-dependent Cl^- secretion induced by forskolin, a rapid increase in the transepithelial potential difference was observed (see Fig. 2A). The value indicated corresponds to the maximal effect of forskolin.

cose analogs specific for the sodium/glucose cotransporter (AMG) or for the facilitated glucose transporter (DOG). As shown in Fig. 3A, HT-29-D4-UD cells possess a facilitative glucose transport since the uptake of DOG was important and sensitive to cytochalasine B (an inhibitor of this transport). In contrast, the activity of this transporter was very weak in HT-29-D4-D cells.

The presence of the sodium/glucose cotransporter on HT-29-D4 cells was studied by measuring the uptake of AMG under three experimental conditions: in the absence or presence of sodium chloride or in the presence of sodium chloride and 100 μM phlorizin (a specific inhibitor of the cotransport function). As shown in Fig. 3B, HT-29-D4-UD cells do not express the sodium/glucose cotransport function since the uptake of AMG was (i) independent of the presence of sodium, and (ii) phlorizin insensitive. In contrast, fully differentiated HT-29-D4 cells possess the sodium/glucose cotransporter since AMG uptake was both sodium-dependent and phlorizin-sensitive. Thus, we concluded that the differentiation process was associated with a decrease in facilitative glucose transport capacity and with the appearence of the sodium/glucose cotransport function.

Then we evaluated the effect of HIV-1 infection on the activity of these glucose transporters during the course of HT-29-D4 differentiation. HT-29-D4-UD cells were infected by HIV-1(LAI) and then induced to differentiate as described under Materials and Methods. The evolution of the two glucose transporters expressed by infected and noninfected control cells was studied at different times of culture. In noninfected HT-29-D4 cells, the facilitative glucose transport function decreased from 26.04 ± 1.27 to 6.03 ± 0.24 nmol/mg protein after 16 days of differentiation (Fig. 4A). A similar evolution of facilitative glucose transport during the course of cell differentiation was observed for infected cells (24.88 ± 1.23 nmol/mg protein vs 7.37 ± 0.43 nmol/mg protein). Thus, the activity of the facilitative glucose transport, which decreased during cell differentiation, was not affected by HIV-1. In contrast, AMG uptake was decreased in infected cells, showing that HIV-1 affected the kinetics of appearence of the sodium/glucose cotransport function during the differentiation of HT-

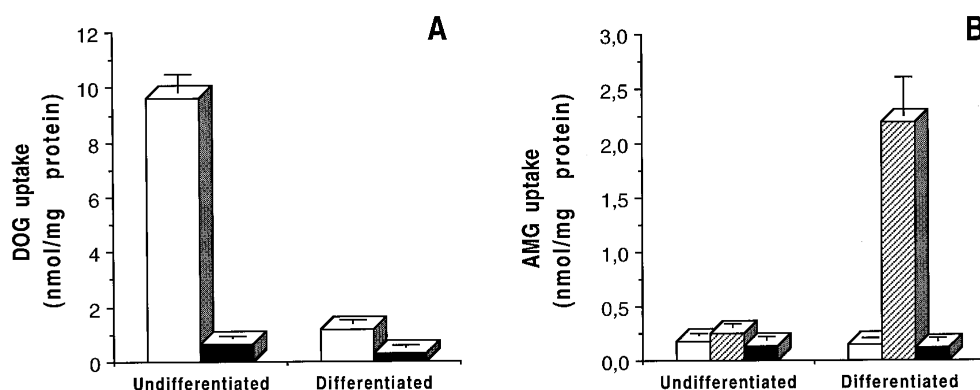


FIG. 3. Characterization of glucose transporters in undifferentiated and differentiated HT-29-D4 cells. (A) HT-29-D4-UD and HT-29-D4-D cells were cultured in 24-well plates. The uptake of 0.1 mM DOG was measured at a single time point (10 min) in either the absence (white bars) or the presence (shaded bars) of 100 μM cytochalasin B. Values are means ± SD of three independent measurements. (B) The uptake of 0.1 mM AMG was measured at 10 min in the absence (white bars) or the presence of sodium (hatched bars) or sodium and 100 μM phlorizin (shaded bars). Values are means ± SD of three independent measurements.

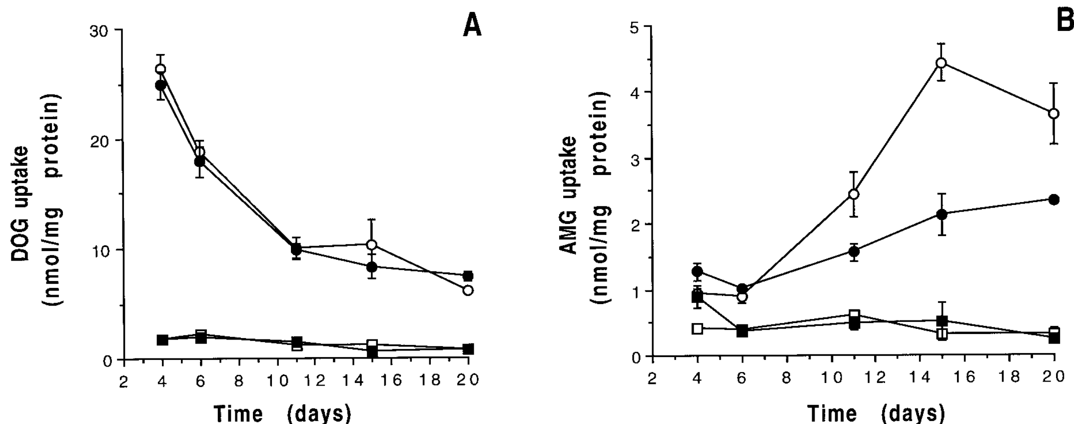


FIG. 4. Effect of HIV-1 on glucose transport capacities of HT-29-D4 cells. (A) Uptake of 2-DOG. Noninfected control cells (open symbols) and HIV-1-infected cells (closed symbols) were washed and incubated with 0.1 mM DOG with (squares) or without (circles) 100 μM cytochalasin B for 20 min. After thorough washing, the cell-associated radioactivity was counted in a beta counter. Values are expressed as the means of four independent determinations ± SD. (B) Uptake of AMG. Similar experiments were done with AMG in the presence (squares) or absence (circles) of 100 μM phlorizin on noninfected control cells (open symbols) and HIV-1-infected cells (closed symbols). Values are expressed as the means ± SD of four independent determinations.

29-D4 cells (Fig. 4B). The effect of HIV infection was significant after 11 days of differentiation (2.43 ± 0.33 nmol vs 1.56 ± 0.15 nmol of AMG/mg protein, for uninfected and infected cells, respectively) and persisted for 20 days (3.64 ± 0.47 nmol vs 2.33 ± 0.04 nmol of AMG/mg protein, for uninfected and infected cells, respectively). It is important to note that this effect was specific since there was not a significant difference between control and infected cells for AMG uptake in the presence of phlorizin. Moreover, the decrease in sodium/glucose cotransport was not due to an effect on cellular proliferation as assessed by determination of cellular protein content (data not shown). The specificity of this down-regulation was further confirmed by the fact that DOG uptake was similar in uninfected and infected HT-29-D4 cells. Thus, one can reasonably conclude that HIV-1 infection specifically perturbed the appearence of the sodium/glucose cotransport function during cell differentiation.

Effect of HIV-1 on microtubules

Experiments were conducted to elucidate the mechanisms by which HIV-1 could affect the process of intestinal epithelial cell differentiation. Microtubule-disrupting drugs such as nocodazole cause the missorting of several apical proteins in epithelial cells (Achler *et al.*, 1989). In this respect, we found that after 48 h of treatment with 100 μM nocodazole, the activity of the sodium/glucose cotransporter was inhibited by 50% (data not shown). Thus, we analyzed the effect of HIV-1 on the organization of the cytoskeleton in HT-29-D4 cells. In these experiments, actin filaments were visualized in red by using rhodamine–phalloidin, and microtubules appeared in green after indirect immunofluorescence staining with an anti-α-tubulin mAb and secondary fluorescein-conjugated anti-mouse IgG. The staining of actin and α-tubulin in differentiated HT-29-D4 cells is shown in Fig. 5 (a and c) at two levels of magnification. Following short-term exposure to HIV-1, the cells displayed two major changes in cytoskeletal organization: (i) a dramatic decrease in the staining of α-tubulin (Figs. 5b and 5d), and (ii) the occurrence of lumina or cysts heavily stained with rhodamine–phalloidin (Fig. 5b). Similar effects were observed after incubating the cells for 1 h with either recombinant gp120 (Fig. 5e) or anti-GalCer antibodies (Fig. 5f), suggesting that the mechanism of HIV-1-induced microtubule disruption involves gp120 binding to GalCer on the surface of HT-29-D4 cells.

DISCUSSION

Gastrointestinal manifestations of AIDS are one of the most striking characteristics of HIV infection (Sharpstone and Gazzard, 1996). During the progression of the disease, the patients suffer chronic diarrhea, dehydration, and malabsorption, leading to substantial weight loss (Dworkin, 1992). These symptoms are particularly acute in Africa, where HIV-1 infection is often associated with a severe chronic diarrhea commonly referred to as "slim disease" (Serwadda *et al.*, 1985). Histological studies of the intestinal epithelium from HIV-1-infected patients generally reveal low-grade small bowel atrophy associated with a maturational defect of enterocytes (Gillin *et al.*, 1985; Ullrich *et al.*, 1989, 1992a). Moreover, ultrastructural analysis of these cells shows evidence of morphological alterations such as intracellular lumina, Golgi hypertrophy, and accumulation of fat vacuoles (Benhamou *et al.*, 1994; Dobbins and Weinstein, 1985; Mathan *et al.*, 1990; Takyia *et al.*, 1991). Although these findings may explain the functional disturbances of enterocytic absorptive capacities, the etiology of HIV-associated intestinal symptoms has been the subject of controversy in the

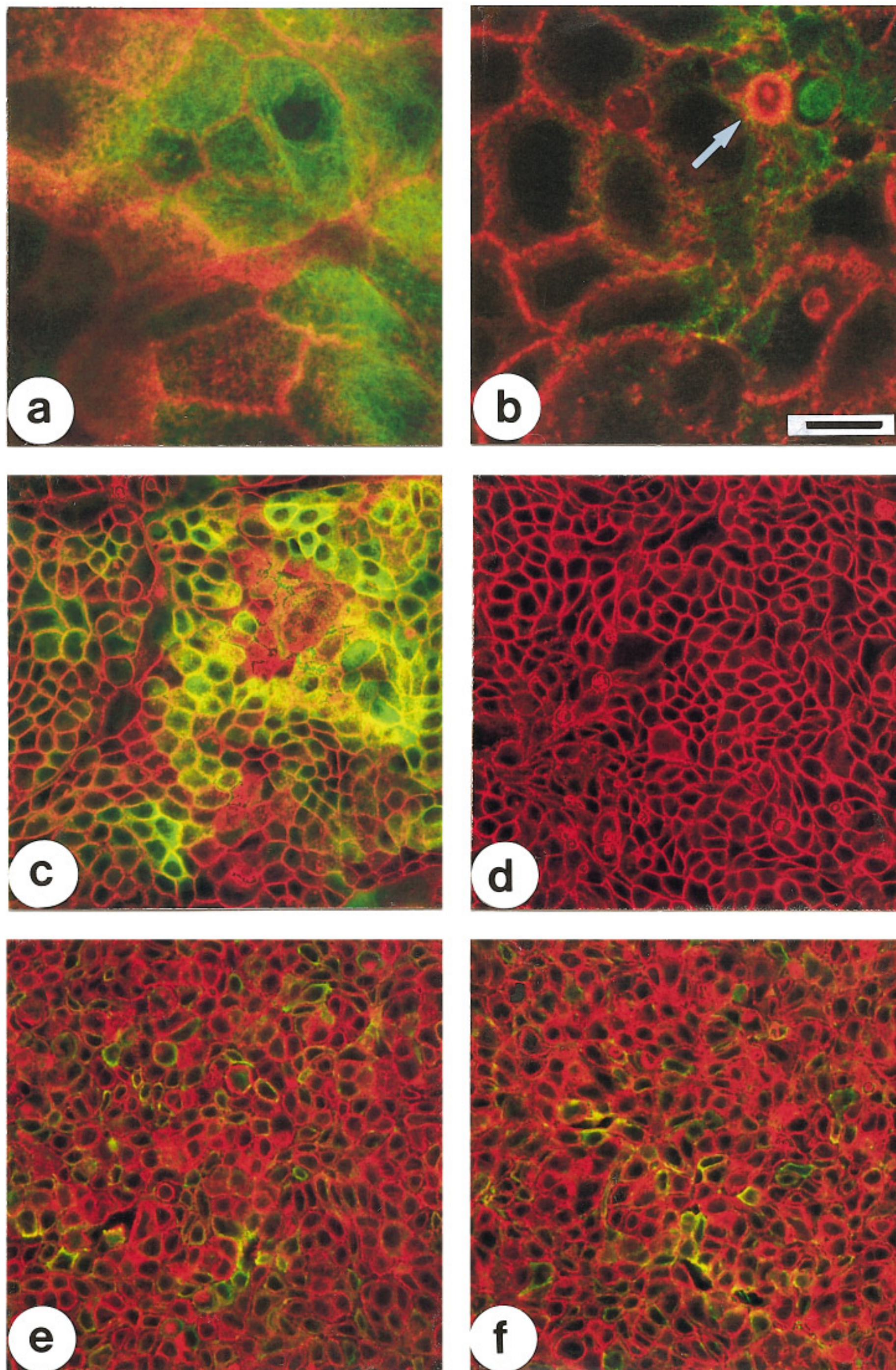


FIG. 5. Effect of HIV-1 on actin and α-tubulin distribution in differentiated HT-29-D4 cells. Differentiated HT-29-D4 cells were analyzed by confocal microscopy at two levels of magnification: ×100 (a, b) and ×40 (c-f). Cells exposed to HIV-1 for 16 h (b, d) displayed a marked decrease in α-tubulin labeling when compared with control cells (a, c). A similar effect was observed after incubation of the cells for 1 h with either 50 nM recombinant gp120 (e) or 50 μg/ml anti-GalCer mAb (f). Scale bar = 20 μm (a, b) or 50 μm (c–f).

past few years. Indeed, several mechanims are probably involved in the gastrointestinal manifestations of AIDS: opportunistic infections (Sharpstone and Gazzard, 1996), secretion of cytokines in response to inflammation (Kotler *et al.*, 1991), and HIV-1 infection per se (Ullrich *et al.*, 1992b). The concept of a primary, HIV-1-associated enteropathy has emerged in the mid-1980s as a tentative explanation for the lack of detection of enteric pathogens in the intestine of as many as 30–40% of HIV-1-infected people (Ullrich *et al.*, 1989). This concept has been further

supported by the observation that intestinal HIV-1 infection correlated with decreased activity of enterocytic enzymes, which reverted toward normal on antiretroviral therapy (Ullrich *et al.,* 1992a). This was confirmed by Kotler *et al.* (1997), who recently established a correlation between the viral load decrease induced by antiretroviral combination therapy in the intestinal mucosa and the clinical benefit. Moreover, a secretory IgA response to HIV-1 antigens has been consistently detected in infected patients with diarrhea (Mathewson *et al.,* 1994). Although these data provide strong evidence of an etiologic role for HIV in the intestinal disorders associated with AIDS, the mechanisms involved in these pathologies have remained elusive.

The objective of this research was to clarify the role of HIV-1 in the intestinal complications of AIDS. Our approach was to determine whether HIV-1 could directly alter the differentiation process of the HT-29-D4 cell clone, which mimicks the enterocytic maturation along the intestinal crypt–villus axis (Fantini, 1992). As previously reported, these cells could be productively infected by various isolates of HIV-1 (Fantini *et al.,* 1991, 1993). In the present study, HT-29-D4 cells were first infected with HIV-1 and then analyzed during the course of epithelial cell differentiation. Under these conditions, the HT-29-D4 cell clone showed a delay in the kinetics of epithelial differentiation as assessed by electrophysiological analysis of the transepithelial resistance. Since the value of this parameter reflects the degree of organization of tight junctions over the cell monolayer (Karnaky, 1992), this result indicates that HIV-1 has a direct effect on the barrier function of a model intestinal epithelium. Although variations of tight junction permeability occur in normal physiological processes (Madara *et al.,* 1992), an altered permeability in infectious diseases may contribute to the development of an inflammatory response (Parkos *et al.,* 1991) and diarrhea (Philpott *et al.,* 1996).

Sugar malabsorption is frequently reported in HIV-1-infected individuals (Ott *et al.,* 1993). In mature enterocytes, the sodium/glucose cotransporter is the main sugar transport system (Wright *et al.,* 1996). Thus, we studied whether HIV-1 could modulate this glucose transport function, which gradually appears during the course of enterocytic differentiation along the crypt–villus axis of the intestinal epithelium (Hwang *et al.,* 1991). We found that the activity of the sodium/glucose cotransporter was significantly lower in HIV-1-infected HT-29-D4 cells than in noninfected control cells. The decreased activity of this cotransporter on the apical membrane of HIV-1-infected cells may result not only in sugar malabsorption, but also in diarrhea, since the sodium/glucose cotransporter has the properties of a water channel (Wright *et al.,* 1996). In this respect, it has been estimated that, in the human small intestine, secondary active transport of water through the sodium/glucose cotransporter can account for as much as 5 liters of water absorption per day (Wright *et al.,* 1996). A diminished transepithelial water absorption may therefore contribute to the diarrhea syndrom. The presence of domes in HIV-1-infected, earlier than in noninfected HT-29-D4 cells (Fig. 1), may also be related to functional alterations of ionic transport, as generally admitted (Lever, 1982). Taken together, these data support the concept of an etiologic role for HIV-1 in the malabsorption and diarrhea syndrome associated with AIDS.

At the morphological level, the defect of cell differentiation induced by HIV-1 was characterized by the accumulation of lumina in several areas of the culture, indicating the inability of these cells to organize into a regular epithelial monolayer (Fig. 1). These lumina, which have been previously observed in HIV-1-infected HT-29 cells, are representative of a blocking of transport of vesicles to the apical membrane, resulting in a defect of the assembly of brush-border components (Fantini *et al.,* 1992). Indeed, a similar accumulation of intracellular vesicles is also found in the supranuclear cytoplasm of colonocytes from children with Davidson's disease, a microvillus inclusion syndrome causing malabsorption and diarrhea (Cutz *et al.,* 1989). In Davidson's disease, the morphological and functional perturbations of enterocytes are due to a dysfunction of the microtubular network, which is unable to transport the vesicles to the apical plasma membrane (Cutz *et al.,* 1989). The correct targeting of apical proteins, including the sodium/glucose cotransporter, is dramatically affected by microtubule-disrupting drugs such as nocodazole (Van Den Bosh *et al.,* 1990). These data prompted us to test whether nocodazole treatment of HT-29-D4 cells could mimic the morphofunctional perturbations induced by HIV-1 infection of these cells. Morphological analysis of nocodazole-treated cells showed the presence of numerous lumina ressembling those observed in HIV-1-infected cells. Moreover, nocodazole treatment of HT-29-D4 cells was associated with a decrease of the sodium/glucose cotransport activity. Therefore, the chemical disruption of microtubules of intestinal epithelial cells by nocodazole elicited morphofunctional perturbations that mimicked those induced by HIV-1.

There are several potential mechanisms by which HIV-1 can affect the integrity of the microtubule network. For instance, it has been shown that the HIV-1 protease encoded by the *pol* gene could hydrolyze certain cytoskeletal proteins such as vimentin (Höner *et al.,* 1991). An effect of the viral protease would be possible only after integration and expression of the HIV-1 provirus in HT-29-D4 cells. However, morphological changes and, in particular, the appearance of lumina occurred shortly (<4 h) after exposure of HT-29-D4 cells to HIV-1 (data not shown). Thus, we postulate that virus binding, rather than viral replication, is responsible for the perturbations observed in HT-29-D4 cells. In this respect, we have recently reported that the binding of recombinant surface envelope glycoprotein gp120 on the GalCer receptor of HT-29-D4 cells evoked a transient increase in intracellu-

lar calcium concentration (Dayanithi *et al.,* 1995, 1996). Such a rise in calcium concentration is likely to affect the integrity of the microtubular network, as previously shown for oligodendrocytes stimulated by anti-GalCer antibodies (Dyer and Benjamins, 1990). Based on these data, one can draw the following sequence of events to explain how HIV-1 can directly interfere with the maturation process of enterocytes: (i) binding of gp120 (either virion-associated or free) to the plasma membrane of enterocytes through GalCer; (ii) increase in intracellular calcium concentration; (iii) depolymerization of microtubules leading to an accumulation of transporting vesicles containing brush-border proteins, and reorganization of the actin cytoskeleton at the level of junctional complexes (Madara *et al.,* 1986), resulting in increased paracellular permeability. One should note that this mechanism does not involve HIV-1 infection of enterocytes. Instead, we suggest that HIV-1 attachment on the plasma membrane is sufficient to alter the morphological and functional maturation of intestinal epithelial cells. This concept of a viral toxin-like effect exerted during virus–cell contact is supported by recent data showing that rotavirus diarrhea is probably caused by nonreplicating viral particles (Shaw *et al.,* 1995). Two lines of evidence suggest a similar mechanism for HIV-1 in the intestinal epithelium. On one hand, we observed that the binding of HIV-1 particles to differentiated intestinal cells, through a GalCer/gp120 interaction, resulted in an important redistribution of cytoskeletal elements, including microtubule depolymerization (Fig. 5). On the other hand, the decrease in the transepithelial resistance induced by HIV-1 in HT-29-D4 cells was observed only during the acute phase of replication of the virus. Indeed, the data in Table 2 show that after 20 days of culture, noninfected and HIV-1-infected cells displayed a similar electrical resistance (1188 ± 166 and 1117 ± 109 ohms·cm2, $n = 3$, respectively). At this time, viral production was minimal, as assessed by *in situ* hybridization of HIV-1 mRNAs (data not shown). In the intestinal mucosa of HIV-1-infected patients, the permanent production of HIV-1 particles from various infected cell types (i.e., lymphocytes, macrophages, and/or epithelial cells) may alter the maturational process of absorptive intestinal cells, resulting in chronic diarrhea and malabsorption.

Further studies are in progress to identify whether other enterocytic functions are altered by HIV-1. The data reported in the present study provide the first experimental support of the hypothesis that HIV-1 may directly alter intestinal functions, probably through virus–cell contact.

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