"FEDERICO II"
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"Human Reproduction, Development and Growth"

Director
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PhD Thesis

"Relationship between transepithelial ion transport and growth and differentiation in human enterocyte"

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To the readers
who will are pleased
reading my PhD thesis.

Vittoria

Chapter 1 Introduction

1.1 Ion transport in the gastrointestinal (GI) tract

Proximal to distal intestine segments, from the duodenum to the distal colon, have differential mechanisms for either electrolytes absorption and secretion. Fluid transport across intestinal epithelial cells is a finely balanced process with net absorption predominating under normal conditions. However a basal level of fluid secretion is necessary for accomplishing the digestive functions.

Large water volumes are secreted and reabsorbed through the small intestinal epithelium during the digestive processes. The cyclic AMP-dependent chloride channel defines as the cystic fibrosis transmembrane conductance regulator or CFTR is located on the brush border. This channel is responsible for water secretion in basal conditions and under secretagogue stimulation (Bradbury 2001).

In the intestine, water secretion is a passive process driven by the active ion secretion, predominantly chloride (Cl^-) (Kunzelmann 2002). Chloride is uptaken by the cell across the basolateral membrane via sodium/potassium/2 chloride cotransporter type 1 (NKCC1), in an electroneutral manner (Fig. 1). Chloride accumulation is a passive process driven by sodium concentration gradient maintained by the basolateral Na,K-ATPase. Two distinct potassium channels are located into the basolateral membrane allowing for potassium recycling and thus preventing cellular depolarization, ultimately preserving the electrical driving force for chloride exit from the cell. Therefore, chloride accumulates until apical chloride channels are opened. The bulk of chloride output occurs via the cAMP-dependent CFTR chloride channel. However, recent data also points to the precence of one additional class of chloride channels, the calcium-activated chloride channels (CaCC), that are expressed in the enterocyte apical membrane. These channels may drive chloride secretion induced by agonists that raise cytosolic calcium (Barrett 2000).

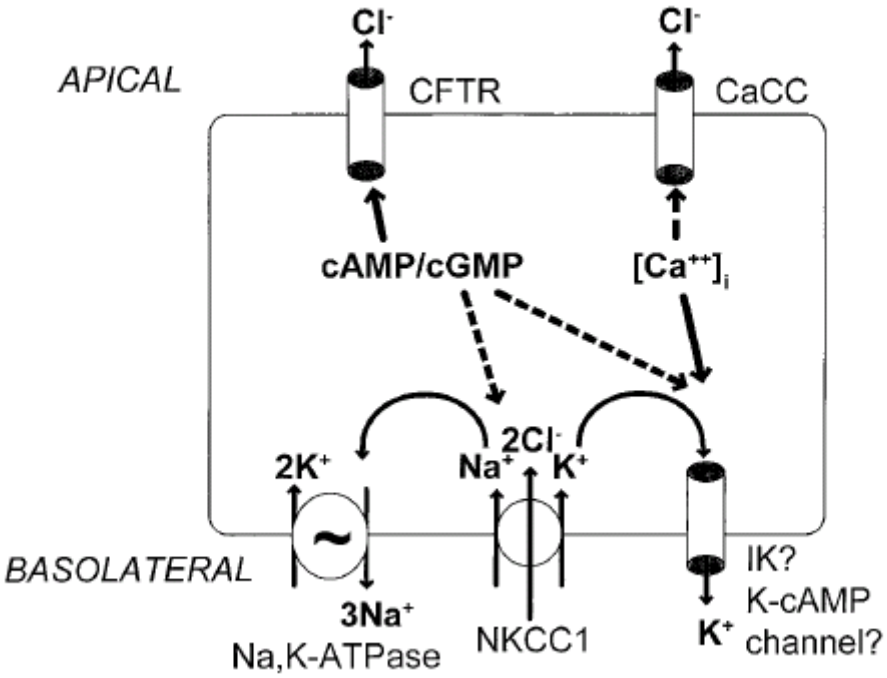

Fig.1 Model of the chloride secretory mechanisms in intestinal epithelial cells. Secretion can be stimulated by increases in either cyclic nucleotide (cAMP/cGMP) or cytosolic calcium ($[Ca^{2+}]i$). The major targets for these intracellular messengers are indicated with solid arrows, with additional postulated sites of action indicated with broken arrows. The identity of basolateral potassium channel(s) involved in either cyclic nucleotide- or calcium-mediated chloride secretion remains unknown. Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; CaCC, calcium-activated chloride channel; NKCC1, sodium/potassium/2 chloride cotransporter type 1; IK, intermediate conductance potassium channel; K-cAMP channel, putative potassium channel regulated by cAMP. (from Barrett 2000)

Epithelial fluid secretion may be upregulated in response to a wide range of physiological stimuli, such as the distension of the intestinal wall and mucus production. In normal conditions, increased fluid secretion is needed for the progression of digested materials and the clearance of harmful substances from the intestinal tract. However, intestinal conditions such as inflammatory bowel diseases and enteric infections can result in a "secretory state". Intestinal ion transport is usually regulated by hormones, neurotransmitters, and inflammatory mediators, acting through specific receptors located on the enterocyte surface that in turn increase the intracellular levels of second messengers. These include: 1) cAMP, 2) cGMP, 3) nitric oxide (NO) and 4) calcium (Ca^{2+}) (Fig.1). Physiological cAMP concentrations activate CFTR resulting in a mild secretion of chloride whereas abnormal activation of CFTR in crypt

cells results in the secretory diarrhoea. Several bacterial strains produce enterotoxins that activate the adenylate cyclase (AC) in crypt enterocytes, leading to elevated levels of cAMP that opens CFTR. The result is a massive water secretion typical of severe diarrhea. Cholera toxin, is the enterotoxin prototype but other bacteria produce similar toxins (Fasano 2002).

Also cGMP is able to phosphorylate and activate CFTR by cGMP dependent protein kinase II, resulting in chloride and bicarbonate secretion (Vaandrager 1997a, 1997b). cGMP generally results in a more potent, though shorter, chloride secretion than that induced by cAMP (Golin-Bisello 2005).

Intracellular Ca^{2+} concentrations are generally very low (approximately 100nM). Neurohormonal substances or toxins can increase intracellular Ca^{2+} by altering the permeability of intracellular stores thereby activating CaCC and promoting chloride secretion (Keely 2000).

There is evidence that NO takes part in the regulation of intestinal ion transport with effects that involved enteric nervous system, suppression of prostaglandin formation, and opening of K^+ channels (Izzo 1998). An important role in intestinal epithelial cells was supported by the demonstration of electrogenic secretion induced by NO substrate L-arginine (Rolfe 1999) and NO syntase inhibitor L-NAME in an *in vitro* model (Berni Canani 2003a). However NO may exert an absorptive effect on transepithelial ion fluxes depending on concentration. Wapnir et al. (Wapnir 1997) have shown that low concentration of L-arginine stimulates water and electrolyte absorption in the rat jejunum whereas higher L-arginine concentrations induced a secretory shift of electrolyte transport. Therefore NO may play a dual role in determining the proabsorbitive or secretive tone depending on its concentration.

MAP Kinases are also involved in the control of intestinal ion transport. Several evidences demonstrated a specific role of two kinases, ERK1/2 (or p42/44) and p38 in the regulation of Ca^{2+}-dependent chloride secretion (Keely 2003). Keely et al. hypothesized that either ERK1/2

and p38 function as physiological brakes to prevent excessive electrolyte loss. It was also demonstrated that the proabsorbitive and anti-secretive effects by growth hormone (GH) on intestinal epithelial cells is mediated by a specific activation of ERK1/2 and p38 (Chow 2003).

1.2 Intestinal cell growth and differentiation

The turnover of epithelial cell lining intestinal villi is a continous process. The small intestine mucosa is composed by villi, projecting into the lumen (Fig.2). A single layer of columnar cells lines the crypts and villi. As the crypt columnar cells migrate toward the tip of the villus, they differentiate into the specific small intestine cell types (absorptive cells, goblet cells and enteroendocrine cells).

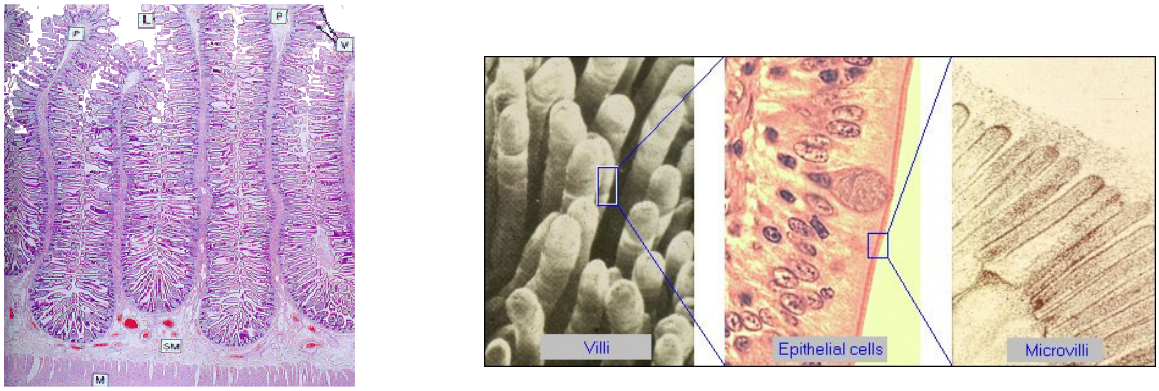

Fig. 2 Intestinal villi are tiny, finger-like structures that protrude from the wall of the intestine; they have additional extensions called microvilli which protrude from epithelial cells lining villi increasing the absorptive area of the intestinal wall.

Columnar cell migration from the crypt base to the villus tip takes 5 to 6 days in the human proximal small intestine and 3 days in the human ileum (Becciolini 1996). As cells exit the crypt and enter the villus, they stop cycling and are trapped in the G1 phase of the cell cycle as a result of down-regulation of cyclin D1 and cyclin-dependent kinase 2 (cdk2) (Chandrasekaran 1996). After 2 to 3 days, they reach the villus tip undergoing apoptosis.

Apoptosis, or programmed cell death, plays an important role in determining the architecture of intestinal epithelia.

The small intestinal epithelium performs highly specialized functions responsible for most digestive and absorptive processes. Specific hydrolases located on the enterocyte brush border, such as maltase, lactase and sucrase, cleave disaccharides to monosaccharides allowing their absorption through specific sodium-dependent transporters (GLUT-1, GLUT-5). Once entered the enterocyte, glucose, galactose and fructose are transported to the basolateral membrane through another hexose transporter called GLUT-2 and then reach the capillary blood along a concentration gradient (Thorens 1996).

Although the digestive enzyme activities increase along the crypt-villus axis (Fan 2001), proliferation and differentiation are at least in part independent processes that need to be finely tuned.

The control of cell cycle and differentiation is mediated by a complex array of signalling molecules at the cell surface which lead to long-term changes in gene expression. The mitogen-activated protein kinases (MAPKs) cascade is required for intestinal cell cycle progression and differentiation of human intestinal cells. MAPKs p42 and p44, also called ERK2 and ERK1, are involved in the regulation of intestinal cell proliferation and differentiation. An interesting feature of this kinase family is that they require dual phosphorylation of threonine and tyrosine residues for their activation. MAPKs activation is mediated by a dual specificity kinase termed MAP kinase kinase (MEK). Upon stimulation, p42/44 MAPKs translocate to the nucleus where they phosphorylate nuclear transcription factors thereby regulating gene expression. Activated MAPKs can regulate a number of downstream targets, including additional kinases, receptors, and transcription factors such as Elk-1, ATF-2, c-Jun, and CHOP (Aliaga 1999).

1.3 Factors implicated in functional modulation of intestinal cell growth and ion transport

Intestinal homeostatic mechanisms are activated in response to a wide pattern of conditions such as feeding pattern (fasted or fed), diet composition, site of nutrient presentation (luminal vs vascular, small bowel vs. colon, jejunum vs. ileum), development stage (suckling, lactation, olden age), or disease states (malnutrition, sepsis, infection, bowel resection ecc.). Increasing evidence suggests that specific nutrients and endogenous molecules can directly influence intestinal mucosal turnover, repair and adaptation. Several of the mechanisms of these effects are unknown.

In addition, intestinal mucosa is sensitive to the action of endocrine and paracrine molecules and exogenous factors able to modulate specific intestinal functions (Tab. 1). The interactions between the latter and intestinal epithelial cells form a complex molecular network regulating ion transport and cell growth.

Tab.1 Intestinal modulators

Agonists and antagonists of intestinal chloride secretion

Secretagogues	
Neurotransmitters	Acetylcholine VIP
Immune Mediators	Histamine Adenosine Prostaglandins Cytokines (IL-1)
Endocrine Mediators	Uroguanylin
Paracrine Mediators	Guanylin 5-HT
Exogenous Agents	Microbial enterotoxins

Inibitors of chloride secretion	
Neuropeptides	NPY PYY
Growth factors	EGF TGF-α Insulin IGF-I GH
Trace elements	Zinc

Intestinal growth modulators

Positive modulators	
Nutrients	Aminoacids (taurine) Minerals (Ca^{2+}, Cu^{2+}, Fe^{2+}, Se^{2+}, Zn^{2+}) Vitamins
Hormones	Insulin TSH Prolactin GH T_3 and T_4
Growth factors	EGF Gastrin IGF-I, IGF-II HGF NGF FGF KGF GLP-2
Attachment factor	Fibronectin
Binding proteins	Lactalbumin Transferrin Casein
Polyammines	Spermine Spermidine Putrescine
Cytokines	TGF-α , TGF-β

Negative modulators	
Cytokines	INFγ IL-4 IL-13
Exogenous agent	Microbial cytotoxins

Abbreviations: EGF, Epidermal Growth Factor; FGF, Fibroblast Growth Factor; GH, Growth Factor; GLP-2, Glucagone-like Peptide-2, HGF, Hepatocyte Growth Factor; 5-HT, 5-Hydroxytryptamine; IGF, Insuline like Growth Factor; IL, Interleukin, INFγ, Interferon; KGF, Keratinocyte Growth Factor; NGF, Nerve Growth Factor; NPY, Neuropeptide Y; PYY, Peptide YY; T_3, Triiodothyronine; T_4, Thyroxine; TGF, Transforming Growth Factor; TSH, Thyroid Stimulating Hormone; VIP, Vasoactive Intestinal Polypeptide.

1.4 Aims of this PhD thesis

The aim of this PhD thesis is to study the physiological modulation of intestinal functions with specific focus on ion transport and enterocyte growth and differentiation. The working hypothesis is that a strict relationship exists between ion absorption and cell growth and, conversely, between ion secretion and impairment of cell growth. To study the effects on either intestinal processes we evaluated the specificities, similarities and differences between agonists and antagonists of either cell growth and ion absorption, selecting moieties of completely different origin. Namely a typical growth factor, a trace element and the most abundant protein in human milk were studied. Caco-2 cell line was used because this is an established human-derived intestinal cell line that differentiates into mature human enterocytes generating monolayers of polarized cells (Grasset 1984).

We also explored the opposite pathway, i.e. ion secretion and cell growth impairment. Intestinal homeostatic pathways can be disturbed by molecules that induce functional and structural damage. Pro-inflammatory cytokines are an example of endogenous molecules that induce an alteration of ion equilibrium within enterocyte and in parallel negatively influence the normal cell cycle. Enterotoxins produced by pathogenic microrganisms are an example of exogenous negative factors. To further investigated the interplay between ion secretion and impairment of cell growth, the effects induced by the transactivating factor protein (Tat) produced by HIV-1 and by heat-stable enterotoxin (ST) produced by enterotoxigenic E.coli as well as their mechanisms were also investigated.

Chapter 2 Methods

2.1 Cell line

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, Maryland). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM Gibco, Grand Island NY, USA) with a high glucose concentration (4.5 g/L) supplemented with 10% FBS, 1% non-essential amino acids, penicillin (50 mU/mL), streptomycin (50 mg/mL) and kept in 5% CO_2-95% air. Cells were used between the 20^{th} and 40^{th} passage and the medium was changed daily.

2.2 Ion transport studies

Cells were grown on uncoated polycarbonate transwell filters (Costar Italia, Milan, Italy) for 15-18 days after confluence. The filter area was 4.9cm^2. Each filter was mounted in an Ussing chamber (WPI, Sarasota, FL) as a flat sheet between the mucosal and the serosal compartment. Each compartment contained 5ml of Ringer solution with the following composition (in millimoles per liter): 114 NaCl, 5 KCl, 1.65 Na_2HPO_4, 0.3 NaH_2PO_4, 1.25 $CaCl_2$, 1.1 $MgCl_2$, 25 $NaHCO_3$, 10 glucose, constantly gassed with 5%CO_2-95%O_2, and maintained at 37° C through a termostat-regulated circulating pump. The following electrical parameters were measured: transepithelial potential difference (PD), short-circuit current (Isc), and tissue ionic conductance (G). Isc is expressed as microamperes per square centimeter (μA/cm^2), G as millisiemens per square centimeter (mS/cm^2), and potential difference as millivolts (mV). Cell viability was evaluated by measuring the electrical response to the serosal addition of theophylline (5mM) at the end of each experiment.

In GH experiments: GH was added to the serosal side of epithelial cells. Pre-incubation of 30 minutes with specific MAPKs inhibitors, was performed to investigate the role of p42/44 and p38 on GH-induced electrical response (PD098059 40μM and SB203580 5μM, respectively). To investigate the role of NO in GH-induced modifications of ion fluxes the specific cNOS inhibitor

Nω-nitro-L-arginine methyl ester (L-NAME) (2×10^{-4} mol/L) was added on the mucosal and serosal sides.

In Zinc experiments: Zinc (35μM) was added to the serosal or mucosal side of epithelial cells.

In HIV-1 Tat experiments: Tat protein was added, at the concentration of 0.1nM, to the serosal or mucosal side of epithelial cells. In experiments performed to investigate the role of Cl^- in the electrical response, SO_4^- substituted Cl^- at an equimolar concentration. To investigate in greater details the role of Cl^- in the electrical effect of Tat, we used the Cl^- channel inhibitor 5-nitro-2-3-(3-phenylpropylamino) benzoic acid (NPPB). Cells were incubated with NPPB (100 nM), Tat was then added, and electrical parameters were monitored. Bay K8644, a specific agonist of L-type Ca^{2+} channels, was used to investigate the role of L-type Ca^{2+} channels in the electrical effects exerted by Tat. Caco-2 cell monolayers were incubated for 20 min with Bay K8644 (1μM, on the serosal side), after which Tat (0.1nM) was added on the serosal side.

In VEGF experiments: VEGF ion transport effect was evaluated as described in Tat experiments. Neutralization experiments were performed using specific anti-Tat polyclonal antibodies developed in rabbit using purified synthetic Tat protein as immunogen. VEGF (0.1nM) was incubated at 37°C for 1h with the antibodies (6ng/ml), then added to Caco-2 cells in Ussing chambers.

In ST experiments: To assess the efficacy of specific ERK1/2 inhibitor, PD098059 (40 μM) was added 30 minutes before the stimulation with ST.

2.3 Intestinal cell growth studies

^{3}H-thymidine uptake: Caco-2 cells were seeded onto 96-well microtiter plates (10^4 cells/well) and cultured for 3 days in DMEM with 10% FBS. After 24h of serum-starvation, cells were exposed to testing substances for 48h in DMEM FBS-free. ^{3}H-thymidine (0.5μCi/well, ICN Biomedicals, Irvine, CA, USA) was added 18h before harvesting the cells with a semiautomatic cell harvester

(Skatron Instruments, Lier, Norway). The filters were dried and beta radioactivity was counted with a Packard scintillation spectrometer.

In GH experiments: GH was added to Caco-2 cells at increasing concentrations. Before GH addition, pre-incubation of 30 minutes with the specific MAPKs inhibitors (PD098059 40µM and SB203580 5µM), was performed to investigate the role of p42/44 and p38 on GH-induced proliferation effect.

Cell counting: Cells were plated into 24-well tissue culture plates (10^4 cells/well), using two wells for each experimental condition, and were grown in DMEM supplemented with 10% FCS and antibiotics for 72h. Cells were then deprived of serum for 24h. Cells were harvested with 1% trypsin, 48h after the addition of each testing substance. The resulting cell suspensions were randomly assigned to another investigator and cells were counted in a blinded fashion. Cell viability was determined by trypan blue exclusion. The difference in paired counts did not exceed 10%.

In VEGF experiments: Neutralization experiments were performed using specific anti-Tat polyclonal antibodies developed in rabbit using purified synthetic Tat protein as immunogen. VEGF (0.1nM) was incubated at 37°C for 1h with the antibodies (6ng/ml), then added to Caco-2 cells in cell growth experiments.

Bromodeoxyuridine incorporation: DNA synthesis was assayed by a 2-h pulse with 100 µM BrdUrd, and incorporation was monitored by using the *in situ* cell proliferation kit FLUOS (Roche Applied Science).

2.4 Intestinal cell differentiation studies

Disaccharidase activity assay: Cells were collected after 24h of stimulation and lactase and sucrase enzymatic activities were measured by modified Dahlqvist method (Messer 1966). Briefly, cells were rinsed in cold PBS and scraped into cold Maleate buffer 0.1M pH 6.0. Samples were sonicated

3 times for 15 sec each, using a Labsonic 2000 (B.Braun Biotech Inc., USA) and total cell lysates were incubated at 37 °C with 50mmol/L lactose for 60 min or 50mmol/L sucrose for 30 min. The glucose generated by enzymatic activity was measured using a glucose oxidase assay.

RNA extraction and Reverse Transcription: Preconfluent Caco-2 cells were collected after 24h of stimulation and total RNA has been extracted from Caco-2 cells by TRIzol ®Reagent protocol (Invitrogen, Life Technologies). The amount of extracted RNA was quantified by measuring the absorbance at 260nm. Reverse Transcription of RNA was performed using High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA).

Quantitative real-time RT-PCR: Real-time RT-PCR was performed according to the recommendations supplied by Applied Biosystems (http://europe.appliedbiosystems.com/). Primers for sucrase (Hs00356112_m1) and lactase (Hs00158722_m1) were purchased from Applied Biosystems. A 25µl PCR reaction volume was prepared using about 40ng of cDNA as template. Reactions were run in 96-well optical reaction plates using an Applied Biosystems 7300 Real-Time PCR System. Thermal cycles were set at 95°C (10 min) and then 35 cycles at 95°C (15 sec) and 60°C (1 min) with auto ramp time. For data analysis the threshold line was set automatically and it was in the linear range of the amplification curves for all mRNA in all experimental runs. All reactions were performed in triplicate. The abundance of target mRNAs was calculated relative to a reference mRNA (GAPDH). Relative expression ratios were calculated as $R=2^{(Ct(GAPDH)-Ct(test))}$, where Ct is the cycle number at the threshold and the test stands for the tested mRNA. The confidence interval was fixed at 95%.

2.5 Determination of intracellular cyclic nucleotide concentrations

cAMP and cGMP concentrations in Caco-2 cells were determined with commercial kits (Biotrak cyclic AMP and Biotrak cyclic GMP assay system; Amersham International, Amersham, UK).

Caco-2 cell monolayers were grown on plates and used at 15 days postconfluence. After the addition of each testing substance, cells were scraped and lysed by the addition of the lysis buffer provided by the kit. Lisates were centrifuged at 2000g for 3 min at 4°C, and the supernatant was collected and evaporated to dryness under vacuum (Speed VAC 110; Savant Instruments, Farmingdale, NY). Dried samples were redissolved in 0.5M acetate buffer, pH 5.8 with 0.01% sodium azide, and cyclic nucleotide concentrations were measured according to the manufacturer's instructions. Results were expressed as picomoles of cGMP or cAMP, normalized for protein content and expressed as fold increase over basal level.

In GH experiments: cNOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) (2×10^{-4} mol/L) was added on Caco-2 cells 20 min before GH stimulation.

2.6 Determination of intracellular nitric oxide concentrations

The combined concentrations of NO_2^- and NO_3^-, the degradation products of NO in the culture medium, were determined by the Griess reaction after nitrate reduction. Total NO_2^-/NO_3^- production is therefore referred as NO production. Caco-2 cell monolayers were grown on plates and used at 15 days postconfluence. Experiments were also performed in Ca^{2+}-free Ringer's solution to investigate whether costituttive NO syntase (cNOS), which is the Ca^{2+}/ calmodulin-dependent NOS form, rather than the iNOS form was involved. The modified Ca^{2+}-free Ringer's solution had the following composition (in mmol/liter): Na_2HPO_4, 1.65; NaH_2PO_4, 0.3; $NaHCO_3$, 15; NaCl, 53; KCl, 10; Na_2SO_4, 30.5; $MgCl_2$, 2.35; glucose, 19; and EDTA, 0.5.

In GH experiments: cNOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) (2×10^{-4} mol/L) was added on Caco-2 cells 20 min before GH stimulation.

2.7 Determination of intracellular calcium concentrations

$[Ca^{2+}]_i$ was measured using a microfluorimetric technique. Briefly, cells grown on glass coverslips

were loaded with 5μM fura-2 AM in Krebs-Ringer saline solution for 1h at 22°C. After loading, the coverslip was introduced into a microscope chamber (Medical System Co., Greenvale, NY) on an inverted Nikon Diaphot fluorescence microscope. Cells were kept in Krebs-Ringer saline solution throughout the experiment. All substances tested were introduced into the microscope chamber by fast injection. A 100-watt Xenon lamp (Osram, Frankfurt, Germany) with a computer-operated filter wheel bearing two different interference filters (340 and 380 nm) illuminated the microscopic field with UV light, alternating the wavelength at an interval of 500ms. The interval between each pair of illuminations was 2 s, and the interval between filter movements was 1s. Consequently, $[Ca^{2+}]_i$ was measured every 3 s. Emitted light was passed through a 400-nm dichroic mirror, filtered at 510 nm, and collected by a CCD camera (Photonic Science, Robertsbridge, UK) connected to a light amplifier (Applied Imaging Ltd, Dukesway Gateshead, UK). Images were digitized and analyzed with a Magiscan image processor (Applied Imaging Ltd, Dukesway Gateshead, UK). Using a calibration curve, the AUTOLAB software (RBR Altair, Florence, Italy) calculated the $[Ca^{2+}]_i$ corresponding to each pair of images from the ratio between the intensity of the light emitted when cells were illuminated at both 340 and 380 nm. At the end of each experimental session, the calibration was performed according to the procedure described by Grynkievicz et al. In particular, cells were lysed with ionomycin (2-10μM), in the presence of 1.5mM extracellular Ca^{2+}. Ionomycin addition produced a rapid increase in fluorescence intensity that allowed to calculate the R_{max} value. To determine the R_{min} value, cells were subsequently exposed to a Ca^{2+}-free solution containing 1-20mM EGTA. Given that K_d for Ca^{2+} of fura-2 AM is 224 nM at 37°C, the R_{min}, R_{max} values were introduced into the Grynkievicz formula to convert the values of fluorescence ratio between 340 and 380 nm into $[Ca^{2+}]_i$. The background values fluorescence obtained from images taken from a region of the coverslip devoid of cells were subtracted. No interference was detected between any of the compounds utilized in the present study and the excitation or the emission spectra of fura-2

AM.

2.8 Western Blot

MAP Kinases, constitutive NO syntase (cNOS) and caspase-3 expressions were estimated by Western blot analysis. After the exposure to testing substances, cells were scraped into PBS buffer and lysed in the following buffer (KCl, 60mM; β-mercaptoethanol, 14mM; EDTA, 2mM; HEPES pH 7.9, 15mM; sucrose, 0.3M; aprotinin, 5μg/mL; leupeptin, 10μg/mL; pepstatin, 2μg/mL; phenylmethylsulfonyl fluoride, 0.1mM) containing 1% Tergitol (Nonidet P-40). Total extracts were centrifuged at 1500 *g* for 20 min at 4°C. Protein content was determined by the Bradford method (Bio-Rad Laboratories, Munich, Germany). The supernatant containing the solubilized proteins was boiled for 5 min in Laemmli buffer (62.5mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue). Cell protein (50μg/lane) was added to SDS-PAGE and transferred to a nitrocellulose membrane (BioBlot-NC-Costar; Corning Incorporated, Canada). Blots were blocked with T-TBS buffer (Tris-HCl pH 8.8, 10mM; NaCl, 150mM; Tween 20, 0.05%) containing 3% albumin, and probed for 1h with specific antibodies. Bound antibody was detected with anti-rabbit or anti-mouse immunoglobulin horseradish peroxidase–linked whole antibody and developed by chemiluminescence reaction (Amersham Pharmacia Biotech, U.K.). All incubations and washes were carried out at room temperature with gentle shaking.

For cNOS, cellular extracts were probed with affinity-purified anti-human cNOS rabbit polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). For p42/44, cellular extracts were probed with specific phospho-p42/44 mouse monoclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). For p38 protein, a specific phosho-p38 MAPK mouse monoclonal IgG (Cell Signaling Technology, Inc., MA, U.S.A.) was used. For caspase-3, a specific caspase-3 rabbit polyclonal antibody (Cell Signalling, Danvers, MA, U.S.A) was used. Normalization was performed by probing stripped filters with specific p42/44 or p38 total protein mouse monoclonal

IgG (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) or specific tubulin mouse monoclonal IgG (Sigma Chemical St. Louis, MO, USA). Nitrocellulose membranes were developed by ECL (Amersham Pharmacia Biothec. Buckinghamshire, U.K.).

2.9 Caspase-3 apoptotic assay

A spectrophotometric apoptosis assay kit from BioVision (BioVision, Mountain View, CA,U.S.A.) was used to determine the caspase-3 activity. Cells that have been induced to undergo apoptosis were collected by centrifugation. The supernatant was gently removed and discarded whereas the cell pellet was lysed by the addition of the lysis buffer provided by the kit. The protease activity in the cell lysate can be measured by the addition of a specific peptide substrate for caspase-3. The cleavage of the peptide by the caspase releases the chromophore pNA, which can be quantitated spectrophotometrically at a wavelength of 405 nm.

2.10 Glucose uptake studies

Caco-2 cells were grown on 24-well plates. After 15 days post-confluence cells were incubated for 30 minutes with the non-metabolizable radiolabeled glucose analogue [^{14}C]-α-Methyl-L-D-glucopyranoside (AMG, 0.1 mM). The cells were lysed in 0.1N NaOH. An aliquot was assayed for protein content (Bradford method, Bio-Rad Laboratories, Munich, Germany) and another for [^{14}C]AMG content using a Packard scintillation spectrometer. To verify the presence of SGLT-1 activity in the cell line, the same experiment was performed in the presence of the selective competitive inhibitor of SGLT-1 phlorizin (100 μM) or in Na^+-free buffer for 1 hour (using choline chloride and K_2HPO_4 in place of NaCl and Na_2HPO_4 adjusted to pH 7.4 with KOH). Tat was added at increasing concentrations (from 0.01 to 1.0 nM) for 1 hour, in the presence or

absence of anti-Tat polyclonal antibodies (10 to 1 weight/weight ratio) or the specific L-type Ca^{2+} channels agonist, Bay K8644 (1μM). All data was expressed as cpm/mg of protein.

2.11 Reagents

Growth Hormone, zinc chloride, human native LF from human milk, VEGF, guanylin and all reagents were purchased from Sigma Chemical (St. Louis, MO, USA). HIV-1 Tat, and rabbit polyclonal antibody anti-Tat were purchased from Tecnogen (Piana di Monteverna, Italy). ST was kindly provided by Dr. Ralph Giannella (Division of Digestive Diseases, University of Cincinnati College of Medicine, Cincinnati, USA).

2.12 Statistical analysis

Each experiment was run in triplicate and was repeated at least three times. Results are expressed as means ± SD. Significance was evaluated by the Student test. Results were considered significant at $p<0.05$.

Chapter 3 Intestinal positive modulators

3.1 Intestinal growth factor: growth hormone

Background

Growth hormone (GH) exerts a direct trophic effect in the intestine. It stimulates enterocyte growth and differentiation in rats which have undergone small bowel resection (Shulman 1993), it is required for growth and differentiation of fetal rat intestinal transplant (Cooke 1986), and it induces a trophic effect in human mucosa cultured in vitro (Challacombe 1995).

However GH also plays a role in intestinal ion transport processes. It decreases the short-circuit current (Isc) in the unstripped rat intestine mounted in Ussing chambers, consistent with an ion absorption (Guarino 1995). The absorptive effects are exerted throughout the rat intestine, but their intensity decrease in a proximal-to-distal direction (Berni Canani 1996). This region-specific distribution resembles the pattern of the trophic effect induced by GH in the intestine (Ulshen 1993).

In addition, GH effects observed in vivo on ion transport and proliferation are reproducible in vitro in a human intestinal cell line (Fig.3). In addition, they are both dependent on tyrosine kinase activity, suggesting a possible coupling of the two biological responses (Berni Canani 1999).

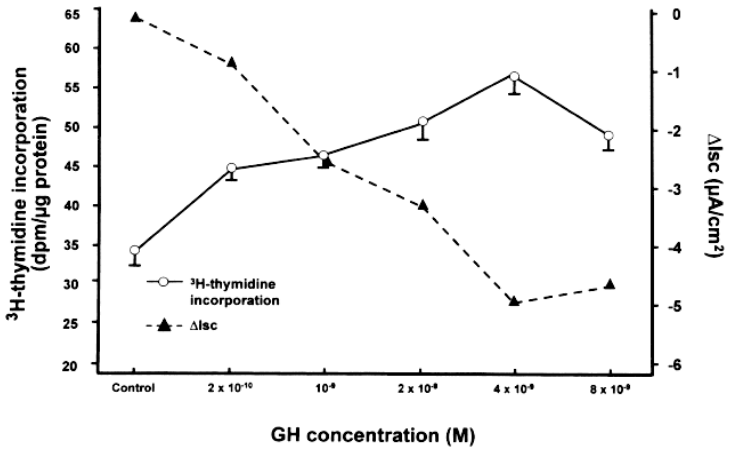

Fig. 3 Effects on proliferation and ion transport by increasing concentrations of growth hormone (GH) in Caco-2 cells (from Berni Canani 1999).

The earliest event in the intracellular signaling mechanisms subsequent to GH binding is the activation of a tyrosine kinase defined as Janus kinase 2. The activation of JAK2 triggers several signalling pathways, including phosphatidylinositol-3'-kinase (PI3K) and mitogen-activated protein kinases (MAPKs) and their upstream activators in the Ras-Raf-MEK1 pathway. The signalling molecules that are activated by GH receptor-JAK2 complex include signal transducers and activators of transcription (Stat) factors, the adapter protein Shc, and the insulin receptor substrates (IRSs) 1 and 2. The recruitment and activation of these signalling intermediates leads to the activation of enzymes such as MAP kinase, PI3K, protein kinase C (PKC), and phospholipase A2 and to the release of second messengers such as diacylglycerol (DAG), Ca^{2+} and nitric oxide (NO) (Dinerstein-Cali 2000).

Results

Role of MAP kinases

Previous findings showed that MAPKs play a crucial role in the regulation of chloride secretion (Keely 2003). Experiments were performed by adding GH on Caco-2 cell monolayer in Ussing chambers, alone or after the preincubation with PD098059 and SB203580, the specific inhibitors of ERK1/2 and p38 respectively. Results showed that the

preincubation with either inhibitors abolished GH proabsorbitive effect indicating that either ERK1/2 and p38 activation are involved in the basal absorption of water and electrolytes mediated by GH (Fig.4A). To evaluate the involvement of MAPKs in GH trophic effect, cell growth experiments were performend adding GH to Caco-2 cell monolayer, alone or in combination with PD098059 and SB203580. Results showed that only PD098059 inhibits GH trophic effect indicating that ERK1/2, but not p38, activation is necessary for GH trophic effect in epithelial intestinal cells (Fig. 4B).

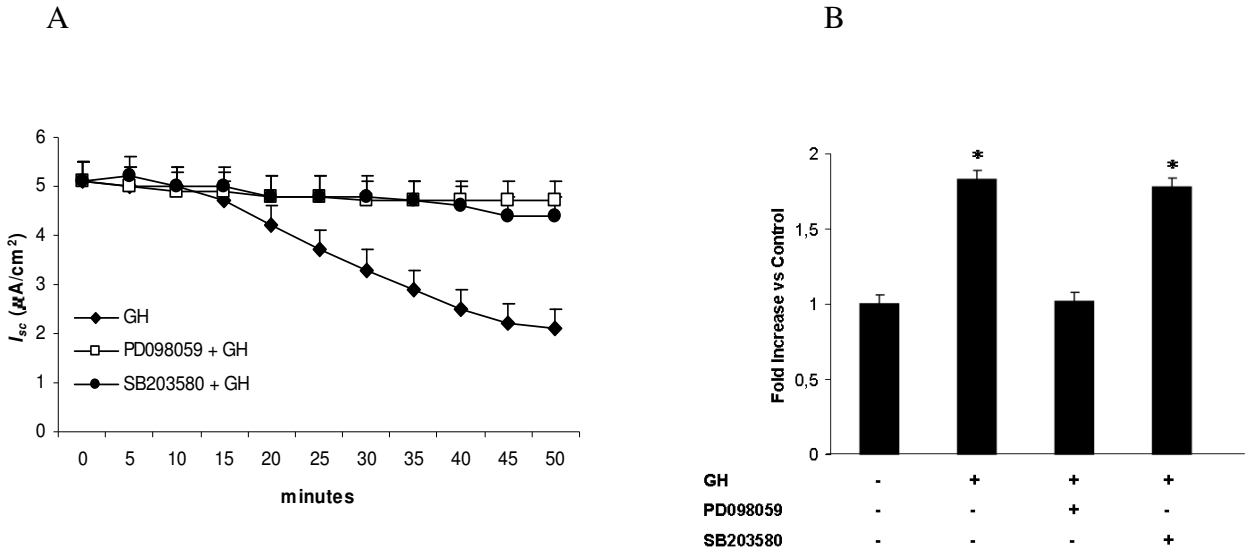

Fig.4 The inhibition of ERK1/2 and of p38 by preincubation with specific inhibitors (PD098059 and SB203580 respectively) abolished completely the GH proabsorbitive effects (A).The inhibition of ERK1/2 but not p38 abolished GH trophic effect (B). P<0.01 vs control. The maximal effective dose of GH was 100 ng/ml.

Role of nitric oxide

There is evidence that NO acts as a second messenger of several GH effects on human metabolism (Campbell 1997). NO production is decreased in patients with untreated GH deficiency, while treatment with recombinant human growth hormone increases NO formation (Boger 1999). The hypothesis that NO play an important role in pro-absorbitive

tone of enterocytes was tested. Experiments were performed to see that the intracellular NO concentration produced in the enterocyte is essential to regulate either basal transepithelial ion equilibrium specifically maintaining the absorptive tone as well as for increasing ion absorption upon stimulation by external stimuli (*Berni Canani 2006a*).

In ion transport studies, NO synthase (NOS) inhibitor L-NAME was used. The addition of L-NAME induced an increase of Isc suggesting that the intracellular NO concentration produced by the enterocyte plays a role in maintaining the basal transepithelial ion transport. In addition GH proabsorbitive effect is abolished by the presence of L-NAME (Fig.5).

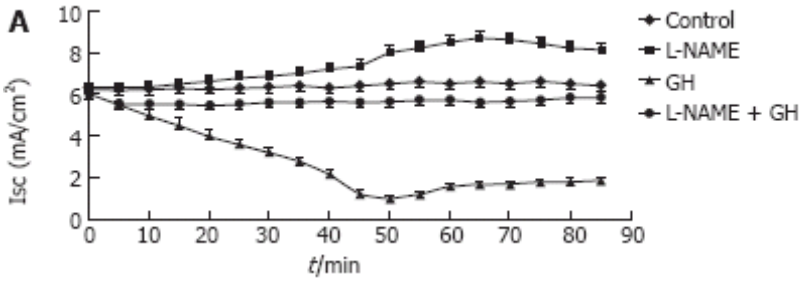

Fig. 5 Time course of the effect on short-circuit current (Isc) of GH (100 ng/ml) and NOS inhibitor, L-NAME ($2x10^{-4}$ mol/L), alone or in combination, in Caco-2 monolayer (from *Berni Canani 2006a*).

In addition a direct involvement on intracellular NO by GH was evaluated. GH was added on Caco-2 cell monolayer and NO concentration was evaluated by Griess reaction. Results showed a significant increase of NO production under GH stimulation in standard condition (Fig.6). To evaluate whether NO increase induced by GH was produced by cNOS the same experiments was performed in Ca^{2+}-free medium. In this condition GH did not induce NO increase (Fig.6).

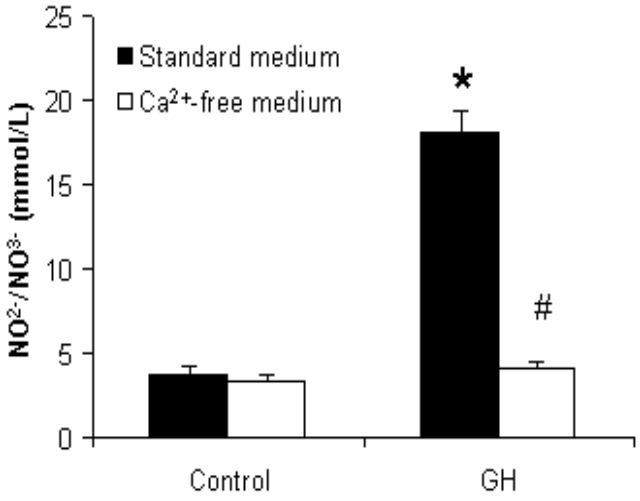

Fig. 6 Total NO production in Caco-2 cells under basal conditions (control) and after stimulation with GH (100ng/ml), in standard or in Ca^{2+}-free medium (modified by *Berni Canani 2006a*). *p<0.01 vs control standard medium #p<0.01 vs GH standard medium

In addition an increase of constitutive NOS form (cNOS) levels induced by GH was observed with western blot method (Fig.7).

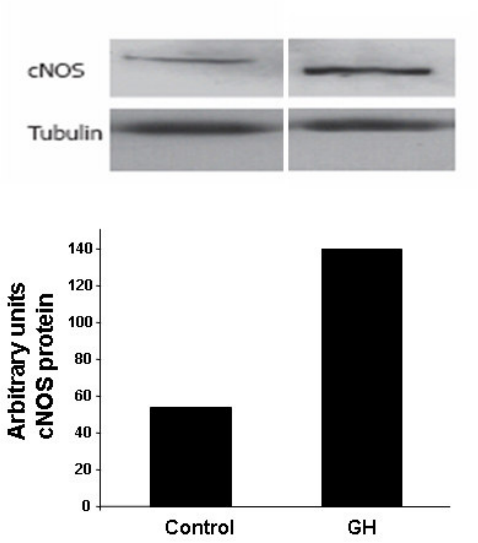

Fig. 7 The upper side of the figure shows cNOS protein expression in Caco-2 cells in basal condition (control) and after 1h of incubation with GH (100ng/ml) as compared to tubulin expression. In the lower side of the figure an optical densitometric analysis of the bands is also reported (modified by *Berni Canani 2006a*).

These data indicate that the observed effects of GH are the result of cNOS activation as shown by the abolished effect in the absence of Ca^{2+} and the upregulation of cNOS protein expression. These results suggest that NO plays a key role in response to external stimuli driving ion fluxes toward an absorption pattern.

Role of cAMP

The hypothesis was tested that under basal conditions, the intracellular cAMP concentration, the CFTR-dependent messenger of chloride secretion, is downregulated by NO (Berni Canani 2003a). The results of the experiments performed in Caco-2 cells showed that cell exposure to NOS inhibitor (L-NAME) is associated with an increase in intracellular cAMP levels in basal conditions. In addition, incubation with GH resulted in significant reduction of basal cAMP but the addition of L-NAME resulted in a total abrogation of GH effect (Fig. 8).

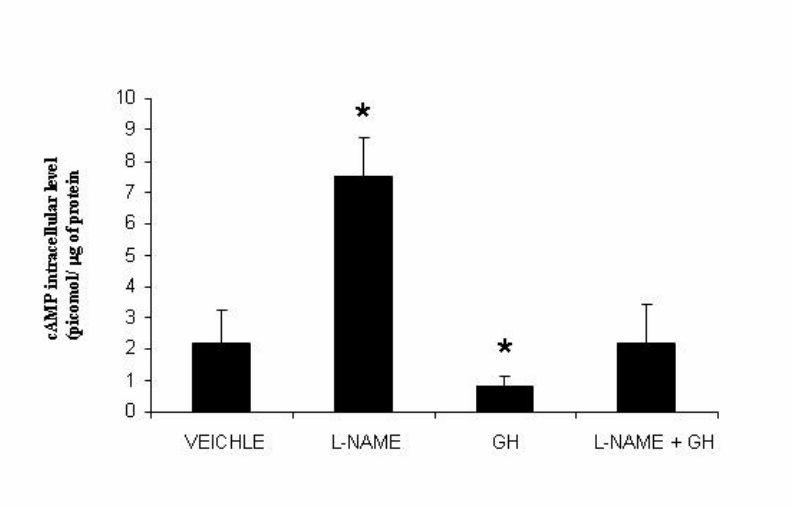

Fig. 8 Modification of intracellular cAMP concentration in Caco-2 cells after incubation with GH,in the presence or in the absence of L-NAME. ∗ p< 0.05 vs control (veichle). (*Berni Canani 2006a*)

This suggests that GH downregulates intracellular cAMP levels through a direct interaction with NO pathway. To support this, the role of NO was investigated in condition of active ion secretion.

Cholera toxin (CT) induces chloride secretion by increasing cAMP intracellular levels. Either cholera toxin or cAMP analogue induced a rapidly progressive, Ca^{2+}-dependent increase in NO concentration, suggesting a homeostatic up-regulation of the constitutive form of NO synthase. Namely in the presence of cAMP-dependent stimulated secretion, NO functions as a braking force of ion secretion (Berni Canani 2003a). These results provided further and direct evidence that the enterocyte regulates its own ion transport processes, either in basal condition or in presence of active secretion, through the activation of NO syntase-NO pathway, functioning as a braking force of cAMP-induced ion secretion (Berni Canani 2003a).

cNOS-NO-cAMP pathway is also implicated in the anti-secretory effect induced by GH in the intestinal cell line (*Berni Canani 2006a*).

Conclusions

In conclusion GH promotes enterocyte ion absorption and proliferation. The effect on intestinal ion transport involves either MAP Kinase ERK1/2 and p38, whereas the effect on intestinal cell proliferation selectively involves ERK1/2 suggesting that the pathways of GH effects are only in part common. NO also plays an active role in ion absorption.

Overall, the cNOS-NO system could be viewed as a regulator of ion transport acting on the enterocyte via three distinct pathways: (1) to keep cAMP production at a low level in basal conditions, in order to maintain an intestinal ion proabsorptive tone; (2) in conditions of excess of ion secretion, to homeostatically downregulate chloride secretion

and (3) in response to external pro-absorptive stimuli, acting as a second messenger (Fig.9).

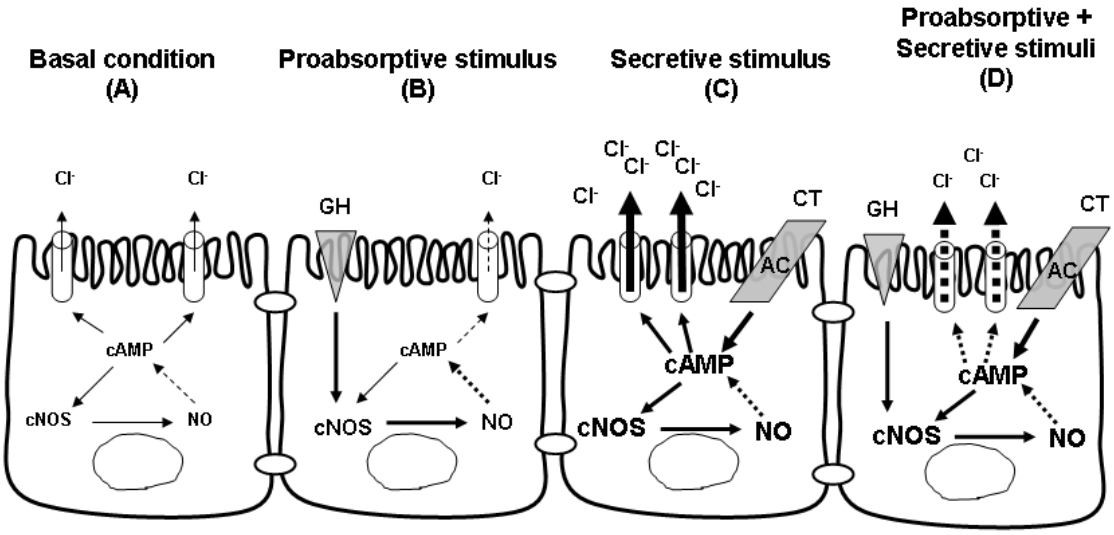

Fig. 9 cNOS-NO-cAMP pathway plays a key role on the enterocyte fluid absorptive/secretory processes. cNOS-NO-cAMP homeostatic pathway is essential to maintain a mild secretion under basal condition (A). Proabsorptive stimulus (GH) upregulates cNOS-NO signalling to reduce cAMP intracellular levels resulting in a decrease of chloride secretion (B). Secretive stimulus (CT) increases cAMP intracellular levels by activating adenylate cyclase (AC) resulting in a potent chloride secretion. cNOS-NO pathway was upregulated functioning as a braking force to reduce chloride secretion (C). This mechanism was further upregulated by proabsorptive stimulus reducing the enterotoxic effect of CT (D).

3.2 Zinc: a trace element with effects on ion transport and cell growth

Background

Zinc is a trace element necessary for a variety of physiological and biochemical functions, including the integrity of intestinal barrier and gut-associated immune function, reduction of oxidative stress, and inhibition of apoptosis (Blanchard 2001). The gastrointestinal tract is the major site of zinc uptake. When zinc intake decreases, zinc transport across intestinal mucosa through specific zinc transporters increases, resulting in a higher efficiency of zinc absorption (Cousins 1999). Zinc is directly involved in crypt cell production (Duff 2002) while zinc deficiency is associated with suppression of colonocyte proliferation (Lawson 1988). Zinc deficiency in malnourished children is associated with severe diarrhea (Bhutta 2000) with generalized malabsorption due to mucosal atrophy. Finally diarrhea induces loss of zinc, which inhibits mucosal turnover and repair, leading to further malabsorption of zinc and other nutrients (Wapnir 2000).

Results

Effects of zinc on intestinal ion transport

Experiments were performed to test the hypothesis that zinc promotes ion absorption through a direct interaction with the enterocyte (*Berni Canani 2005*). $ZnCl_2$ addition at the final concentration of 35μM, to the mucosal side of Caco-2 cell monolayer mounted in Ussing chambers, induced a decrease in Isc entirely due to an effect on PD, without affecting G values. The peak effect was observed 25 min after zinc addition (Fig.10A). $ZnCl_2$ addition to the serosal side induced a decrease in Isc entirely similar to that observed with mucosal addition, although the magnitude of the response was slightly reduced compared to that

observed with mucosal addition (Fig.10A). The decrease in Isc induced by both the M and S addition of zinc indicates ion absorption. No effect on G values was observed.

The effect on Isc was dose-dependent being detected at a zinc concentration as low as 10 µM, peaking at 35µM and decreasing with higher concentrations (Fig.10B). A toxic concentration (200µM) of $ZnCl_2$ induced an increase in Isc to a value above that of the untreated control cells, indicating ion secretion. (Fig.10B).

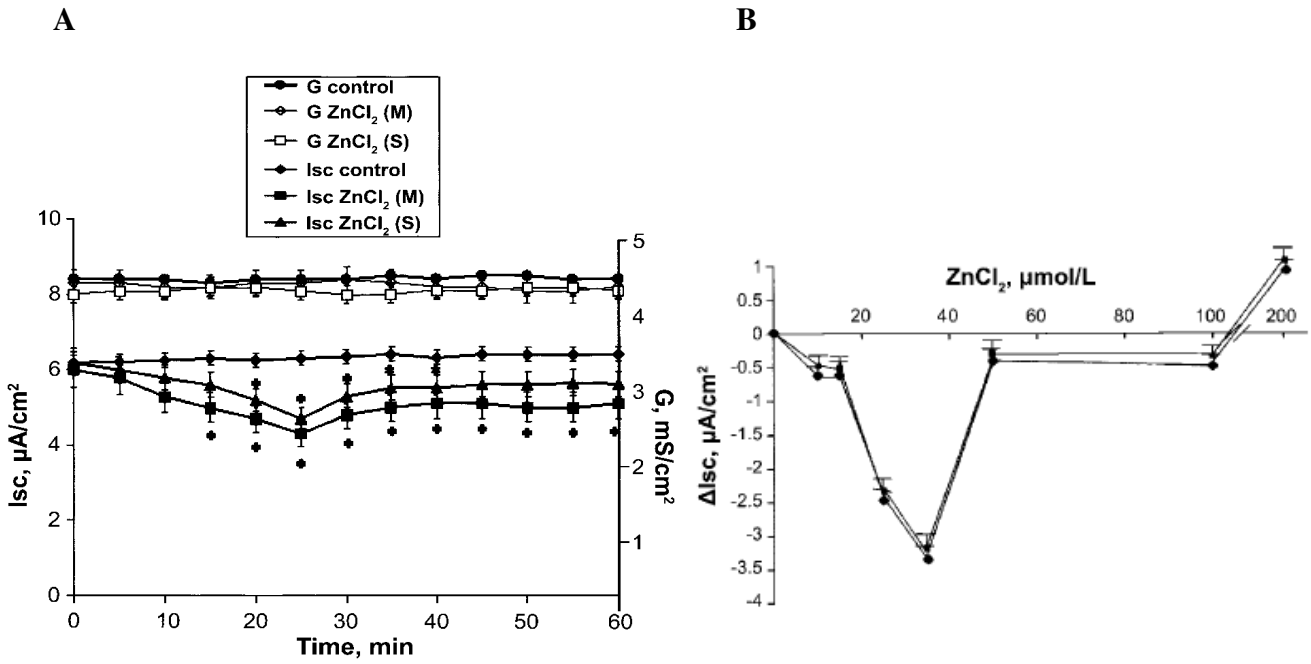

Fig. 10 (A) Time course of the effects of the mucosal (M) and serosal (S) addition of $ZnCl_2$ (35 µM) on short-circuit current (Isc) and tissue ionic conductance (G) in Caco-2 cells mounted in Ussing chambers. *p<0.05 vs control. (B) $ZnCl_2$ induced a dose-dependent decrease in Isc, in response to mucosal (●) or serosal (■) addition, peaked at 35µM. (from *Berni Canani 2005*)

Effects of zinc against active secretion triggered by enterotoxins

The hypothesis was tested that the proabsorptive effect induced by zinc (alike GH effect) may be effective against secretagogues. Preincubation with zinc reduced ion secretion induced by cAMP secretagogues in the Cholera toxin model in Caco-2 cells (Tab.2) measured in Ussing chambers. In addition, preincubation with $ZnCl_2$ resulted in a significant reduction of Isc

elicited by γ–Interferon, a NO activator, and by Carbachol, a Ca^{2+} activator (Tab.2). Zinc significantly inhibited the intracellular increases of cAMP, Ca^{2+} and NO, in response to Cholera toxin, Carbachol and γ–Interferon, respectively (Tab.2). In contrast, zinc was not effective against cGMP-dependent ion secretion upon E.coli ST stimulation (*Berni Canani 2005*).

Tab.2 Effects of exogenous zinc on ion secretion and relative mechanisms

SECRETOGOGUE	**Reduction in % of Isc increase measured after zinc preincubation (*)**	**INTRACELLULAR MEDIATOR**	**Reduction in % of intracellular mediator increase measured after zinc preincubation (*)**
CT ($6x10^{-8}$ M)	- 65%	**cAMP**	-80%
CARBACHOL (10^{-9} M)	- 68%	**Ca^{2+}**	- 68%
IFN-γ (50.000 U/mL)	- 100%	**NO**	– 97%
ST (10^{-6} M)	-------	**cGMP**	----------

Abbreviations: CT, Cholera toxin; INFγ, Interferon; ST, *E.coli* heat-stable enterotoxin.
*zinc preincubation was performed with $ZnCl_2$ 35μM 25 min before secretagogue addition

To further explore the antisecretive properties of zinc, experiments were performed using Tat, a HIV protein able to elicit chloride secretion through a Ca^{2+}-mediated pathway. Zinc was effective in inhibiting of ion secretion induced by HIV-1 enterotoxin, Tat protein (*Berni Canani 2007*). As shown in Fig.11, pre-incubation of human enterocytes with zinc resulted in the total inhibition of Tat-induced ion secretion, as reflected by the intensity of short circuit current. These results suggest that zinc is able to prevent intestinal fluid secretion induced by Tat.

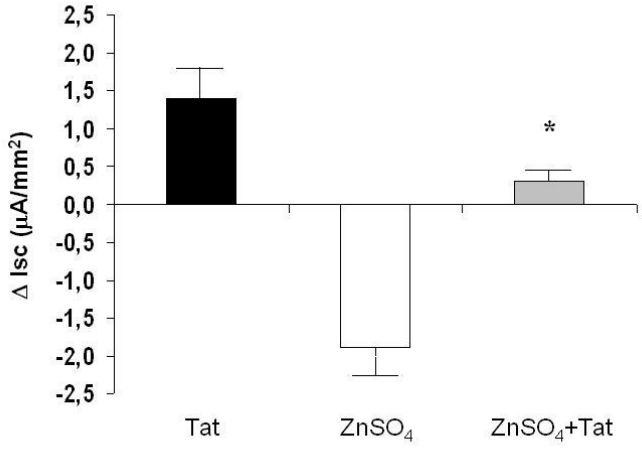

Fig. 11 Effects of HIV-1 Tat protein, alone or in the presence of zinc sulphate ($ZnSO_4$) on short-circuit current intensity (Isc) measured in Caco-2 cell monolayer mounted in Ussing chambers. Tat induced an Isc increase indicating a secretory effect on transepithelial ion trasport. $ZnSO_4$ determined a pure pro-absorptive effect (i.e., a decrease in Isc). Pre-incubation for 20 min with $ZnSO_4$ was able to significantly reduce the secretory response elicited by Tat at intestinal level (*Berni Canani 2007*). *$p<0.01$ vs Tat

Role of nitric oxide

Because NO is implicated in the proabsorbitive effect induced by GH and most data obtained with GH resembled those seen with zinc, NO production was also evaluated in basal condition and upon zinc addition. We observed an increase of nitric oxide induced by zinc in a dose dependent manner after 60 min of stimulation in undifferentiated Caco-2 cells (Fig.12).

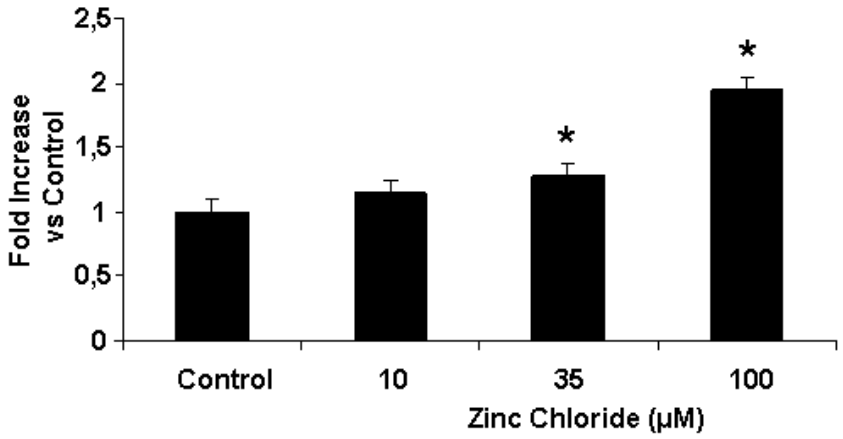

Fig. 12 Caco-2 undifferentiated cells (3 days post-plating) were exposed to increasing concentrations of $ZnCl_2$ (micromol/L) and nitric oxide was dosed by Griess reaction. The maximal effect was observed after 30 min of incubation. *p<0.01 vs control

Role of intracellular calcium

To test the hypothesis that zinc increases intracellular Ca^{2+} levels, microflorimetry experiments were performed.

In basal condition was registrated a constitutive level of free calcium ion; when zinc ($ZnCl_2$ 35µM) was added in cell milieu the increase of signal intensity was revelead indicating an increase of intracellular calcium levels (Fig. 13).

Basal condition Exogenous zinc

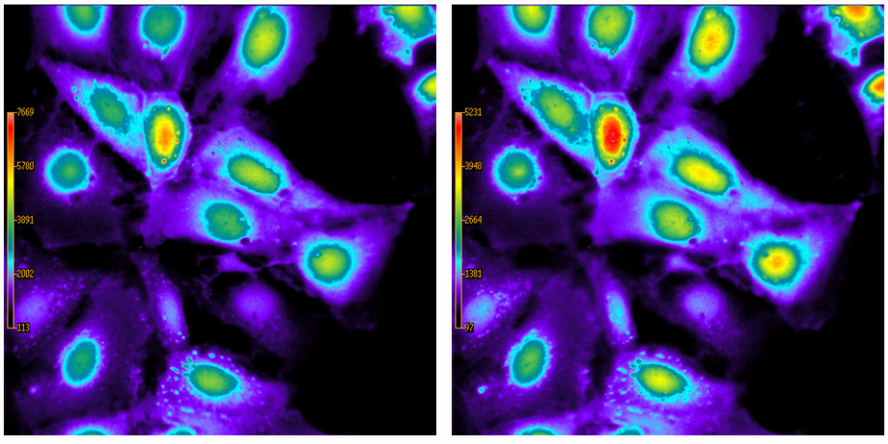

Fig. 13 Effect of $ZnCl_2$ on intracellular calcium levels in a microfluorimetry experiment.

These data all together show that, through a direct interaction with the enterocyte, zinc reduces ion secretion induced by three out of the four established intracellular signal transduction pathways that are responsible for enterotoxin diarrhea: cAMP; Ca^{2+} and (NO). In contrast zinc does not prevent cGMP-mediated ion secretion, although it may still have a protective effect through its action on basal ion transport (*Berni Canani 2005*).

Effects of zinc on intestinal cell growth

To investigate the trophic effect of zinc on intestinal epithelial cells, experiments were performed by adding zinc to cell culture medium and determining cell proliferation. In undifferentiated Caco-2 cells an increase of ^{3}H-thymidine incorporation in response to increasing zinc concentrations was observed (Fig.14).

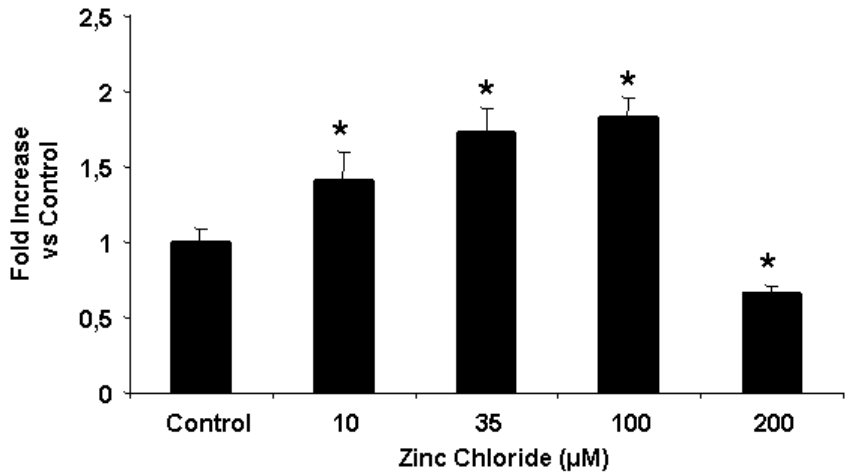

Fig. 14 Caco-2 undifferentiated cells (3 days post-plating) were exposed to increasing concentrations of zinc and cell proliferation were evaluated as decribed in Method section. *p<0.01 vs control

The maximal effective dose corresponded to that observed in ion transport experiments. However higher zinc concentrations were toxic for cells, as previously observed by other authors (Zodl 2003).

Role of MAP kinases in the zinc induced effects

Because zinc effects resemble GH effects in the same conditions and GH involves the activation of MAP Kinases to induce enterocyte proliferation, we investigated whether the pathways of either ion transport and cell growth effects were also similar. Upregulation of ERK1/2 (Fig. 15) but not p38 (data not shown) was induced by zinc in undifferentiated and Caco-2 cells evaluated with the western blot.

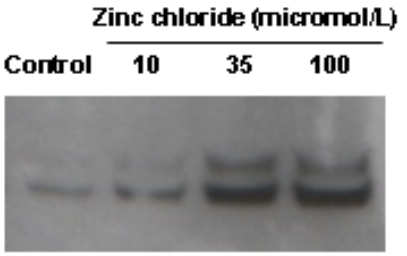

Fig. 15 Caco-2 undifferentiated cells (3 days post-plating) were exposed to increasing concentrations of zinc for 15 min. ERK1/2 activation was evaluated by western blot with the specific p-ERK1/2 antibody. Normalization was performed with the antibody vs the total form of ERK1/2 (data not shown).

Effects of zinc on intestinal cell differentiation

Cell differentiation is an important process at intestinal level. Genetic programming and selected factors play a role in intestinal differentiation in modulating brush border disaccharidases expression. Sucrase and lactase are markers of enterocyte differentiation and increase during cell migration from the crypt to villus tip. Their activities were dosed in preconfluent cells exposed to increasing zinc concentrations. Fig. 16 shows that both sucrase and lactase activities were significantly increased under 10 and 35µM $ZnCl_2$ addition, suggesting that zinc induces enterocyte differentiation.

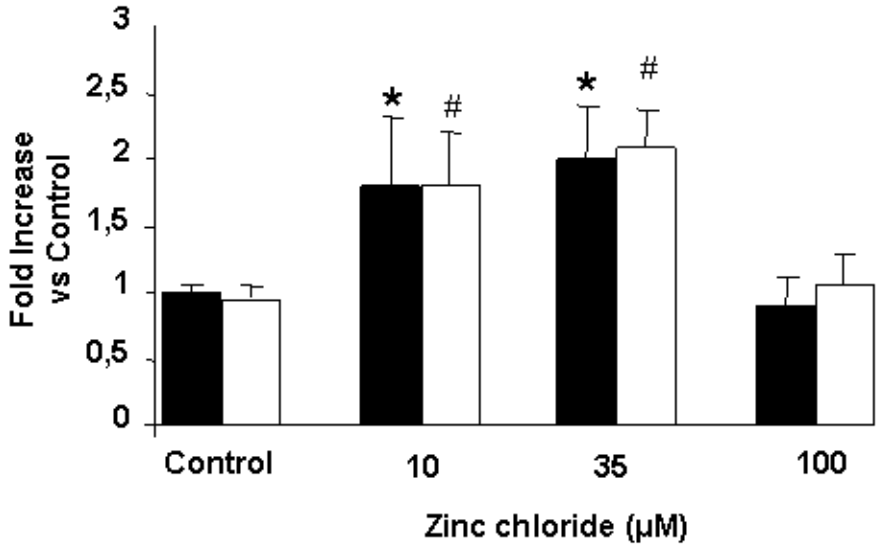

Fig. 16 Caco-2 undifferentiated cells (3 days post-plating) were exposed to increasing concentrations of $ZnCl_2$ (µM) and sucrase (■) and lactase (□) activities were evaluated by Dalqvist method as described in Method section. *p<0.01 vs sucrase control. #p<0.01 vs lactase control.

3.3 Lactoferrin: a human milk protein with effects in cell growth and differentiation

Background

Colostrum and mature human milk contain many growth factors including EGF, IGF-I and HGF, whose concentrations change with time during lactation (Qin 2004, Hirai 2002, Yamada 1998). Intestinal length doubles in the last phase of pregnancy and is maximal at birth (Babyatsky 2003). Intestinal permeability is an indirect measure of intestinal epithelial development and it decreases in the first days of age (van Elburg 2003). Newborn infants fed human milk vs formula had decreased permeability at 28 days of age (Shulman 1998), indicating a more rapid maturation of intestinal epithelium, which may well be the result of growth factors contained in human milk.

Lactoferrin (LF), an iron-binding 80-kDa glycoprotein, is found in amniotic fluid and mammalian milk in either iron-saturated and iron-unsaturated forms (Farnaud 2003). Its concentration in human milk is related to infant age. It peaks in colostrum and rapidly decreases in mature milk (Hirai 1990). Lactoferrin is a major protein component of human milk and exerts a broad spectrum of physiologic activities, such as enhancement of immune function, defence against pathogenic bacteria and viruses, stimulation of healthy microflora (Vorland 1999). The hypothesis was tested that LF functionally modulates intestinal ion transport and nutrient absorption functions.

Results

Effects of lactoferrin on intestinal ion absorption and cell growth

Experiments performed in Ussing chambers did not result in an effect of LF on ion transport in Caco-2 cells monolayer (data not shown). Experiments were performed to test the hypothesis that lactoferrin promotes intestinal cell growth through a direct effect on the enterocyte.

Human LF induce a trophic effect on Caco-2 cells in a dose dependent manner (*Buccigrossi 2007*). ^{3}H-thymidine incorporation was increased in Caco-2 cells exposed to LF after 3 days post-plating, with a maximal effective dose of 100 µg/ml. The effect was progressively lost in older cells (Fig. 17). These effects are consistent with an indirect stimulation of nutrient absorption by lactoferrin.

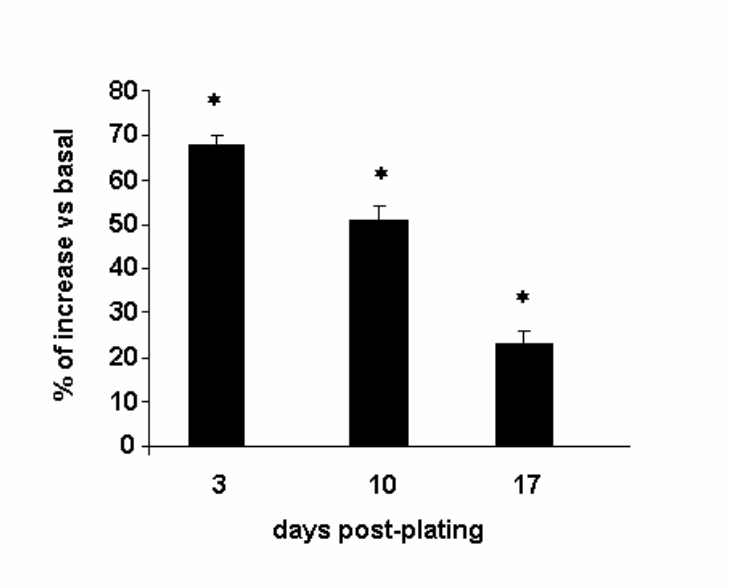

Fig.17 Effects of LF on Caco-2 cell growth at different stage of differentiation. Cell growth was evaluated in Caco-2 cells at 3, 10 and 17 days post-plating using uptake of ^{3}H-Thymidine method after LF (100 µg/ml) stimulation. Data were expressed as percentage vs basal condition (from *Buccigrossi 2007*). *p<0.01 vs basal

Role of MAP kinases

To investigate the molecular mechanisms involved in LF-induced cell growth, the activation of MAPKs was measured. Caco-2 cells at two distinct differentiation stages were stimulated with different concentrations of human native LF and ERK1/2 was visualized by western blot (Fig. 18). In undifferentiated enterocytes, the basal phospho-ERK1/2 activity increased after LF exposure in a concentration range of 1-100 µg/ml. In differentiated cells also, LF increased of phospho-ERK1/2 levels, but the magnitude of stimulation was reduced compared

to that observed in undifferentiated cells. Intracellular levels of activated p38 did not increase upon LF stimulation in either differentiation stage (Fig. 18). This data indicate that p42/44 is implicated in LF trophic effect and that immature enterocytes are its preferred target.

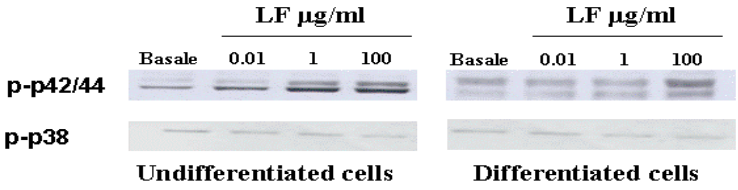

Fig.18 Effects of LF on p-p42/44 and p-p38 levels in Caco-2 cells at different stage of differentiation. Three LF concentrations were used for stimulation of 15 minutes, then activated forms of p42/44 and p38 (p-p42/44 and p-p38 respectively) was evaluated by western blot method. Normalization was performed with total form of proteins and no differences was observed between basals and stimulated cells (data not shown).

Effects of lactoferrin on intestinal cell differentiation

In basal conditions, sucrase and lactase activities progressively increased in growing Caco-2 cells. LF induced a dose-dependent increase of sucrase and lactase activities, with a peak at 100 ng/ml. These effects were strictly depended on the time of LF addition. Exposure of more immature (younger) cells to LF corresponded to the maximal effect. In parallel experiments we added LF to the cells at 3, 6, 10 and 15 days post-plating. The effect was strongest in cells exposed to LF at 3 days for sucrase activity and in those exposed to LF at 6 days for lactase activity (Fig. 19). Thus LF tends to induce a more potent effect in immature cells.

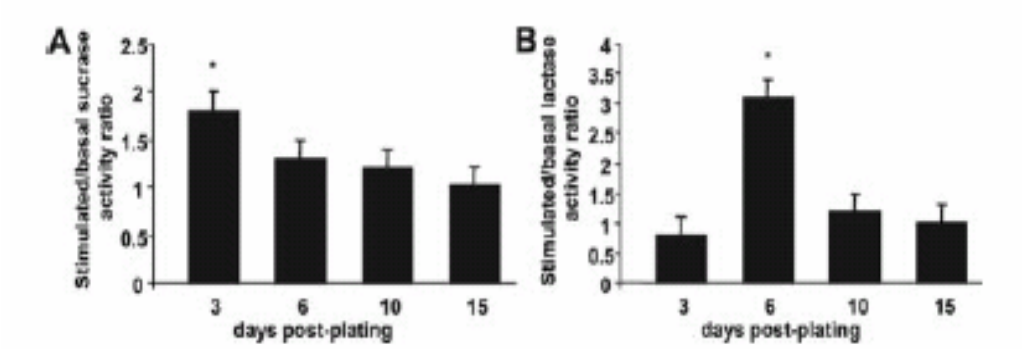

Fig.19 Modulation of disaccharidase activities by LF in Caco-2 cells at different stage of differentiation. Disaccharidase activities were evaluated in Caco-2 cells at 3, 6, 10 and 15 days post-plating. LF (100 ng/ml) induced a significant increase of sucrase (A) and lactase (B) activities 3 and 6 days post-plating respectively (from *Buccigrossi 2007*).*$p<0.05$ vs basal activity

These effects were exerted at transcriptional level as judged by a significant increase of sucrase and lactase mRNA expression observed upon LF stimulation (*Buccigrossi 2007*).

It should be noted however that enzyme activities were expressed per mg of cell protein, therefore the observed increase was unrelated to the stimulation of cell growth.

3.4 Summary

The enterocyte is the target of moieties of different nature, all merging to induce similar effects on ion absorption and enterocyte growth and differentiation that may be modulated at enterocyte level. GH and zinc have an important role on the regulation of basal proabsorptive tone of enterocyte in physyiological condition and reduce the secretive effects induced by enterotoxic factors, directly acting on intracellular mediators. We also found effects on cell growth for either factors, and differentiation for zinc. On the contrary, LF only induces intestinal cell growth and differentiation of immature enterocytes and it does this in a concentration-dependent fashion, providing an explanation for the age-dependent concentration pattern of LF in human milk. At high concentrations LF acts as an optimal intestinal growth factor, while at low concentrations, it induces intestinal differentiation and a strong inhibition of cell growth (Fig. 20).

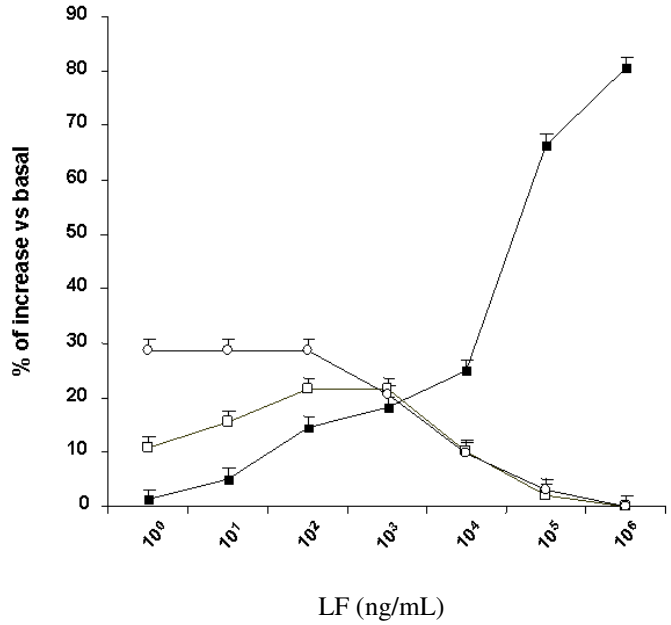

Fig. 20 LF concentrations functionally modulate intestinal processes in a dose dependent fashion. High doses of LF induce a rapid increase of intestinal cell proliferation (■), whereas low doses are effective increasing lactase (○) and sucrase (□) activities.

The signal transduction of the effects by GH, zinc and lactoferrin were the same. The similarities of the effects and pathways need further comments: a close relationship exists

between ion absorption and cell growth. This pattern may be seen as part of the evolution of a positive phenomenous, since it protects from diarrhea (one of the major danger in term of evolution) and allows body growth. Another comment is the broad pattern of moieties capable to induce the same effects. Finally it is somehow surprising that the target of those moities, i.e. their signal transduction pathways, were the same.

Chapter 4 Intestinal negative modulators

4.1 HIV-1 transactivating factor protein (Tat)

Background

The digestive tract is a major target of HIV. Children with severe immune impairment have a high rate of severe gastrointestinal problems, in part due to opportunistic infections (*Guarino 2004*). Chronic diarrhea, dehydration and malabsorption lead to progressive weight loss, which contributes to morbidity and mortality in HIV-1-positive individuals (Sharpstone 1996). However the etiology of diarrhea is unknown in at least one-third of cases, and it has been suggested that HIV-1 itself could cause diarrhea and intestinal damage (Seidman 2000). Partial villus atrophy associated with a maturational defect of enterocytes has been reported in HIV-1 infected patients and is known as HIV enteropathy. A pathogenic role of HIV-1 is supported by the detection of viral proteins and/or nucleic acids in enterochromaffin, in intestinal epithelial and goblet cells (Seidman 2000). However, several HIV-1 effects are not mediated by the lytic propagation of viral particles, rather by secreted viral factors. Thus, HIV-1 may alter the morphological and functional maturation of intestinal epithelial cells without necessarily infecting enterocytes (Kotler 1999). In addition to structural and enzymatic proteins, HIV-1 encodes for a group of at least six auxiliary regulatory proteins, including Tat, a trans-activator peptide essential for HIV-1 replication. Tat exerts its effects by activating L-type Ca^{2+} channels (Gallo 1999, Zocchi 1998) and/or by mobilizing intracellular calcium stores (Haughey 1999). Despite its nuclear localization, Tat is secreted from HIV-1-infected cells and acting as paracrine agent in neighboring uninfected cells. Tat is found in the sera of AIDS patients even in the absence of a massive lysis of infected cells and is involved in many pathological processes that may contribute to immune and non-immune dysfunctions associated with HIV-1 infection (Rubartelli 1998).

Tat protein induced ion secretion in Caco-2 cells and in human colonic mucosa (Berni Canani 2003b). The maximal effective dose was 0.1nM, the same concentration detected in the sera of HIV-1-infected patients (Albini 1996a). Chloride secretion was associated with an increase in intracellular Ca^{2+}, as a result of both extracellular Ca^{2+} entrance and intracellular stores mobilization.

In addition to ion secretion, Tat induced a potent inhibition of intestinal cell proliferation as judged by ^{3}H-thymidine uptake and cell count models (Berni Canani 2003b). Therefore Tat has a double effect: it increases fluid secretion and impairs cell growth. This double pattern adds to the concept that a relationship exists between ion transport processes and cell growth.

Results

Effects of Tat protein on intestinal apoptosis

To further explore this relationship, I investigated the role of caspase-3, an apoptotic marker. The specific hypothesis was that the Tat inhibition of cell growth is due to an increase of apoptosis (Fig. 21).

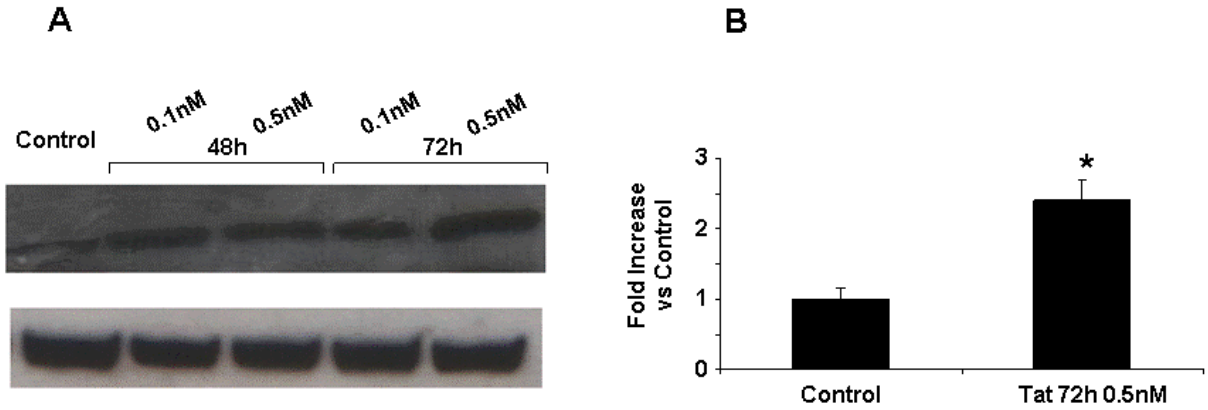

Fig.21 Activation of caspase-3 was evaluated after Tat stimulation by two methods: a specific polyclonal antibody anti cleaved caspase-3 in western blot experiment (A) and a caspase-3 activity commercial kit (B) as decribed in Methods section. *p<0.01 vs Control.

The upregulation of caspase-3 cleaved protein (Fig. 21A) and its increased activity level under Tat stimulation (Fig. 21B) demonstrated the activation of the apoptotic pathway.

Effects of Tat protein on intestinal glucose absorption

Sugar malabsorption is the most frequent and severe feature of AIDS-related intestinal dysfunction, and it contributes to AIDS-associated malnutrition (Miller 1991). In the human intestine, and in Caco-2 cells, glucose absorption is coupled with Na^+ absorption through the Na^+-D-glucose symporter 1 (SGLT-1) located on the enterocyte apical membrane. The transporter GLUT-2, which is located on the basolateral membrane, carries intracellular glucose to the bloodstream (Kellett 2001). The working hypothesis was that Tat inhibits SGLT-1 activity in the intestinal epithelium, thereby inducing glucose malabsorption in AIDS patients.

Tat addition to Caco-2 cells dose-dependently inhibited glucose uptake (Fig. 22). This effect was prevented by anti-Tat polyclonal antibodies and by L-type Ca^{2+} channels agonist Bay K8644. Western blot analysis of cellular lysates and brush-border membrane preparations showed that Tat induced SGLT-1 missorting. Tat also caused a dramatic decrease in α-tubulin staining, which indicates dysruption of the cytoskeleton organization (*Berni Canani 2006b*), which certainly contributes to nutrient malabsorption.

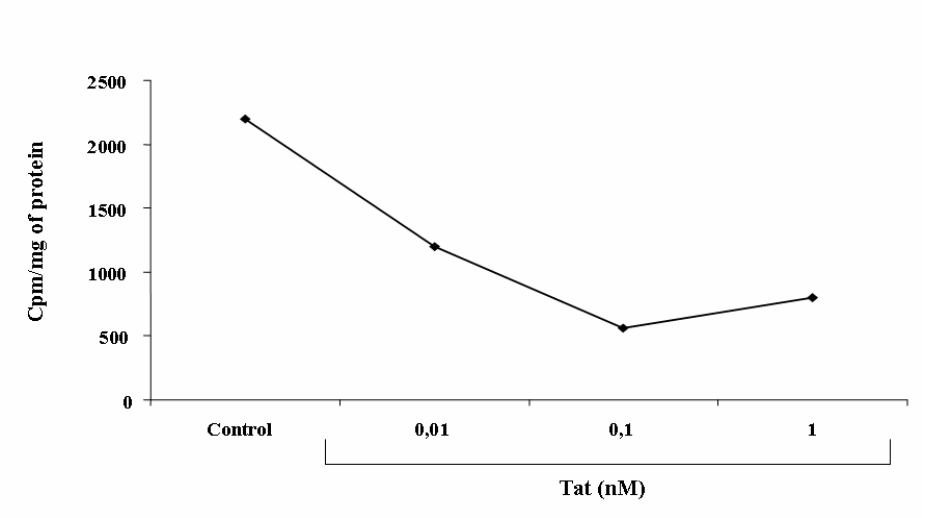

Fig.22 Glucose uptake was significantly inhibited by incubation for 1 hour with Tat. The effect was dose-dependent and saturable with a maximal effective concentration of 0.1 nM. The magnitude of the maximal inhibitory effect induced by Tat was comparable to that observed with the maximal effective dose of phlorizin (70% *vs.* control cells) (from *Berni Canani 2006b*).

The intestinal pathogenic effects by Tat: an example of moleculat mimicry

High affinity binding by Tat to vascular endothelial growth factor receptors (VEGRs) was previously described. VEGFR-2 (Flk-1) was activated by Tat in vascular endothelial cells (Albini 1996a) and in a Kaposi's sarcoma cell line (Ganju 1998) whereas VEGFR-1 (Flt-1) was activated by Tat in monocytes (Mitola 1997). HIV-Tat protein shows a arginine-lysine rich domain that resembles a poly-basic sequence of angiogenic factors such as VEGF (Albini 1996b). Basic domains of Tat mimic VEGF-like activities such as monocyte chemotaxis (Benelli 1998) and angiogenesis (Scheidegger 2001). Based on this data, the hypothesis was raised that Tat is a functional mimetic-peptide of VEGF. VEGF is a heparin binding glycoprotein that functions as an endothelial-cell-specific mitogen, a potent angiogenic, permeability and fibrosis factor. VEGF may be synthesized by a wide variety of cells, including keratinocytes, fibroblasts, macrophages, smooth muscle cells, epithelial cells, tumor cells, eosinophils, and neutrophils. Inducers of VEGF synthesis include

proinflammatory cytokines (IL-6, TNF-α), growth factors (PDGF, EGF), and tissue hypoxia. VEGF receptors are also present on intestinal epithelial cells.

I performed comparative experiments with VEGF and HIV-Tat to investigate the similarities and differences of their effects. The addition of VEGF to the serosal side of the cell monolayer induced chloride secretion with the same time- and dose-dependent pattern observed for Tat protein (Fig.23A). Similar to Tat, mucosal addition of VEGF did not change electrical parameters. The similarities were also observed in the inhibition of cell proliferation as shown in Fig. 23B.

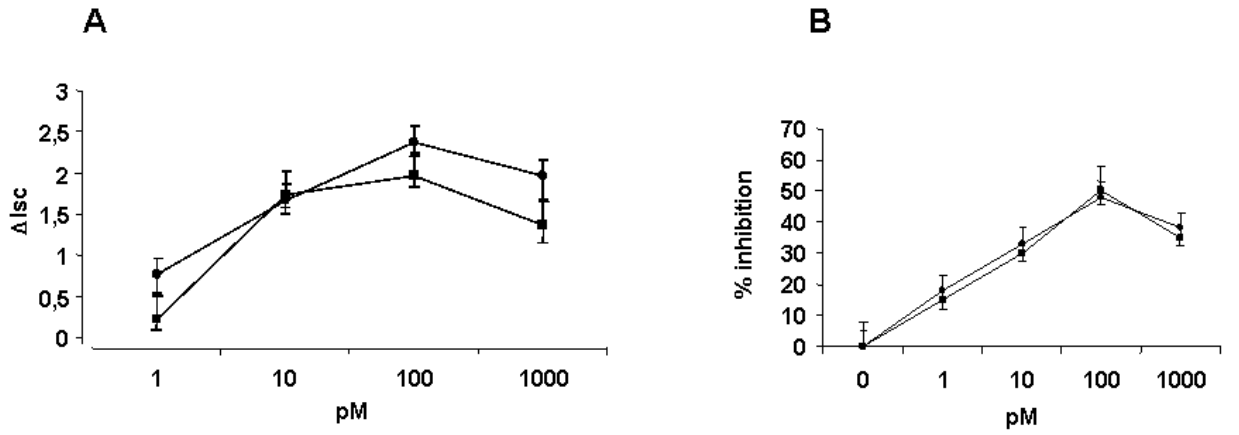

Fig. 23 Effects of increasing concentrations of VEGF and Tat protein on short circuit current (ΔIsc) (A) and on ^{3}H-thymidine incorporation (B) in Caco-2 cells.

Both effects on ion transport and on cell proliferation elicited by VEGF were neutralized by Tat antibodies providing further compelling proof that VEGF is the endogenous analogue of the Tat protein (Fig. 24).

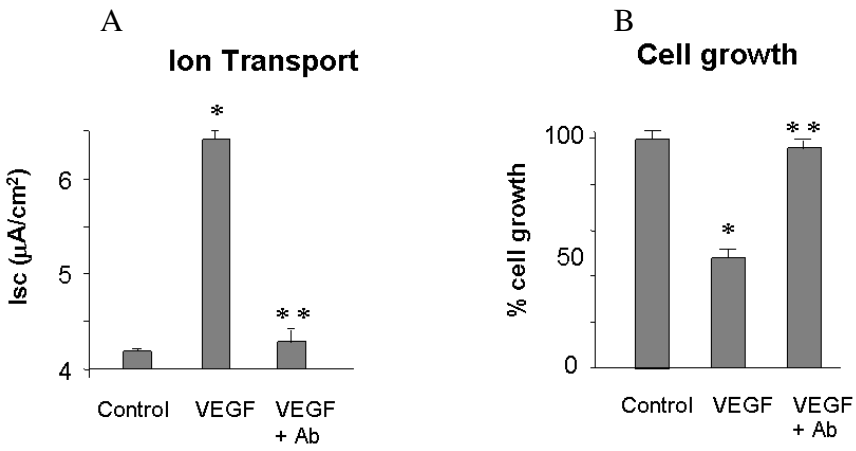

Fig. 24 Effects of VEGF (100 pM) on short circuit current (A) and ^{3}H-thymidine incorporation (B) after preincubation with specific antibodies against Tat protein * = p <.01 *vs* control; ** = p <.01 *vs* VEGF alone.

4.2 E. coli heat-stable enterotoxin (ST)

Background

Enterotoxigenic *E. coli* strains elaborate two classes of enterotoxins, namely the heat-labile and the heat-stable enterotoxins (LT and ST, respectively). LT is homologous to cholera toxin and induces chloride secretion by increasing the intracellular cAMP levels. ST causes an increase of intracellular cGMP to induce chloride secretion and diarrhea (Guarino 1989). ST is a small peptide pathogenic for humans (Giannella 1995). The jejunum is a major target of ST-elicited anion secretion that is mediated by CFTR (Vaandrager 1997a). ST binds to its receptor guanylate cyclase C (GCC) on the apical surface of enterocytes, resulting in the generation of cGMP. This in turn activates a cGMP-dependent kinase (cGKII) leading to the phosphorylation of the CFTR on the apical membrane (Vaandrager 2000), and in the consequent inhibition of Na^+ absorption on the apical membrane of jejunal enterocytes. This result shifts the ion fluxes toward net fluid secretion (Lucas 2000, Vaandragen 2002).

Previously it was demonstrated that the addition of ST to the basolateral side of intestinal epithelial cell monolayer induced a similar but less intense secretory effect compared with ST mucosal stimulation and the same pattern was observed in increasing cGMP levels (*Albano 2005*). However intestinal cGMP pathway could be involved in enterocyte proliferation as well. It was hypothesized that the activators of GCC induce an antiproliferative effect on intestinal cell cancer growth (Pitari 2003, Shailubhai 2000, Wang 2000). On the contrary, the absence of normal GCC expression with a reduction of cGMP levels resulted in a decrease of intestinal cell growth of which MAPK ERK1/2 is the main signaling pathway (Aliaga 1999). The interaction between ERK1/2 and cGMP pathway is therefore unclear (Rao 2004, Saha 2007).

The hypothesis has been raised that GCC activation controls intestinal cell proliferation and ion secretion in a compartmentalized manner (Jin 1999). In other words, ST may act on either the mucosal and serosal side of the polarized enterocyte inducing two distinct effects through two distinct patways, namely cGMP and ERK1/2.

Alternatively, the mucosal or the serosal addition could induce a more potent effect on either fluid secretion or cell growth. The mechanism of such quantitative difference could involve the signal transduction, i.e. ST could preferentially acts on a specific serosal messenger (either cGMP or ERK1/2) depending on the enterocyte compartment i.e. the apical or basolateral side of ST addition. Compartimentalization is supported by the fact that the enterocyte is a strongly polarized cell, with two distinct membranes (apical vs basolateral) that are structurally and functionally different. In addition a strong evidence of polarization is transepithelial PD, which is generated by changed ion fluxes.

Results

Effects of ST on intestinal cell growth and ion transport

The addition of ST to Caco-2 monolayer results in an increased BrdUrd incorporation (indicating a proliferative effect) with a more potent effect upon ST addition to the serosal side, than that observed with ST addition to the mucosal side. However, ST simultaneous addition to both sides did not correspond to the sum of the single compartments (Fig. 25).

ST mucosal addition induced a more potent chloride secretion than ST serosal stimulation. Also for this effect, the simultaneous addition to both compartments resulted in an effect that was not significantly increased compared with that observed with the mucosal addition (Fig. 25). Since all electrical modifications were inhibited in chloride-free buffer (data not shown) the observed effect is consistent with anion secretion.

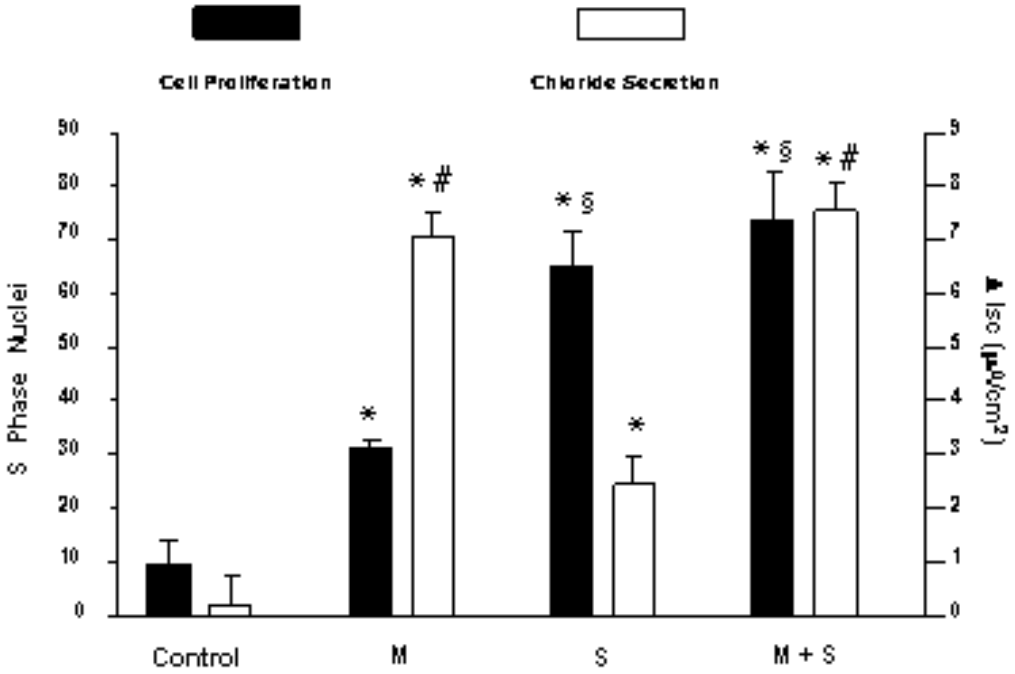

Fig. 25 ST serosal addition (S) to Caco-2 monolayers induced an increase in BrdUrd incorporation, indicating a proliferative effect. Mucosal addition (M) induced a less evident proliferation. ∗p<0.05 vs Control. On the contrary, ST mucosal addition induced a more potent chloride secretion vs serosal stimulation. # p<0.05 vs Control.

Role of MAP kinases p42/44 and cGMP

As shown in a representative western blot activation of ERK1/2 was more potent when ST was added to the serosal than to the muosal side of intestinal epithelium. The densitometric acquisition of western blots showed 3-and 4-fold increases for mucosal or serosal ST addition respectively. Conversely, an increased cGMP production was observed upon ST mucosal rather than serosal addition to Caco-2 monolayers. These data parallel the effects induced by ST addition on either side of Caco-2 cell monolayer (Fig. 26).

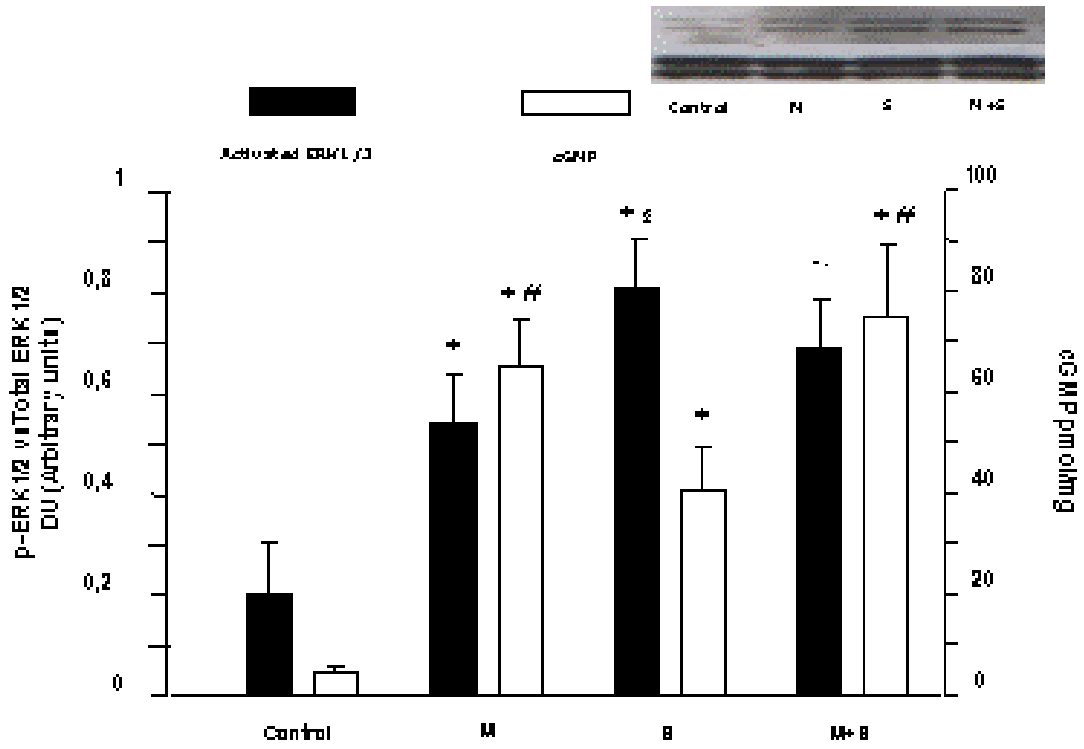

Fig. 26 ERK1/2 was more strongly activated upon serosal (S) vs mucosal (M) ST stimulation. # $p<0.05$ vs Control. A representative western blot was shown in inset. Maximal ST-stimulated cGMP production was observed upon mucosal addition (M) to Caco-2 monolayers. *$p<0.05$ vs Control.

Because ERK1/2 is essential to maintain the basal proabsorptive tone in the enterocyte, the hypotesis was tested that ERK1/2 activation in response to serosal ST is involved in downregulating chloride secretion induced by ST. Cells were pre-incubated with the specific inhibitor of ERK1/2, PD098059, for 30 minutes before the serosal addition of ST. In standard condition ST serosal addition resulted in a weak chloride secretion that strongly increased with a preincubation with PD98059 (Fig. 27). These experiments indicate that when ERK1/2 is inhibited, the electrical response (i.e. ion secretion) induced by the serosal addition of ST is strongly increased.

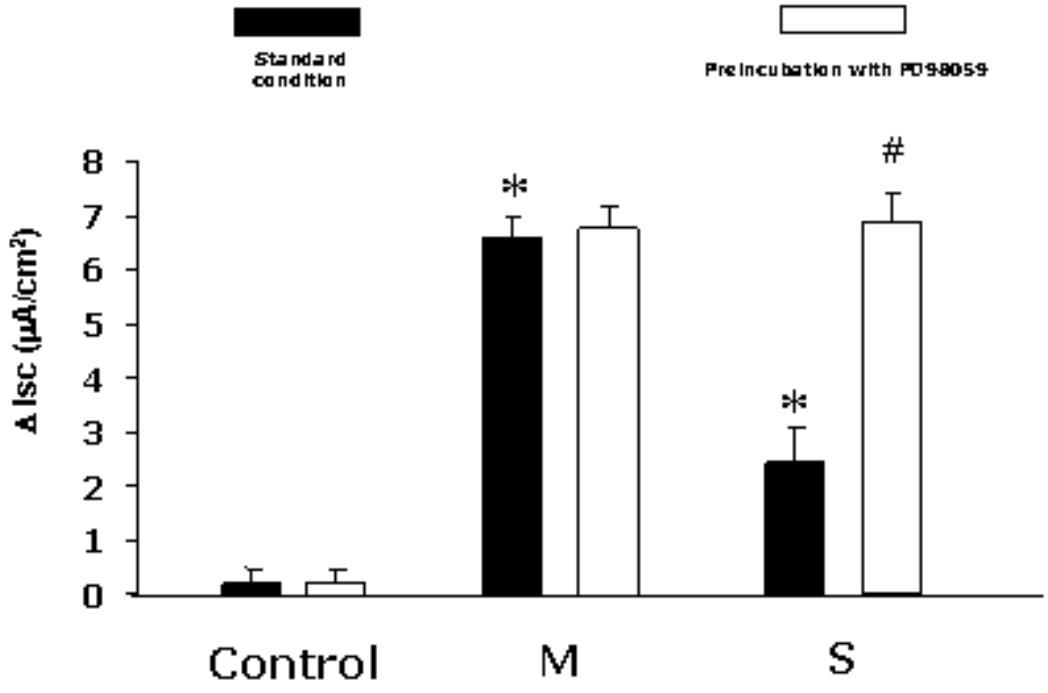

Fig. 27 PD98059 strongly increased chloride secretion in response to the serosal (S), but not mucosal ST addition (M). ∗ $p<0.05$ vs Control in Standard condition. #$p<0.01$ vs ST serosal addition in Standard condition.

These results give to ERK1/2 a double role in the enterocyte physiology. ERK1/2 is essential for cell growth and it also acts as a braking force of stimulated ion secretion. This is the first demonstration that ERK1/2 controls ion transport through a compartimentalized mechanism and is consistent with the observation that ERK1/2 is mainly located in basolateral membrane of intestinal epithelial cells (Boucher 2003). Again, this data provide an additional proof of a link between ion transport and cell growth.

The intestinal pathogenic effects by ST: another example of moleculat mimicry

Bacterial and viral enterotoxins are usually known to induce functional damage to the host. Hence the association of enterotoxic and proliferative effects is a peculiar feature of ST.

This feature may depend on cGMP pathway activation and GCC binding. The physiological ST receptor in the intestine (i.e. GCC) was unknown until Currie et al (Currie 1992) extracted and purified from the rat small intestine a peptide that is homologous to ST. This endogenous peptide, named guanylin, shares 50% homology with ST and competes with the ST for intestinal binding sites stimulating chloride secretion (Carpick 1993). The endogenous ligands of GCC receptor, guanylin and uroguanylin are produced predominantly in the intestine although uroguanylin is also expressed in the kidney (Whitaker 1997, Cohen 1995). Proguanylin and prouroguanylin are secreted into the intestinal lumen but they are also detected in the blood stream as 11 – kilodalton prohormones and are each cleaved to the active 15 amino acid carboxy termini that bind GCC (Hamra 1996). Circulating uroguanylin induces natriuresis, kaliuresis, and diuresis in isolated perfused rat kidney. It has been suggested that uroguanylin represents a gut-to-kidney signaling hormone that upon ingestion of high-salt meals causes natriuresis in anticipation of increased intestinal salt absorption (Fonteles 1998). However, guanylin and uroguanylin may have other physiological roles (Zhang 1998, Cohen 1998) including the activation of a cGMP signal transduction pathway that may take part in the regulation of the turnover of epithelial cells by continuous replenishment of the epithelial cells (Shailubhai 2000, Wang 2000).

Albano et al. (*Albano 2005*) demonstrated that ST is more potent than guanylin in stimulating intestinal secretion and cGMP production and these effects are more intense on the luminal rather than basolateral membranes of intestinal epithelial cells (*Albano 2005*). These new data demonstrate that guanylin induce the same effects and involved the same intracellular mechanisms of ST but with a decreased intensity compared to ST (Tab. 3), providing an example of molecular mimicry.

Tab.3 ST and Guanylin effects

Intestinal effect				
	Chloride Secretion		Cell Proliferation	
ST	M	9.2 ± 2.1∗	M	3.2 ± 1.6
	S	3.1 ± 1.2∗	S	7.3 ± 2.1
Guanylin	M	5.2 ± 2.1 ∗	M	2.9 ± 1.8
	S	1.5 ± 1.7∗	S	6.5 ± 1.7

Intracellular mechanisms				
	cGMP levels		ERK1/2 activated	
ST	M	14.1 ± 1.8 ∗	M	2.9 ± 1.3
	S	8.2 ± 1.3∗	S	4.1 ± 1.2
Guanylin	M	8.2 ± 1.2 ∗	M	3.0 ± 1.8
	S	5.3 ± 1.4∗	S	3.9 ± 1.4

Abbreviations: M, mucosal side and S, serosal side of Caco-2 monolayer.
All data are expressed as fold increase vs basal condition.
∗Data from *Albano 2005*

4.3 Summary

In the first section of this PhD thesis I have decribed a link between ion transport and cell growth based on the combination of absorptive and proliferative effects exerted by the same moieties. In this second section this concept is further supported by the data obtained with Tat, essentially consisting in the association of its enterotoxic and cytotoxic effects. Tat protein functionallly and structurally damages epithelial intestinal cells, inducing a secretive diarrhea, apoptosis and sugar malabroption.

An exception to the view that couples ion absorption with growth and, conversely, ion secretion with cell growth inhibition is obtained with E.coli ST.

Indeed ST toxin induces secretive diarrhea but increases intestinal cell proliferation.

The explanation is given by the presence of the target receptors and molecular mimicry: the sequence omology allows these toxins to mimic the effects of physiological factors. Therefore VEGF and ST are examples of molecular mimicry where bacteria have developed a strategy that exploits mechanisms active in intestinal ion functions.

Chapter 5 Discussion

In order to maintain electrolyte homeostasis, a complex interaction between secretory and absorptive processes is necessary. Secretion and absorption are two distinct processes that occur simultaneously in crypt and villus cells (Kockerling 1993) and are controlled by a complex array of endocrine, paracrine, autocrine and neuronal stimuli.

These functions also depend on the constant turnover of the intestinal epithelium, one of the most rapidly replicating tissues in human body (Yen 2006). Small and large intestinal cell turnover depend on gut mucosal stem cell proliferation, their migration along the crypt-villus axis and cell apoptosis (Booth 2000).

There is a close relationship between the degree of enterocyte maturation and absorptive functions. Mature villus cells are actively absorbing whereas more immature crypt cells are in a basal secretive state.

In this PhD thesis I investigated the relationship between transepithelial ion transport and growth and differentiation of epithelial intestinal cells, choosing the effects of different moieties on either process and also exploing their mechanisms.

I found several lines of evidence of a close relationship between cell growth and ion transport. Firstly GH has a dual role on intestinal epithelial cells. GH induces a trophic effect, whereas the ion transport is tuned up in a proabsorbitive condition. The effects were obtained at the same concentration and share, at least in part, the same intracellular mechanisms. ERK1/2 was involved in either effect as demonstrated by pre-incubation experiments with a specific inhibitor.

GH proabsorbitive effect was an early event just as activation of ERK1/2 and it's well known that this activation drives the cell cycle toward the mitosis phase. Therefore ERK1/2 could be the molecular link of the relationship between ion transport and cell growth. To support this hypothesis I tested a second proabsorbtive factor, zinc, and again I observed an effect on ion

absorption coupled with cell growth and both were related to the activation of ERK1/2. Another possible key molecule implicated in this interaction is nitric oxide. Several studies demonstrated that high NO levels induce apoptosis in cancer cell lines (Gao 2005, Kashfi 2005), but the specific role by NO in cell growth is unknown. Both the proabsorbitive factors tested were effective in increasing NO levels by activating cNOS. This event was strictly correlated with ion transport as indicated by the results obtained with NO inhibitor L-NAME. Further experiments are needed to esthablish the role of NO in intestinal cell growth. In addition, althought lactoferrin induced intestinal cell growth and promotes ERK1/2 activation, no effect on Isc was observed. This is probably due to the activation of different proliferative pathways. Alternatively lactoferrin could modulate the ion transport with a more long term effect. However these data support the hypothesis that an ion proabsorbitive event triggers intestinal cell proliferation. To partial support this hypothesis there are evidences that ion secretion results in inhibition of intestinal cell growth. HIV-Tat protein induced chloride secretion and inhibition of cell growth through an increased apoptosis, providing an example of a toxin that induces either functional and structural damages in the intestinal mucosa. An exception to the observed pattern combining ion absorption with cell growth one side and ion secretion with cell damage on the other, was observed with E. Coli ST. ST induced a rapid and potent chloride secretion but this was associated with a proliferative effect. The opposite effect had a peculiar feature in their being polarized, i.e. closely linked to basolateral and luminal side of ST addition to enterocyte. This feature finds an explanation in the ST molecular mimicry with a physiological endogenous secretagogue, guanylin. Differential activation of guanylin receptors by serosal and mucosal side of enterocyte induces different physiological effects.

However data showed in this thesis demonstrated a relationship between transepitelial ion transport and growth in the intestinal epithelium linked by specific pathways. Therefore

generally proabsorbitive factors promotes cell growth but not all growth factors are able to induce absorption.

These concepts, in a perinatal interpretation, have important implications on knowledge of intestinal physiology in infant age and particularly for the early stages of intestinal development.

Survival depends on the ability of neonates to clear intestinal content (meconium), to absorb nutrients immediately after birth, to reduce intestinal permeability and to balance ion transport. All these processes undergo dramatic changes at birth, when there is the need of immediate adaptation. Structural and functional events occurs at intestinal level and are influenced by exogenous and endogenous factors and their timing is essential.

Almost all term infants expell meconium within 48 h of life with no differences between breast or formula-fed infants (Metaj 2003). The mechanisms involved in this process are known only in part. CFTR has an important role in intestinal water secretion and is involved in meconium output (Eggermont 1996) and factors regulating CFTR have an important role in the physiological meconium production and output. My results suggest that two distinct molecules play a role in intestinal ion secretion: guanylin and VEGF. They are involved in chloride secretion through the activation of CFTR and CaCC, respectively. Guanylin serum concentrations are unknown in newborns but the intestine has the highest concentration of guanylin receptors GCC in the first 3 days of life (Guarino 1987). It is logical to speculate that guanylin effects peak at this age when an increased fluid secretion helps expelling meconium. To support this, there is a high incidence of meconium ileus in cystic fibrosis, a disease characterized by a structural and functional abnormality of CFTR (Kerem 1995).

VEGF induces Ca^{2+}-dependent chloride secretion by activating CaCC. VEGF receptors are present on intestinal epithelial cells (Vuorela 2000) but the mechanism to upregulate intestinal

effects depends on ligand concentrations: VEGF is present at high levels in colostrum (Siafakas 1999) and in infant serum (Malamitsi-Puchner 2000).

Interestingly either guanylin and VEGF share the ability of modifying cell proliferation. Guanylin promotes intestinal proliferation mainly acting on the serosal side of crypt cells whereas VEGF at high concentration reduces cell proliferation probably increasing apoptosis of mature enterocytes. However the combination of increased cell proliferation and apoptosis promots a rapid enterocyte turnover from the the crypt to the villus tip. In addition a more rapid enterocyte migration from the crypt to the villus tip by the maturing enterocyte is linely associated with a less pronounced “ion absorptive state”. Rather the relative abundant crypt cells in their typical secretory state may help expelling meconium. These apparently opposite effects occur simultaneously. Indeed intestinal cell turnover is maximal in this phase. Intestinal epithelium protects the host from invading pathogens, creating a barrier between microbes within the intestinal lumen the blood stream: factors contributing to disrupt this barrier may expose the host to bacterial translocation and to development of systemic inflammatory diseases. In newborns the immaturity of the mechanical barrier and the incomplete development of immune system may contribute to the pathogenesis of necrotizing enterocolitis (NEC) (Anand 2007).

Intestinal ion secretion and the rapid enterocyte turnover occur in the first days of life and depend on cGMP and Ca^{2+} pathways as discussed previously. After the intestinal washing, which is needed to expel meconium, secretion must be reduced rapidly. Experimental data demonstrated that MAPKs reduce chloride secretion: ERK1/2 and p38 reduced Ca^{2+}-dependent chloride secretion but ERK1/2 was selectively effective in reducing cGMP-dependent secretion. In addition a distinct second messenger, nitric oxide, acts as a brake to CFTR-dependent secretion.

Afterwards, the newborn intestine undergoes the adaptative processes induced by endogenous and environmental factors. Timely modifications of transepithelial ion transport continue toward the proabsorbitive homeostatic final condition.

Human milk is a rich source of substances able to modulate intestinal functions. In particular I found that two factors present in human milk, namely GH and zinc, are able to activate MAPKs and NO pathways involved in the proabsorbitive tone.

Another important function in infant intestine is related to the proliferation of intestinal cells in order to reduce intestinal permeability and develop a barrier against bacteria and antigen traslocation. This event is supported by the combined action of several factors, such as GH, zinc and lactoferrin present in human milk. Lactoferrin, in particular, is able to differentially modulate intestinal functions in a concentration dependent manner. Lactoferrin at high doses such as in colostrum promotes intestinal growth but at lower doses such as in mature mother milk, promotes intestinal differentiation acting directly at transcriptional level. This is an example of functional modulation and adaptation induced by a single factor.

Differently from lactoferrin, zinc exerts a dual effect at the same concentration, inducing both proliferation and differentiation. This process may shorten the time of passage of enterocytes along crypt-villus axis allowing a rapid turnover highly needed at this age.

Finally, the coordination of these events results in the gradual maturation of newborn intestine, reaching a final condition in which the main intestinal functions are defined: the proabsorbitive status, the barrier function and the ability to digest and absorb nutrients.

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Chapter 6 Publications

Original papers produced by the student during PhD program and cited in the text:

- ☆ **Buccigrossi V**, De Marco G, Bruzzese E, Ombrato L, Bracale I, Polito G, Guarino A. Lactoferrin induces concentration-dependent functional modulation of intestinal proliferation and differentiation. Pediatr Res 2007; 61(4): 410-14

- ☆ Berni Canani R, Ruotolo S, **Buccigrossi V**, Passariello A, Porcaro F, Siani MC, Guarino A. Zinc fights diarrhoea in HIV-1-infected children: in-vitro evidence to link clinical data and pathophysiological mechanism. AIDS 2007; 21(1):108-10

- ☆ Berni Canani R, Cirillo P, Mallardo G, **Buccigrossi V**, Passariello A, Ruotolo S, De Marco G, Porcaro F, Guarino A. Growth hormone regulates intestinal ion transport through a modulation of the constitutive nitric oxide synthase-nitric oxide-cAMP pathway. World J Gastroenterol 2006; 12(29):4710-5

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- ☆ Albano F, De Marco G, Berni Canani R, Cirillo P, **Buccigrossi V**, Giannella RA, Guarino A. Guanylin and E. coli heat-stable enterotoxin induce chloride secretion through direct interaction with basolateral compartment of rat and human colonic cells. Pediatr Res 2005; 58(1):159-63

- ☆ Berni Canani R, Cirillo P, **Buccigrossi V**, Ruotolo S, Passariello A, De Luca P, Porcaro F, De Marco G, Guarino A. Zinc inhibits cholera toxin-induced, but not Escherichia coli heat-stable enterotoxin-induced, ion secretion in human enterocytes. J Infect Dis 2005; 191(7):1072-7

- ☆ Guarino A, Bruzzese E, De Marco G, **Buccigrossi V**. Management of gastrointestinal disorders in children with HIV infection. Paediatr Drugs 2004; 6(6):347-62 Review.

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Lactoferrin Induces Concentration-Dependent Functional Modulation of Intestinal Proliferation and Differentiation

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ABSTRACT: Human milk stimulates intestinal development through the effects of various moieties. Lactoferrin (LF) is a glycoprotein of human milk whose concentration is highest in colostrum decreasing in mature milk. LF promotes enterocyte growth in intestinal cell lines. We tested the hypothesis that LF induces a distinct effect on enterocyte proliferation and differentiation, depending on its concentration. We examined the dose-related effects by human-native LF (N-LF) in Caco-2 (human colon adenocarcinoma) cells. At high concentrations, N-LF stimulated cell proliferation in immature Caco-2 cells, as judged by ^{3}H-thymidine incorporation. In contrast, sucrase and lactase activities were increased at low but not high LF concentrations and their mRNA were also increased, indicating a transcriptional effect. Because iron binds specific LF sites, we compared the potency of N-LF and iron-saturated LF (I-LF) and found the native form more potent. Finally, we tested the effects by bovine LF (bLF) in the same system and found the latter more potent than the human isoform in inducing cell growth and lactase expression. These results suggest that LF directly induces enterocyte growth and proliferation, depending on its concentration, thereby regulating the earlyx postnatal intestinal development. bLF could be added to infant formula as a growth factor in selected intestinal diseases. (*Pediatr Res* 61: 410–414, 2007)

Intestinal epithelial development changes immediately after birth, with an age-dependent enterocyte proliferation and differentiation pattern (1). Intestinal cell growth peaks at birth (2,3) upon the stimulation exerted by growth factors in amniotic fluid and in human milk (4,5), but also as a consequence of dietary changes (6). Intestinal epithelial growth and development is faster in breast-fed than in formula-fed infants. Brush border enzymes such as lactase and sucrase also show an increased activity and correlate with epithelial differentiation (7–10). Human colostrum possesses a potent growth-promoting activity, which decreases within a few days, and milk formula has no such effect (3). Colostrum and human mature milk contain many growth factors, including EGF, IGF-I, and HGF, the concentrations of which change during lactation (4,5,11). LF, an iron-binding 80-kDa glycoprotein (12), is found in amniotic fluid and mammalian milk in iron-saturated and iron-unsaturated forms (13,14). Its concentration in human milk is related to infant age: it peaks in colostrum and rapidly decreases in mature milk (15). It has been reported that LF resists proteolysis through the infant's digestive tract (16) and binds to a specific receptor located on the enterocyte brush-border (17). Human LF induced intestinal proliferation and differentiation in the Caco-2 intestinal cell line (18). Although digestive enzyme activities increase along the crypt villus axis (19), proliferation and differentiation are not simultaneous processes and need to be finely tuned, particularly in newborn infants, in whom the enterocytes have not yet acquired the features of mature, ion-absorptive cells. The aim of this study was to test the hypothesis that LF induces concentration-dependent functional modulation of intestinal epithelial proliferation and differentiation, thereby contributing to the fine tuning of early development of intestinal epithelium.

In human milk, native LF is a combination of 10% iron-saturated LF and 90% iron-unsaturated isoforms (15). This ratio is stable. Because evidence has been produced that different LF isoforms exert different biologic effects (18,20), we tested the effects of N-LF (10% iron-saturated isoform or native LF) in comparison with I-LF (100% iron-saturated isoform). We investigated the effects of a wide range of LF concentrations on proliferation and on differentiation markers in rapidly growing intestinal Caco-2 cells. Caco-2 is an established human-derived intestinal cell line that differentiates into mature human enterocytes generating monolayers of polarized cells (21–23). To monitor cell differentiation, we determined sucrase and lactase activities. The latter increases with age in the first weeks of life and it was increased in milk-fed more than in formula-fed infants (6,7).

Finally, because bLF shows a strong sequence amino acid homology with human LF (NCBI protein-protein BLAST) (24), we compared the biologic effects of bovine and human LF. This was done to see whether bLF was a potential functional nutrient to be added to infant formula to achieve clinical effects.

MATERIALS AND METHODS

Cell line. Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) with a high glucose concentration (4.5 g/L) supplemented with 10% fetal bovine serum (FBS), 1%

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Abbreviations: bLF, bovine native lactoferrin; Caco-2, human colon adenocarcinoma; I-LF, human iron-saturated lactoferrin; LF, lactoferrin; N-LF, human native lactoferrin

nonessential amino acids, penicillin (50 mU/mL), and streptomycin (50 mg/mL) and kept in 5% CO_2–95% air. Cells were used between the 20th and 40th passage and the medium was changed daily.

Cell growth. Caco-2 cells were seeded onto 96-well microtiter plates (10^4 cells/well) and cultured for 3 d in DMEM with 10% FBS. After 24 h of serum starvation, cells were exposed to increasing doses of LF for 48 h in DMEM FBS-free; then ^{3}H-thymidine (0.5 μCi/well, ICN Biomedicals, Irvine, CA) was added 18 h before harvesting the cells with a semiautomatic cell harvester (Skatron Instruments, Lier, Norway). The filters were dried and beta radioactivity was counted with a Packard scintillation spectrometer (Packard Instrument Co., Meriden, CT). The same experiment was repeated after 10 and 17 d from plating. For cell counts, cells were seeded onto 24-well plates (7×10^4 cells/well), cultured, and stimulated under the same experimental conditions and counted in a Neubauer chamber.

Lactase and sucrase activity assays. Cells were collected after 24 h of LF stimulation and lactase and sucrase enzymatic activities were measured by modified Dahlqvist method (25). Briefly, cells were rinsed in cold PBS and scraped into cold maleate buffer 0.1 M pH 6.0. Samples were sonicated three times for 15 s each, using a Labsonic 2000 (Sartorius AG, Goettingen, Germany) and total cell lysates were incubated at 37°C with 50 mM lactose for 60 min or sucrose for 30 min. The glucose generated by enzymatic activity was measured using a glucose oxidase assay.

Comparative effects of human and bovine LF. Experiments were run in parallel to test the effects of bovine LF on Caco-2 cell growth and differentiation. Bovine LF was used in concentrations equimolar to human LF.

RNA extraction and reverse transcription. Preconfluent Caco-2 cells were collected after 24 h of bLF stimulation and total RNA has been extracted from Caco-2 cells by TRIzol reagent protocol (Invitrogen). The amount of extracted RNA was quantified by measuring the absorbance at 260 nm. Reverse transcription of RNA was performed using a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA).

Quantitative real-time RT-PCR. Real-time RT-PCR was performed according to the recommendations supplied by Applied Biosystems (available at: http://europe.appliedbiosystems.com/). Primers for sucrase (Hs00356112_m1) and lactase (Hs00158722_m1) were purchased from Applied Biosystems. A 25-μL PCR reaction volume was prepared using about 40 ng of cDNA as template. Reactions were run in 96-well optical reaction plates using an Applied Biosystems 7300 Real-Time PCR System. Thermal cycles were set at 95°C (10 min) and then 35 cycles at 95°C (15 s) and 60°C (1 min) with auto ramp time. For data analysis, the threshold line was set automatically and it was in the linear range of the amplification curves for all mRNA in all experimental runs. All reactions were performed in triplicate. The abundance of target mRNA was calculated relative to a reference mRNA (GAPDH). Relative expression ratios were calculated as $R = 2^{(Ct(\beta 2\ microglobulin) - Ct(test))}$, where Ct is the cycle number at the threshold and the test stands for the tested mRNA. The confidence interval was fixed at 95%.

Reagents. N-LF with 10% iron-saturation, iron-saturated isoform from human milk, and all reagents were purchased from Sigma Chemical Co. (St. Louis, MO). N-LF from bovine milk was kindly provided by Prof. P. Valenti (Department of Experimental Medicine, II University of Naples, Naples, Italy).

Statistical analysis. Each experiment was run in triplicate and was repeated at least three times. Results are expressed as mean ± SD. Significance was evaluated by *t* test. Results were considered significant at $p < 0.05$.

RESULTS

Effects of LF on cell growth. ^{3}H-thymidine incorporation was increased in Caco-2 cells exposed to N-LF (Fig. 1*A*) after 3 d postplating. N-LF stimulated cell growth at a concentration as low as 1 μg/mL, as judged by ^{3}H-thymidine uptake. The effect increased in a dose-dependent fashion peaking at 100 μg/mL. Higher concentrations did not induce further proliferation, indicating a saturation pattern of the effect. The experiments were repeated using cell count to monitor cell proliferation. A near-perfect correlation was observed between the two cell proliferation markers (Fig. 1*B*). We repeated the same experiment at d 10 and 17 postplating using LF concentration of 1 and 100 μg/mL (Fig. 2, *A* and *B*, respectively). The proliferative effect induced by N-LF was maximal at 100 μg/mL in preconfluent condition, but it was progressively lost in older cells.

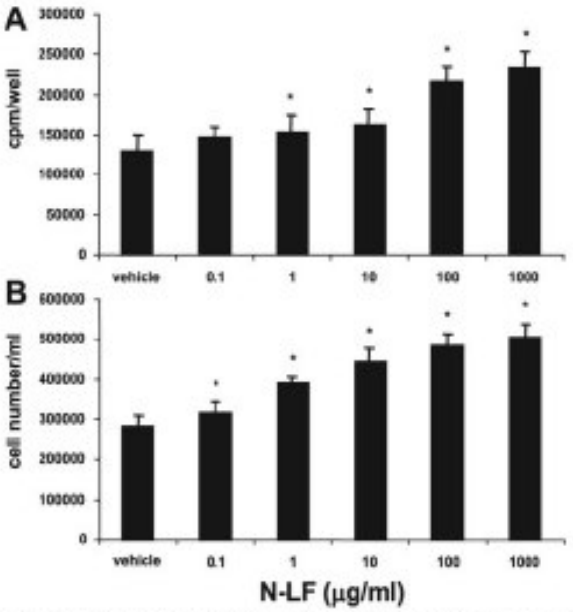

Figure 1. Effects of LF on Caco-2 cell growth. Caco-2 cells were exposed to increasing concentrations of N-LF as described in Methods. Uptake of ^{3}H-thymidine (*A*) and cell count (*B*) were evaluated. Zero dose corresponds to control cells exposed to vehicle in the same conditions of treated cells. Data are mean ± SD of three independent experiments. *Significantly different from 0 μg/mL N-LF ($p < 0.05$).

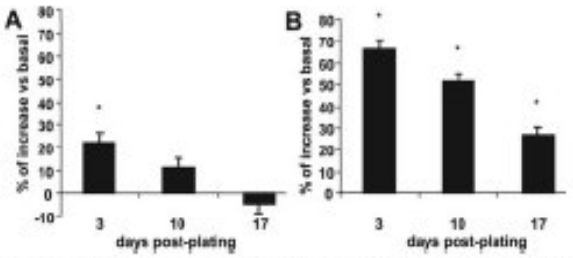

Figure 2. Effects of LF on Caco-2 cell growth at different stages of differentiation. Cell growth was evaluated in Caco-2 cells at 3, 10, and 17 d after plating using uptake of ^{3}H-thymidine method. Caco-2 cells were exposed to 1 μg/mL (*A*) and 100 μg/mL (*B*) of N-LF. Data were expressed as percentage *vs* basal condition and are means ± SD of three independent experiments. *Significantly different from basal ($p < 0.05$).

Effects of LF on sucrase and lactase activities. Under basal conditions, sucrase and lactase activities progressively increased in growing Caco-2 cells. Sucrase activity was measurable at 3 d and peaked between 14 and 16 d after plating. N-LF induced a dose-dependent increase of sucrase activity, with a peak at 100 ng/mL (Fig. 3). N-LF also induced an increase in lactase activity, which, however, was independent on its concentration (Fig. 4). Stimulation of lactase and sucrase activities strictly depended on the time of LF addition. Exposure of more immature cells to LF corresponded to the maximal effect. In parallel experiments we added LF to the cells at 3, 6, 10, and 15 d postplating. The effect was strongest in cells exposed to LF at 3 d for sucrase activity and in those exposed to LF at 6 d for lactase activity (Fig. 5). Thus, LF induces a more potent effect on immature cells.

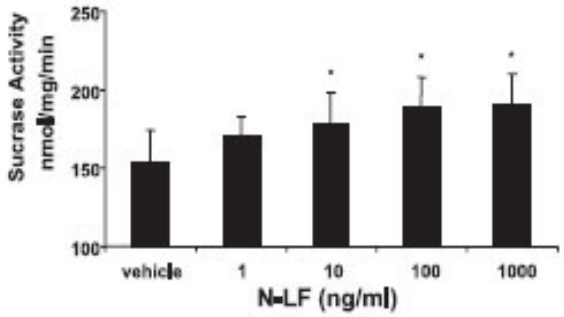

Figure 3. Effects of LF on sucrase activity. Caco-2 cells were stimulated with increasing concentrations of N-LF at 3 d after plating, and sucrase activity was evaluated as described in "Materials and Methods." Zero dose corresponds to control cells exposed to vehicle in the same conditions of treated cells. Data are expressed as nanomoles of glucose generated per minute and normalized for milligram of protein content. Data are means ± SD of three independent experiments. *Significantly different from 0 ng/mL N-LF ($p < 0.05$).

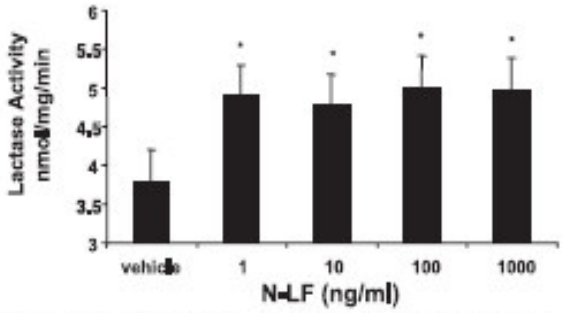

Figure 4. Effects of LF on lactase activity. Caco-2 cells were stimulated with increasing concentrations of N-LF at 6 d after plating and lactase activity was evaluated as described in Methods. Zero dose corresponds to control cells exposed to vehicle in the same conditions of treated cells. Data are expressed as nanomoles of glucose generated per minute and normalized for milligram of protein content. Data are means ± SD of three independent experiments. *Significantly different from 0 ng/mL N-LF ($p < 0.05$).

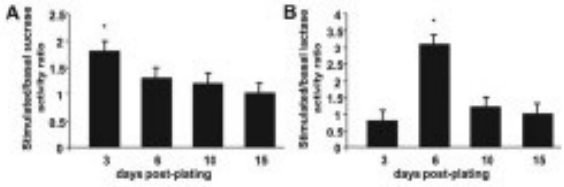

Figure 5. Modulation of disaccharidase activities by LF in Caco-2 cells at different stages of differentiation. Disaccharidase activities were evaluated in Caco-2 cells at 3, 6, 10, and 15 d after plating. LF (100 ng/mL) induced significant stimulation of sucrase (*A*) and lactase (*B*) activities 3 and 6 d after plating respectively. *Significantly different from basal activity ($p < 0.05$).

Biologic effects induced by LF in relation to its iron-saturation status. Because LF exists in iron-saturated and iron-unsaturated isoforms, we investigated changes of cell proliferation, sucrase and lactase activities in Caco-2 cells exposed to N-LF and I-LF. I-LF had a lower effect than N-LF on cell proliferation (Fig. 6). A distinct effect was also detected for differentiation markers. Whereas N-LF induced an increase in sucrase activity in Caco2 cells, I-LF did not (Fig. 7*A*). In contrast, the two isoforms induced lactase activity to a similar extent (Fig. 7*B*). These data suggest that the biologic effects induced by LF depend at least in part, on its iron saturation.

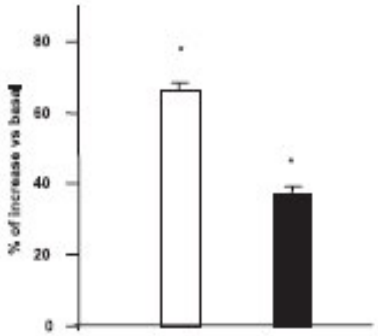

Figure 6. Effect of LF iron-binding status on intestinal growth. Cell proliferation was evaluated in Caco-2 cells after exposure to N-LF (*white column*) and I-LF (*black column*). The concentrations used were 100 μg/mL. The basal level corresponds to control cells exposed to vehicle in the same conditions of treated cells. Data were expressed as percentage *vs* basal condition and are means ± SD of three independent experiments. *Significantly different from basal ($p < 0.05$).

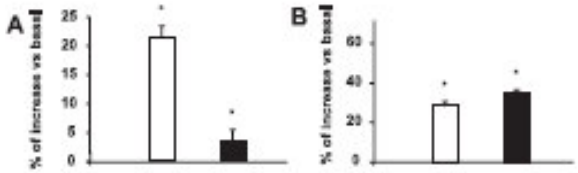

Figure 7. Effect of LF iron-binding status on intestinal differentiation. Differentiation was evaluated in Caco-2 cells after N-LF (*white column*) and I-LF (*black column*) exposure. The concentration used was 100 ng/mL in sucrase (*A*) and lactase activity (*B*). The basal level corresponds to control cells exposed to vehicle in the same conditions of treated cells. Data were expressed as percentage *vs* basal condition and are means ± SD of three independent experiments. *Significantly different from basal ($p < 0.05$).

Effects of BLF on cell growth and differentiation. We observed ^{3}H-thymidine incorporation in Caco-2 cells exposed to bLF (Fig. 8*A*). bLF was used at high and low concentrations equimolar to human LF. At high doses, bLF showed effects similar to N-LF, but at low doses bLF induced a more potent effect on cell growth. A weak stimulation of sucrase activity, similar to what observed with human LF, was observed with 1 ng/mL of bLF. At higher bLF concentrations, there was virtually no effect on sucrase activity (Fig. 8*B*). A reversed pattern of comparative potency by the two LF isoforms was observed for lactase activity. The latter increased more upon bLF than upon human LF stimulation (Fig. 8*C*).

Lactase and sucrase mRNA expression under LF stimulation. To test the hypothesis that LF effects are exerted at transcriptional level, we determined the specific mRNA levels in baseline conditions and in the presence of maximal effective LF concentration. The experiments were performed in Caco-2 cells in preconfluent condition after 24 h of bLF stimulation (Fig. 9, *A* and *B*, respectively). A significant increase of sucrase and lactase mRNA expression was observed with a maximal effect at bLF concentration of 1 ng/mL dose.

DISCUSSION

Intestinal length doubles in the last phase of pregnancy and is maximal at birth (26). Intestinal permeability is an indirect measure of intestinal epithelial development and it decreases

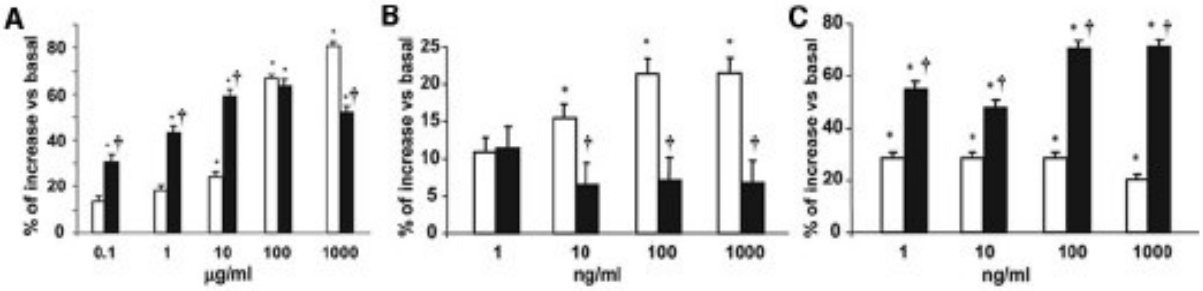

Figure 8. Comparative effects between N-LF and bLF on Caco-2 cell growth and differentiation. Parallel preparations of Caco-2 cells were exposed to increasing concentrations of N-LF (*white column*) or bLF (*black column*) as described in "Materials and Methods." Uptake of ^{3}H-thymidine (*A*), sucrase (*B*), and lactase activities (*C*) were evaluated. The basal level corresponds to control cells exposed to vehicle in the same conditions of treated cells. Data were expressed as percentage *vs* basal condition and are means ± SD of three independent experiments. *Significantly different from basal ($p < 0.05$). †bLF significantly different from N-LF ($p < 0.05$) at the same concentration.

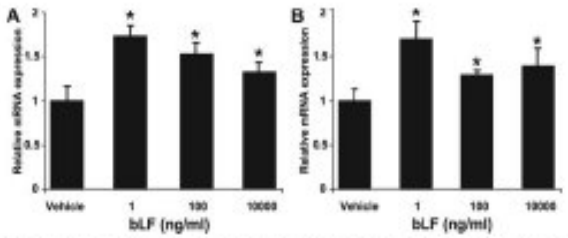

Figure 9. Relative concentration of mRNA for sucrase and lactase in Caco-2 cell line. Relative concentrations of sucrase (*A*) and lactase mRNA (*B*) were determined by real-time quantitative PCR. GAPDH was selected as an endogenous RNA control to normalize for differences in the amount of total RNA. Data are expressed as relative mRNA expression and are means ± SD of two independent experiments. Zero dose corresponds to control cells exposed to vehicle in the same conditions of treated cells. *Significantly different from 0 ng/ml bLF ($p < 0.05$).

in the first days of life (27,28). Newborn infants fed human milk *versus* formula had decreased permeability at 28 d of age (28), indicating a more rapid maturation of intestinal epithelium. This is certainly associated with growth factors in human milk. LF is a major protein component of human milk and exerts numerous physiologic activities, such as enhancement of immune function, defense against pathogenic bacteria and viruses, and stimulation of beneficial gut microflora. Its also promotes gut development and its functions (29). We observed a close relationship between LF concentration and its biologic effects on Caco-2 growth and differentiation. High LF concentrations induced a potent and rapid increase in intestinal epithelial cell proliferation, whereas low LF concentrations induced stimulation of intestinal differentiation. These findings suggest that LF is a key modulator of intestinal epithelium development. They also support the unique properties of colostrum, suggesting that, in addition to its anti-infectious and nutritional effects, it is also involved in the rapid intestinal cell proliferation that is observed immediately after birth. The data on lactase and sucrase activities support the role of LF in the early intestinal development and show that LF directly promotes enterocyte differentiation. Sucrase and lactase show a sugar-dependent rapid increase in the first days of life (6). Interestingly, a rapid increase is observed in disaccharidase activities in jejunal fluid during the first, second, and third weeks of life (30) in parallel with the decrease in LF in human milk (15). We speculate that the higher concentrations of LF in colostrum contributes to the early proliferation of intestinal cells, which then differentiate as a result of its decreased concentration. Oguchi *et al.* (18) investigated the effects of LF on brush border enzymes and found that the iron-saturated form of LF induces sucrase activity but has no effect on alkaline phosphatase activity. However, their experiments were performed in confluent Caco-2 cells undergoing differentiation. Here we show that LF stimulates both lactase and sucrase activity on subconfluent cells, *i.e.* when added at an early phase of differentiation. These findings suggest a positive role of LF in human milk in regulating the levels of lactase activity. The increase of lactase activity during the early weeks of life in preterm infants is greater than the increase in small intestinal mucosal mass (31). Therefore, other factors play a crucial role in stimulating lactase development. Lactase is expressed at higher levels in breast-fed than in formula-fed infants (7). Lactase-specific activity may be regulated *via* transcriptional or post-translational events, as well as by controlling the break-down of lactase protein. Human milk is a rich source of lactose as well as of growth factors and of components of the immune system. Any of these moieties may regulate lactase expression either alone or in combination with lactoferrin. Goda *et al.* (32) suggested a regulatory mechanism of sucrase and lactase gene transcription and protein translation in differentiation process of epithelial intestinal cells. Our data support the concept that LF acts directly on the enterocyte at transcriptional level. It does this in a concentration range typical of mature milk. Therefore, LF is able to modulate as a transcriptional factor mRNA expression in immature intestinal cells typical of intestinal crypt regions. Of course, this last observation should be confirmed in primary cultures obtained from human specimens.

Oguchi *et al.* (18) reported that the iron-saturated LF isoform stimulated the proliferation of confluent (mature) Caco-2 cells, whereas the iron-unsaturated form suppresses it. In contrast, Nichols reported that iron is not required for LF-induced growth of enterocyte (33). Iron saturation of N-LF is 10% in human milk and does not change during lactation (34). Our data indicate that the effects by LF on Caco-2 partially depend on iron saturation. In conclusion, the results of this

research show that LF induces intestinal cell growth and differentiation and it does this in a concentration-dependent fashion, providing an explanation for the age-dependent concentration pattern of LF in human milk. At high concentrations, LF acts as an optimal intestinal growth factor, whereas at low concentrations, it induces intestinal differentiation and a strong inhibition of cell growth. In addition, LF biologic effects are observed in immature Caco-2 only. As a result, there is probably an excess of crypt-type enterocytes that have an ion secretory rather than an absorptive pattern in the very early phase after birth (35). This probably promotes the fluidification and elimination of meconium. Interestingly, the developmental pattern of LF corresponds to that described for guanylate cyclase activity (36), a regulator of enterocyte ion transport channels, and also of cell proliferation, which also peaks in the first 3 d of life (37). Recent data on the guanylate cyclase endogenous ligand, guanylin, suggest that fluidification of intestinal content is regulated developmentally to promote meconium output (38). By promoting rapid proliferation of immature enterocytes that are in a secretive ion transport state, LF could be a key component of this complex interplay.

Finally, our data also have practical implications. They indicate that bovine LF exerts effects on human intestinal cells that are similar to those induced by the human isoform. The comparative experiments showed that bovine isoform is even more potent than human LF in inducing cell growth and lactase expression. LF has been proposed for a number of therapeutic purposes in human disorders, including intestinal inflammation, cancer prevention, and rotavirus infection (39–41). Our findings add to this concept and suggest that bovine LF could be used as a functional component of infant formula to promote intestinal epithelial growth and differentiation. This effect is highly desirable, particularly in premature newborn infants or in intestinal diseases associated with epithelial atrophy.

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and 480 ng/ml (IQR 212–675 ng/ml) in non-responders. Neither indinavir concentrations in the hair, nor trough indinavir concentrations in plasma were correlated with indinavir daily doses. In multivariate analysis, the indinavir hair concentration remained the only factor associated with virological success [$P = 0.04$; odds ratio (OR) 3.88; 95% CI 1.01–14.94], whereas sex (male versus female; $P = 0.06$; OR 5.91; 95% CI 0.90–38.73), baseline protease inhibitor-naive patient status ($P = 0.29$; OR 5.22; 95% CI 0.23–115.93), high baseline HIV-RNA level ($P = 0.06$; OR 0.68; 95% CI 0.47–1.01), and indinavir trough plasma concentration ($P = 0.13$; OR 0.99; 95% CI 0.99–1.01) were not.

As already found for patients receiving non-boosted indinavir, hair concentrations were related to virological success; the concentrations observed in virological responders and non-responders were in the same range as those previously observed in patients with non-boosted indinavir [10]. Furthermore, when also analysing the concomitant plasma concentration and other virological success-associated factors, we found that the determination of the indinavir concentration in hair was more accurate for predicting virological success than a single determination of the indinavir plasma concentration. As we have previously found in analysing the part played respectively by adherence and pharmacokinetic characteristics in the concentration–efficacy relationship, a single plasma concentration determination without concomitant data on adherence within the previous day leads to a misinterpretation of the results [8]. It is particularly striking in the case of the four virological non-responder patients without detected resistance-associated mutations for whom indinavir plasma levels were accurate whereas the indinavir concentrations in hair were low, probably revealing a poor adherence level. Monitoring the indinavir plasma concentration in the hair allows a longitudinal assessment of indinavir impregnation in a large time window, which takes into account the individual pharmacokinetic characteristics, including intraindividual variability, and the patient's adherence history [7]. It has recently been shown in highly adherent patients receiving protease inhibitors with sustained undetectable viral loads in whom extensive repeated plasma levels were measured that the intra-individual coefficient of variability was up to 45%, which may limit the utility of a single measurement in therapeutic drug monitoring for protease inhibitors [7].

Therefore, in the context of therapeutic drug monitoring, ritonavir-boosted indinavir hair sampling may be a useful tool to monitor indinavir impregnation and to help interpret concomitant plasma concentrations allowing an adequate decision based on a same day determination. This combined procedure represents a less traumatic means of patient data collection avoiding repeated plasma sampling and delays in clinical interventions.

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Zinc fights diarrhoea in HIV-1-infected children: in-vitro evidence to link clinical data and pathophysiological mechanism

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Diarrhoea-related morbidity is reduced by zinc supplementation in HIV-1-infected children. The mechanisms of this effect are largely undefined. We

provide evidence for role for Tat (transactivating peptide produced by HIV-1) in the pathogenesis of diarrhoea in AIDS patients. In this study we showed that zinc, preventing Tat-induced fluid secretion, directly limits a specific mechanism of HIV-1-related diarrhoea. Our data support a 'zinc approach' in adjunct to specific antiretroviral therapy in HIV-1-infected children.

A beneficial reduction of watery diarrhoea-related morbidity induced by zinc supplementation in HIV-1-infected children has recently been demonstrated [1]. In the past few years zinc has emerged as a major therapeutic and preventive strategy against diarrhoea [2–4]. These data now open new perspectives for its use, suggesting that zinc could be a safe, simple and cost-effective tool to reduce morbidity and mortality in HIV-1-infected children [1]. Even though zinc is recommended for the treatment of childhood diarrhoea by WHO/UNICEF [3], several important questions remain to be answered, in particular in the HIV-1-infected child, because the study by Bobat *et al.* [1] did not provide evidence on the mechanisms elicited by zinc to limit diarrhoea in these particular patients.

The aetiology of HIV-1-related severe watery diarrhoea is certainly multifactorial and still largely unknown [5]. We have recently provided evidence for a primary role of Tat (the transactivating peptide produced by HIV-1 and essential for its replication) in the pathogenesis of diarrhoea in AIDS patients. Functioning as an enterotoxin, like *Vibrio cholerae* toxin, Tat stimulates active fluid secretion from the serosal to the luminal side of human enterocytes in the classical Ussing chamber in-vitro model used to investigate transepithelial ion transport [6,7]. A similar mechanism has been reported [8] for *Cryptosporidium parvum*, the most frequent and dangerous opportunistic enteric pathogen in AIDS patients. These findings suggest that HIV-1-infected children are at a high risk of secretory diarrhoea. In addition, we have demonstrated that Tat is able to inhibit sodium ion/glucose symporter activity, a major mechanism to absorb fluid at the intestinal level, further determining the occurrence of diarrhoea [7]. Interestingly, the Tat effects on intestinal ion transport were dose dependent, with a maximal effective dose of 0.1 nmol, which is well within the range of what is generally measured in the sera of patients with HIV-1 infection, suggesting that effective Tat concentrations may well be reached *in vivo* [9,10]. On the other hand, zinc is directly active on transepithelial ion transport at the intestinal level, as direct zinc–enterocyte interaction results in net ion absorption, thereby counteracting active secretion, such as that induced by *V. cholerae* toxin in the same experimental model [11]. To determine whether zinc was effective in inhibiting Tat-induced ion secretion, we incubated human derived Caco-2 intestinal cells with chemical synthesized, high-pressure liquid chromatography 96% pure, HIV-1 Tat, (Tecnogen, Piana di Monteverna, Italy), in the presence or absence of zinc ($ZnSO_4$; Sigma Chemical Co., St Louis, Missouri, USA), using the Ussing chamber experimental model [6]. As shown in Figure 1, the pre-incubation of human enterocytes with

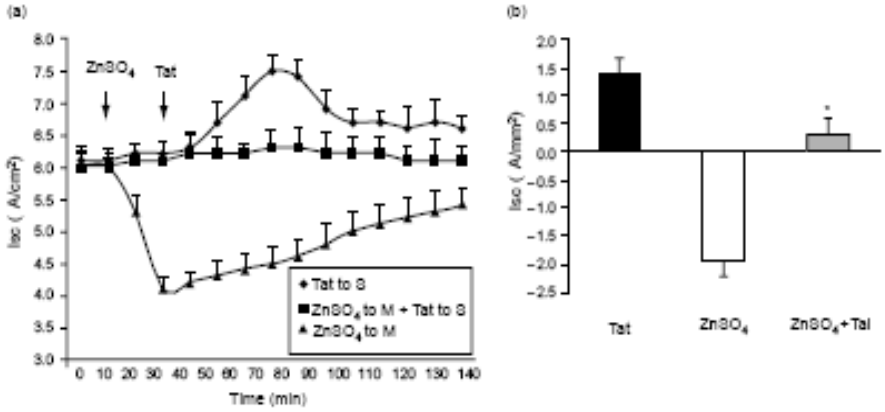

Fig. 1. Zinc inhibitory effect against HIV-1 Tat protein-induced intestinal ion secretion. (a) Time course of the effect of HIV-1 Tat protein and $ZnSO_4$ addition, alone or in combination, on transepithelial ion transport in human enterocyte (Caco-2 cell) monolayer mounted in Ussing chambers (i.e. an established in-vitro model to study ion transport). Tat addition to the enterocytes serosal side (S) induced an increase in the intensity of short circuit current (Isc), indicating the presence of active chloride ion secretion. $ZnSO_4$ determined a pure pro-absorptive effect on transepithelial chloride ion transport (i.e. a decrease in Isc). Pre-incubation for 20 min with $ZnSO_4$ to the mucosal side (M) was able significantly to reduce the secretory response elicited by Tat at intestinal level. The arrows indicate the time of addition either of Tat or $ZnSO_4$. (b) Maximal Isc modifications after Tat and $ZnSO_4$ addition, alone or in combination, to human enterocytes mounted in Ussing chambers. Data are expressed as mean ± SE, and significance was evaluated by the non-parametric, two-tailed Mann–Whitney *U* test. $^*P < 0.001$ Tat alone versus $ZnSO_4$ plus Tat. ◆ Tat to S; ■ $ZnSO_4$ to M plus Tat to S; ▲ $ZnSO_4$ to M.

zinc (35 μmol) resulted in the almost total inhibition of Tat-induced ion secretion, as reflected by the intensity of the short circuit current. These results suggest that zinc is able to prevent intestinal fluid secretion induced by Tat, and is able to interact directly with a specific mechanism of HIV-1-related diarrhoea, explaining well the results obtained in South Africa by Bobat and co-workers [1], and supporting the usefulness of the 'zinc approach' in adjunct to specific antiretroviral therapy in HIV-1-infected children.

An emphasis on the costs and economic benefits of an alternative therapy is an important aspect of health services research. The cost savings and the attractive cost-effectiveness, disposability and thermostability of zinc indicates the need to assess further the role of these micronutrients in the prevention and treatment of diarrhoea in AIDS patients.

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RAPID COMMUNICATION

Growth hormone regulates intestinal ion transport through a modulation of the constitutive nitric oxide synthase-nitric oxide-cAMP pathway

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Abstract

AIM: Growth hormone (GH) directly interacts with the enterocyte stimulating ion absorption and reducing ion secretion induced by agonists of cAMP. Since nitric oxide (NO) is involved in the regulation of transepithelial ion transport and acts as a second messenger for GH hemodynamic effects, we tested the hypothesis that NO may be involved in the resulting effects of GH on intestinal ion transport.

METHODS: Electrical parameters reflecting transepithelial ion transport were measured in Caco-2 cell monolayers mounted in Ussing chambers and exposed to GH and cholera toxin (CT) alone or in combination, in the presence or absence of the NO synthase (NOS) inhibitor, N_{ω}-nitro-L-arginine methyl ester (L-NAME). Similar experiments were conducted to determine cAMP and nitrite/nitrate concentrations. NOS expression was assayed by Western blot analysis.

RESULTS: L-NAME causes total abrogation of absorptive and anti-secretory effects by GH on intestinal ion transport. In addition, L-NAME was able to inhibit the GH-effects on intracellular cAMP concentration under basal conditions and in response to CT. GH induced a Ca^{2+}-dependent increase of nitrites/nitrates production, indicating the involvement of the constitutive rather than the inducible NOS isoform, which was directly confirmed by Western blot analysis.

CONCLUSION: These results suggest that the GH effects on intestinal ion transport, either under basal conditions or in the presence of cAMP-stimulated ion secretion, are mediated at an intracellular level by the activity of cNOS.

Key words: Nitric oxide synthase; Cholera toxin; Intestinal ion secretion; cAMP; Enterocyte

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INTRODUCTION

Intestinal ion fluxes are regulated by several agents including neurotransmitters, hormones, or paracrine agents[1]. We obtained evidence that growth hormone (GH) and nitric oxide (NO) act as modulators in this network[2-5]. GH increases basal intestinal water and ion absorption in *in vivo* and *in vitro* animal models and is also capable of substantially reducing ion secretion induced by agonists of cAMP, cGMP, or intracellular Ca^{2+}, the second messengers of ion secretion[4,5]. Using the human intestinal cell line Caco-2, we showed that the GH effects on ion transport result from direct interaction with the enterocyte[2]. Free radical NO acts as a second messenger of several GH effects on human metabolism[6]. NO production is decreased in patients with untreated GH deficiency, while treatment with recombinant human growth hormone (rhGH) increases NO formation[7]. In the past decade NO has emerged as a signalling molecule mediating a broad spectrum of intestinal processes, such as gastrointestinal motility, inflammatory changes, malignancy, mucosal blood flow and transepithelial ion transport[8,9]. NO is a gas with a half life of less then 5 s generated through a series of regulated electron transfer steps by a family of P450-like enzymes, termed nitric oxide synthases (NOS)[10,11]. Two NOS are continuously present and are termed constitutive nitric oxide synthase (cNOS). These two isoforms are Ca^{2+}/calmodulin-dependent, produce small amounts of NO in

short bursts and are involved in homeostatic processes. A third isoform, which is Ca^{2+}/calmodulin-independent, is induced by intestinal injury and inflammation. This latter isoform, termed inducible nitric oxide synthase (iNOS), requires a lag period of at least 2-3 h and, once expressed, produces large amounts of NO for longer time[1,5,12]. NO can be directly produced by enterocytes through both the constitutive and the inducible NOS isoforms[5,13,14]. An important feature of the NO effect is its concentration-dependence. Leading to the concept that NO often acts as a double-edged sword mediator with beneficial as well as detrimental effects. While at lower concentrations it maintains a basal ion intestinal pro-absorptive tone, it increases in several pathologic states such as inflammatory bowel diseases, toxic megacolon, and infectious gastroenteritis, contributing to ion secretion[8,12,15]. Recently, we showed that under basal conditions the intracellular cAMP concentration ([cAMP]i) is downregulated in the enterocyte by a cNOS-dependent NO production. Furthermore, in the presence of a cAMP-dependent stimulated secretion, cNOS is activated functioning as a breaking force of ion secretion[3]. This raised the hypothesis that the enterocyte is capable of self-regulating its own ion transport process through the activation of the cNOS-NO pathway which is able to modulate the [cAMP]i level[3]. The aim of this study was to determine whether NO is also involved in mediating the ion absorptive effects triggered by an extracellular stimulus. Specifically, we tested the hypothesis that the cNOS-NO-cAMP pathway is implicated in the pro-absorptive and in the anti-secretory effect induced by GH at the intestinal level. We used the Caco-2 in vitro cell model, previously validated for investigating the GH and the NO intestinal effects[2].

MATERIALS AND METHODS

Cell culture

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose concentration (4.5 g/L) supplemented with 10% FCS, 1% nonessential amino acids, penicillin (50 mU/mL), streptomycin (50 mg/mL) and were incubated in 50 mL/L CO_2-950 mL/L air. Medium was changed daily.

Ion transport studies

Cells were grown on uncoated polycarbonate transwell filters as previously described and used for intestinal transport studies at 15 d post-confluence[3]. The filter area was 4.9 cm^2. Each filter was mounted in an Ussing chamber (World Precision Instrument, Sarasota, FL) as a flat sheet between the mucosal and the serosal compartment. Each compartment contained 10 mL of Ringer's solution with the following composition (in mmol/L): NaCl (114), KCl (10), Na_2HPO_4 (1.65), NaH_2PO_4 (0.3), $CaCl_2$ (1.25), $MgCl_2$ (1.1), $NaHCO_3$ (15), glucose (19). In experiments performed to investigate the role of Cl^- in the electrical response, SO_4^- substituted Cl^- at an equimolar concentration. The incubation fluid was circulated by a thermostat-regulated circulating pump and continuously gassed with 95% O_2-5% CO_2. Transepithelial potential difference (PD), short-circuit current (Isc) and tissue ionic conductance (G) were monitored by an automatic voltage-clamp device (DVC 1000, World Precision Instrument, Sarasota, FL) as described elsewhere[16], before and after mucosal or serosal addition of GH, cholera toxin (CT), and the specific NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME). Isc is expressed as microamperes per square centimeter (μA/cm^2), G as millisiemens per square centimeter (mS/cm^2), and PD as millivolts (mV). Caco-2 cell monolayers, pre-incubated for 20 min with GH (4×10^{-9} mol/L) on the serosal side, were exposed to CT (6×10^{-8} mol/L) on the mucosal side, in the presence or the absence of L-NAME (2×10^{-4} mol/L) added to both sides. The maximal effective concentrations of GH, CT and L-NAME were determined by dose-response experiments (data not shown). Cell viability was evaluated at the end of each experiment by measuring the electrical response to the serosal addition of theophylline (5×10^{-3} mol/L).

Intracellular cAMP concentration determination

To test the hypothesis that GH specifically counteracts the CT-induced cAMP increase, we determined the modifications in [cAMP]i after 1 h of incubation with GH and CT, alone or in combination, and in the presence or the absence of L-NAME. [cAMP]i in Caco-2 cells which was measured by using a commercial kit (Biotrak cyclic AMP assay system; Amersham International, Amersham, UK), as previously described[17].

Western blot analysis

Caco-2 cells were stimulated with GH (4×10^{-9} mol/L) for 1, 6 or 24 h. Cells were then scraped into PBS buffer and lysed in a buffer containing 1% Tergitol (Nonidet P-40) with the following composition: KCl, 60 mmol/L; β-mercaptoethanol, 14 mmol/L; EDTA, 2 mmol/L; HEPES pH 7.9, 15 mmol/L; sucrose, 0.3 mol/L; aprotinin, 5 μg/mL; leupeptin, 10 μg/mL; pepstatin, 2 μg/mL; phenylmethylsulfonyl fluoride, 0.1 mmol/L. Whole cellular extracts were centrifuged at 1500 g for 20 m at 4℃. Protein content was determined by the Bradford method (Bio-Rad Laboratories, Munchen, Germany). The supernatant containing the solubilized proteins was then boiled for 5 min in Laemly buffer (tris-HCl pH 6.8, 62.5 mmol/L; SDS 2%; glycerol 10%; 2-mercaptoethanol 5%; bromophenol blue 0.001%). Cell proteins (50 μg/lane) were added to an SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane (BioBlot-NC-Costar, Corning Incorporated, Canada). Blots were blocked with T-TBS buffer (tris-HCl pH 8.8, 10 mmol/L; NaCl, 150 mmol/L; Tween 20, 0.05%) containing 3% albumin, and probed for 1 h with affinity purified anti-human NOS1 (1:2000) dilution ratio, NOS2 (1:200) or NOS3 (1:1000) rabbit polyclonal antibodies. Bound antibody was detected with anti-rabbit immunoglobulin horseradish peroxidase-linked whole antibody and developed by chemiluminescence reaction (Amersham Pharmacia Biotech, UK). Gamma-interferon (50000 U/mL) was used as a positive control in experiments performed using anti-iNOS antibodies.

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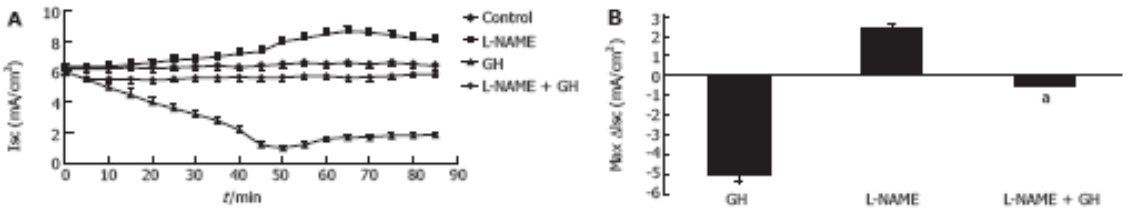

Figure 1 A: Time course of the effect on short-circuit current (Isc) of GH (4×10^{-9} mol/L) and L-NAME (2×10^{-4} mol/L), alone or in combination, to Caco-2 cells mounted in Ussing chambers; B: Isc peak after L-NAME or GH addition, alone or in combination, to Caco-2 cells mounted in Ussing chambers. Data are mean ± SD of 6 different observations. $^{a}P < 0.05$ GH *vs* L-NAME + GH.

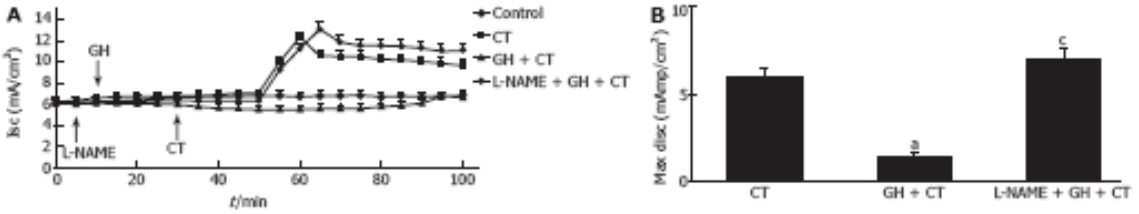

Figure 2 A: Time course of the GH (4×10^{-9} mol/L) effect on CT (6×10^{-8} mol/L)-induced short-circuit a current (Isc) increase in the absence or in the presence of L-NAME (2×10^{-4} mol/L) in Caco-2 cells mounted in Ussing chambers. The arrows indicate the time of addition of each agent; B: Maximal Isc increase after CT addition, alone or in the presence of GH alone or in combination with L-NAME. A total abrogation of the antagonistic effect of GH on the CT-induced electrical response was observed in the presence of L-NAME. Data are mean ± SD of 6 different observations. $^{a}P < 0.05$ CT alone *vs* GH + CT; $^{c}P < 0.05$ GH + CT *vs* L-NAME + GH + CT.

Nitrite/nitrate (NO_2^-/NO_3^-) production

The combined concentration of nitrite and nitrate, the degradation products of NO in the culture medium, was determined by the Griess reaction after nitrate reduction[18]. Total nitrite/nitrate production was referred to NO production. Experiments were performed using normal or Ca^{2+}-free Ringer's solution to investigate the involvement of the cNOS isoform (the Ca^{2+}/calmodulin-dependent isoform or NOS1). The modified Ringer's solution had the following composition (mmol/L): $NaHPO_4$, 1.65; NaH_2PO_4, 0.3; $NaHCO_3$, 15, NaCl, 53; KCl, 10; Na_2SO_4, 30.5; $MgCl_2$, 2.35; glucose, 19; EDTA, 0.5.

Chemicals

All chemicals were of reagent grade and were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). Culture media were from Life Technologies GIBCO BRL (Mascia e Brunelli, Milan, Italy). Transwell filters and supports were from Costar (Costar Italia, Milan, Italy). rhGH was obtained from Serono (Industria Farmaceutica Serono, Rome, Italy). Anti-cNOS and anti-iNOS polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-iNOS polyclonal antibodies were purchased from Transduction Laboratories (ABD Company, Lexington, KY, USA).

Statistical analysis

Each experiment was run in duplicate and repeated at least 3 times. Results are expressed as mean ± SD. Repeated-measures ANOVA were applied using the Bonferroni test for multiple comparisons. The significance was set at 5%. The SPSS software package for Windows (release 11.0.1; SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

RESULTS

Intestinal transport studies

GH (4×10^{-9} mol/L) and L- NAME (2×10^{-4} mol/L) caused opposite effects on basal Isc. GH induced a decrease and L-NAME induced an increase in Isc. Both effects were totally related to PD modifications, as no significant variations of G were recorded. Pre-treatment with L-NAME (2×10^{-4} mol/L) for 5 m almost abolished the electrical response to GH (Figure 1). The addition of CT (6×10^{-8} mol/L) to Caco-2 cells induced an increase in Isc. Both the GH and CT effects were Cl^--dependent as demonstrated in the experiment done in Cl^- free Ringer solution. Thus, in the absence of Cl^- the electrical effects were virtually abolished indicating that they were entirely due to transepithelial Cl^- transport modifications (data not shown). Pre-incubation with GH for 20 m substantially reduced the CT effect on Isc. However, pre-incubation with L-NAME resulted in total abrogation of the antagonistic effect of GH on the CT-induced electrical response (Figure 2).

Intracellular cAMP concentration determination

Incubation with GH (4×10^{-9} mol/L) resulted in significant reduction of basal [cAMP]i. On the contrary, incubation with CT induced an increase in [cAMP]i. The addition of L-NAME produced a significant increase in basal and in CT-stimulated [cAMP]i. Pre-incubation with GH for 20 min resulted in a reduction of the CT-induced [cAMP]i increase. Finally, the addition of L-NAME resulted in total inhibition of the GH-induced decrease in basal [cAMP]i as

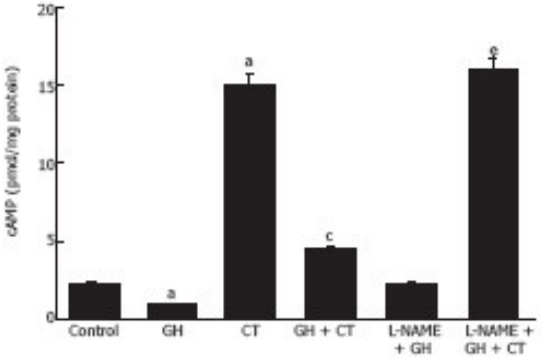

Figure 3 Modification of intracellular cAMP concentration in Caco-2 cells after incubation with GH, CT, alone or in combination, in the presence or in the absence of L-NAME. Data are mean ± SD of 6 different observations. $^{a}P < 0.05$ *vs* control; $^{c}P < 0.05$ CT alone *vs* GH + CT; $^{e}P < 0.05$ GH + CT *vs* L-NAME + GH + CT.

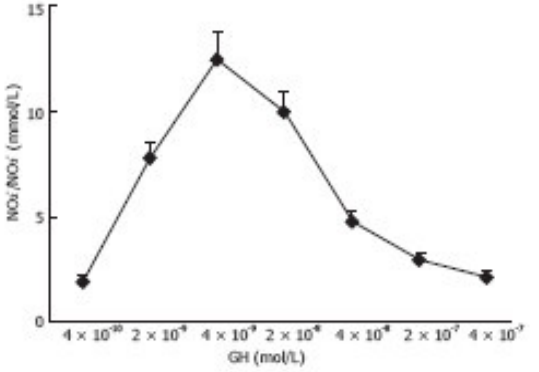

Figure 5 Effects of increasing concentration of GH on NO production in Caco-2 cells. Increasing concentrations of GH were added to Caco-2 cell monolayers and NO production was determined after 1 h of incubation. Data are mean ± SD of 6 different observations.

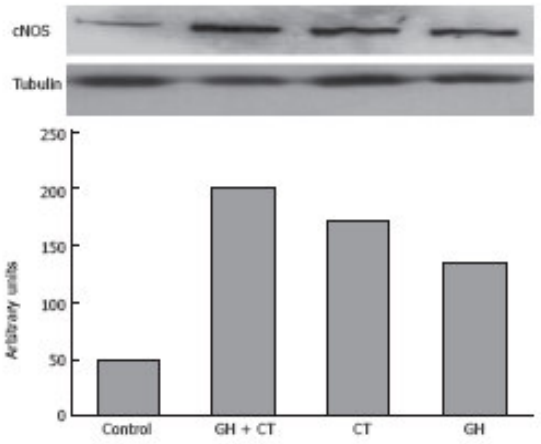

Figure 4 The upper side of the figure shows the cNOS protein expression in Caco-2 cells after 1 h of incubation with GH and CT alone or in combination, as compared to tubulin expression. The cNOS protein expression is revealed by the appearance of 160-kD band that corresponds to human NOS 1 (neuronal NOS). Shown is a representation of 3 separate experiments. In the lower side of the figure an optical densitometry analysis of the bands is also reported.

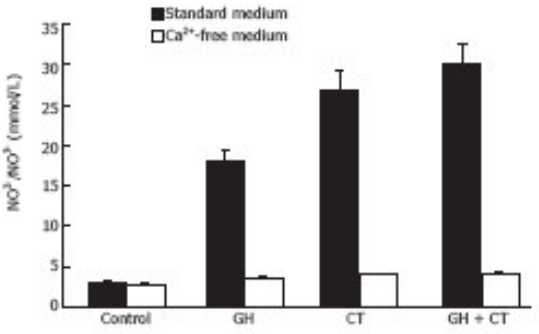

Figure 6 Total NO production in Caco-2 cells under basal conditions and after stimulation with GH and CT, alone or in combination, in standard or in Ca^{2+}-free medium. Data are mean ± SD of 6 different observations. $^{a}P < 0.05$ *vs* control.

well as a total abrogation of the GH effect on CT-induced [cAMP]i increase (Figure 3). These results point to a role of NO either under basal conditions or in response to external stimuli which drive ion fluxes toward an absorption pattern.

Western blot analysis

Caco-2 cells showed low but detectable basal cNOS protein expression (Figure 4). Western blot analysis performed after 1 h of incubation with GH revealed the amplification of a 160 kD band corresponding to human NOS 1. Simultaneous incubation of Caco-2 cells with either GH or CT resulted in further amplification of the NOS 1 band (Figure 4). On the contrary, NOS 2 and NOS 3 protein expressions were undetectable in unstimulated cells and in cells exposed to GH and CT alone or in combination for up to 24 h of incubation (data not shown).

Nitrite/nitrate (NO_2^-/NO_3^-) production

NO production by Caco-2 cells was determined in culture medium after 1 h of incubation with increasing GH doses. As shown in Figure 5 a dose-dependent increase in NO production was detected in response to the hormone. GH doses higher than 4×10^{-9}mol/L did not induce further increase in NO production, indicating a saturation pattern of the effect. Caco-2 cell stimulation with simultaneous exposure to GH and CT resulted in a further increase in NO production compared to each individual substance. The effect was Ca^{2+}-dependent, since in the absence of Ca^{2+}, the NO increase in response to GH addition was abolished. This data suggests an involvement of the constitutive rather than the inducible NOS isoform in the GH effect (Figure 6).

DISCUSSION

We have previously shown that GH is able to increase intestinal fluid absorption under basal conditions and to inhibit ion secretion elicited by the 3 main intracellu-

lar second messengers of bacterial enterotoxins: cAMP, cGMP and Ca^{2+}[2,4]. The data from this study provides new evidence on the ability of GH to regulate water and ion transport and implicates cNOS-NO activity for this effect. A complete abrogation of GH effects on Isc was seen in the presence of the specific NOS inhibitor L-NAME. An increase of cNOS activity and a subsequent Ca^{2+}-dependent production of NO were observed in enterocytes treated with GH. These effects were associated with a Cl-dependent decrease in Isc, consistent with an anion absorptive effect. We have recently demonstrated that the CT-enterocyte interaction results in an enhanced NO production. Such an effect may be interpreted as a homeostatic mechanism operated by the enterocyte and functioning as a breaking force to limit ion secretion[3]. cNOS-NO system is activated by a yet unknown sensing mechanism and reacts to balance the stimulated secretion. The data in this work support and extend this hypothesis and suggest that the modulation of the cNOS-NO activity could also be dependent on extracellular stimuli control, namely on the GH signal transduction. In all instances, the target of cNOS-NO is cAMP. Our results are similar to those obtained using NO donors, which are able to inhibit forskolin-stimulated cAMP production by adenyl cyclase (AC) isoforms AC5 and AC6, in both T84 epithelial cells and mucosal scrapings from mouse colon[19-21], and are in agreement with those previously obtained in isolated cholangiocytes[22].

It has been recently suggested that GH inhibitory effect on intestinal ion secretion is related to the transactivation of epidermal growth factor (EGF) receptor and the subsequent activation of extracellular signal-regulated kinase (ERK, also known as p 44/42 mitogen activated protein kinase or MAPK) activity[23]. Interestingly, a NO stimulation through ERK-dependent upregulation of cNOS gene transcription has been recently demonstrated for proinsulin C-peptide in endothelial cells[24], and similar effects have been shown with the endothelium-derived hyperpolarizing factor (EDHF)[25]. Thus, it is possible to also hypothesize that the NO-mediated GH effects at the intestinal level could involve a MAPK activation. It also remains to be clarified whether GH effects are mediated by Ca^{2+}. Overall the cNOS-NO system could be viewed as a regulator of ion transport acting on the enterocyte via three distinct patterns: (1) to keep cAMP production at a low level under basal conditions, in order to maintain an intestinal ion pro-absorptive tone; (2) during stimulation of ion secretion, such as that trigged by CT, to reduce ion secretion; (3) in response to extracellular pro-absorptive stimuli, namely acting as second messenger of the GH-induced ion absorption. In all these 3 instances the target of cNOS-NO is cAMP, the effect is Ca^{2+}-dependent and involves Cl^- transcellular flux. Thus the cNOS-NO-cAMP pathway plays a key role on the enterocyte fluid absorptive/secretory processes.

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Inhibitory effect of HIV-1 Tat protein on the sodium-D-glucose symporter of human intestinal epithelial cells

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Objective: The pathophysiology of HIV-1-related intestinal dysfunction is largely unknown. We previously found that the transactivator factor peptide (Tat) produced by HIV-1 induces ion secretion and inhibits cell proliferation in human enterocytes. Because sugar malabsorption is a frequent feature in AIDS patients, we evaluated whether Tat inhibits intestinal glucose absorption.

Design and methods: We measured Na^+-D-glucose symporter (SGLT-1) activity and determined its phenotypic expression in Caco-2 cells, in the presence and absence of Tat, in uptake experiments using a non-metabolized radiolabelled glucose analogue, and by western blot analysis, respectively. α-Tubulin staining was used to study the effects exerted by Tat on cell structure.

Results: Tat dose dependently inhibited glucose uptake by human enterocytes. This effect was prevented by anti-Tat polyclonal antibodies and by L-type Ca^{2+} channels agonist Bay K8644. Western blot analysis of cellular lysates and brush-border membrane preparations showed that Tat induced SGLT-1 missorting. Tat also caused a dramatic decrease in α-tubulin staining, which indicates dysruption of the cytoskeleton organization.

Conclusions: Tat acutely impairs intestinal glucose absorption through SGLT-1 missorting. This result indicates that Tat is directly involved in AIDS-associated intestinal dysfunction.

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Keywords: AIDS, intestinal glucose absorption, intestinal dysfunction, diarrhoea

Introduction

Intestinal diseases are a hallmark of HIV-1 infection [1]. During the progression of the disease, chronic diarrhoea, dehydration, and malabsorption lead to progressive weight loss, and so contribute to the morbidity and mortality of HIV-1-positive subjects [2]. Functional and structural changes of gut mucosa may be detected before the onset of opportunistic infections and will eventually be responsible for intestinal dysfunction [1,3]. Carbohydrate and lipid malabsorption, and increased small bowel transepithelial permeability are common in patients who are not on HAART [3]. HAART rapidly improves intestinal sugar absorption [4–6]. The aetiology of HIV-1 associated intestinal dysfunction is largely unknown, and has been variously attributed to opportunistic infections, cytokine secretion in response to chronic inflammation, and a direct role of HIV-1 virus

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itself [1,3,7,8]. The latter concept is supported by the finding that recovery of intestinal digestive-absorptive function paralleled the decrease of viral load in children started on HAART [1,4]. Primary HIV-1 induced enteropathy is also consistent with the detection of viral proteins and/or nucleic acids in the intestinal mucosa of AIDS patients [9]. However, HIV-1 is not invariably found in the intestinal epithelium of AIDS patients. In addition, diarrhoea and nutrient malabsorption do not correlate with the presence of HIV-1 in gut mucosa [10].

Some of the effects induced by HIV-1 are not mediated by lytic propagation of viral particles but are induced by viral factors [11,12]. In addition to structural and enzymatic proteins, HIV-1 encodes a group of regulatory proteins including Tat, a transactivating peptide essential for HIV-1 replication [11,13–15]. Despite its nuclear localization, Tat is secreted from HIV-1-infected cells and taken up by uninfected neighbouring cells. Tat can occur in the sera of AIDS patients in the absence of massive lysis of infected cells, and is involved in many processes that contribute to immune and non-immune changes associated with HIV-1 infection [11,13–15]. Several effects induced by Tat require activation L-type Ca^{2+} channels and/or the mobilization of intracellular Ca^{2+} stores [11,13–15]. We previously reported that the addition of Tat to human enterocytes, and to human colonic mucosa, induces electrolyte secretion similar to that caused by classical bacterial enterotoxins, which suggests that Tat is directly involved in AIDS-related diarrhoea [16]. The finding that Tat induced a potent anti-proliferative effect in human enterocytes, links it to the pathogenesis of HIV-1-related intestinal mucosal atrophy [16].

Sugar malabsorption is the most frequent and severe feature of AIDS-related intestinal dysfunction, and it contributes to AIDS-associated malnutrition [17]. In the human intestine, and in the human intestinal cell line, Caco-2, glucose absorption is coupled with Na^+ absorption through the Na^+-D-glucose symporter 1 (SGLT-1) located on the enterocyte apical membrane. The transporter GLUT-2, which is located on the basolateral membrane, then carries intracellular glucose to the bloodstream [18–21]. The aim of this study was to test the hypothesis that, by inhibiting SGLT-1 activity in the intestinal epithelium, Tat is involved in the pathogenesis of glucose malabsorption in AIDS patients.

Materials and methods

Cell growth

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, Maryland, USA). Cells were grown in Dulbecco's modified Eagle medium with high glucose concentration (4.5 g/l) supplemented with 10% foetal calf serum, 1% non-essential amino acids, penicillin (50 mU/ml), and streptomycin (50 mg/ml) and were incubated in 5% CO_2/95% air. The medium was changed daily.

Glucose uptake studies

Caco-2 cells were grown on 24-well plates. After 15 days post-confluence cells were incubated for 30 min with the non-metabolizable radiolabelled glucose analogue [^{14}C]-α-Methyl-L-D-glucopyranoside (AMG, 0.1 mM). The cells were lysed in 0.1 N NaOH. An aliquot was assayed for protein content (Bradford method, Bio-Rad Laboratories, Munich, Germany) and another for [^{14}C]AMG content using a Packard scintillation spectrometer. To verify the presence of SGLT-1 activity in the cell line, the same experiment was performed in the presence of the selective competitive inhibitor of SGLT-1 phlorizin (100 μM) or in Na^+-free buffer for 1 h (using choline chloride and K_2HPO_4 in place of NaCl and Na_2HPO_4 adjusted to pH 7.4 with KOH). Tat was added at increasing concentrations (from 0.01 to 1.0 nM) for 1 h, in the presence or absence of anti-Tat polyclonal antibodies (10 : 1, w/w) or the specific L-type Ca^{2+} channels agonist, Bay K8644 (1 μM) as reported previously [16]. All data was expressed as c.p.m./mg protein.

Western blot analysis

The phenotypic expression of SGLT-1 was analysed in whole Caco-2 cell and in preparations of brush border membrane (BBM) vesicles. Briefly, cell blots and BBM vesicle preparations, obtained by magnesium precipitation method as described previously [22], were incubated with a rabbit polyclonal antibody, raised against the synthetic peptide corresponding to amino acids 564–575 of rabbit intestinal SGLT-1 sequence. Purified BBM vesicles from Caco-2 cells were pre-incubated with Tat and lysed with a buffer (150 mM NaCl, 10% glycerol, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 10 μg/ml aprotinine, 10 μg/ml leupeptine, 100 mM NaF, 1 mM phenilmethilsulphonyl fluoride, 1% Triton X-100 in 50 mM HEPES buffer, pH 7.5). In parallel experiments, the antibody was preadsorbed with the corresponding antigenic peptide, to confirm hybridization specificity. BBM vesicles were solubilized in Laemmli buffer (23 mmol/l Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 0.001% Bromophenol blue, and 5% 2-mercaptoethanol) and resolved by 8% SDS–PAGE. Proteins were electrotransferred onto nitrocellulose membranes using a transblot apparatus (Bio-Rad, Hercules, California, USA). Non-specific binding sites were blocked with phosphate-buffered saline (PBS) containing 0.05% Tween 20 and 3% bovine serum albumin (BSA). Blots were incubated with the primary antibody at a 1 : 5000 dilution for 16 h at 4°C. In control experiments, nitrocellulose membranes were incubated with the same antibody previously pre-adsorbed with the antigenic peptide. Anti-SGLT-1

antibody was detected by enhanced chemiluminescence (Amersham International, Buckinghamshire, UK) using a peroxidase-conjugated anti-rabbit IgG (Sigma Chemical Co., St. Louis, Missouri, USA) as secondary antibody (1 : 3000 dilution). After detection, hybridization bands were quantified by scanning densitometry.

Immunofluorescence of α-tubulin

Caco-2 cells were seeded in 24-well plates on glass coverslips. At 15 days post-confluence, cells were exposed to increasing Tat concentrations ranging 0.01 to 1.0 nM for 1 h. At the end of incubation, cells were fixed with 4% paraformaldehyde (w/v), then treated with NH_4Cl (50 mM) in PBS for 10 min, and permeabilized with ice-cold methanol. Cells were blocked with 3% BSA (w/v) in PBS for 30 min. For α-tubulin staining, coverslips were incubated in a humid atmosphere with the specific primary antibody (1 : 300) for 1 h at room temperature in blocking medium. Fluorescein-conjugated secondary antibody (1 : 100) was added in 3% BSA in PBS for 1 h at room temperature. After washing, samples were fixed in 90% glycerol (v/v), 0.2% N-propylgallate (w/v) in PBS and observed with a fluorescence microscope (Nikon Eclipse E600, Tokyo, Japan).

Reagents

Non-metabolizable radiolabelled glucose analogous AMG was from Amersham Pharmacia Biotech (Milan, Italy). Phlorizin was from Sigma Chemical Co. Chemically synthesized, high-performance liquid chromatography 96% pure HIV-1 Tat, as well as rabbit polyclonal antibody anti-Tat were from Tecnogen (Piana di Monteverna, Italy). Mouse monoclonal antibody against α-tubulin and fluorescein-conjugated secondary antibody were from Sigma Chemical Co. Bay K8644 was purchased from Calbiochem (La Jolla, California, USA).

Statistics

Data are expressed as mean ± SE, and significance was evaluated by the nonparametric, two-tailed Mann–Whitney *U* test. A P value < 0.05 was considered significant. The SPSS software package for Windows (release 12.0.1; SPSS Inc., Chicago, Illinois, USA) was used for the statistical analysis.

Results

Effects of Tat on intestinal glucose uptake

Firstly, we determined the basal activity of SGLT-1 in Caco-2 cells by measuring [^{14}C]-AMG absorption, in Na^+-free medium or in the presence of phlorizin. As shown in Fig. 1, glucose uptake was significantly inhibited in Na^+-free conditions and in the presence of phlorizin, which demonstrated normal functioning of the Na^+-dependent glucose absorption pathway in Caco-2 cells. We then performed experiments to test the effects of

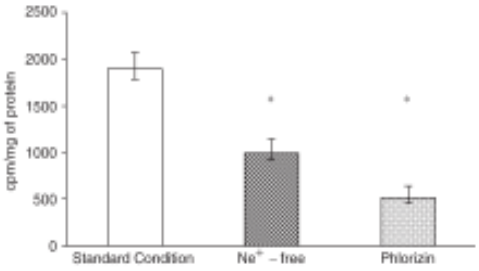

Fig. 1. Characterization of Ne^+-D-glucose symporter activity in differentiated Caco-2 cells. The uptake of 0.1 μM of non-metabolizable radiolabelled glucose analogous AMG was measured at a single time point (1 h) in standard conditions (white bar) or in Na^+-free condition (lined bar) or in the presence of phlorizin (pointed bar). Values are means ± SE of three independent measurements. *$P < 0.01$ versus standard condition.

increasing doses of Tat. Glucose uptake was significantly inhibited by incubation for 1 h with Tat. The effect was dose-dependent and saturable with a maximal effective concentration of 0.1 nM (Fig. 2). The magnitude of the maximal inhibitory effect induced by Tat was comparable to that observed with the maximal effective dose of phlorizin (70% vs. control cells). To investigate the specificity of Tat effects on glucose uptake, neutralization experiments were performed in the presence of specific anti-Tat antibodies. In this condition, the inhibitory effect of Tat on glucose uptake was almost totally abolished (Fig. 3). As Tat activates L-type Ca^{2+} channels, we also tested the effect of the specific L-type Ca^{2+} channels agonist BayK8644 on glucose uptake. BayK8644 alone did not significantly affect Caco-2 cells glucose uptake. However, it significantly inhibited the effect induced by Tat on glucose absorption (Fig. 3).

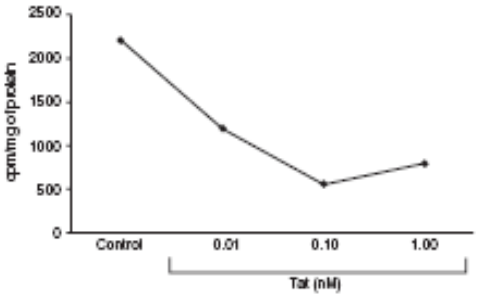

Fig. 2. Effect of increasing concentrations of Tat on non-metabolizable radiolabelled glucose analogous AMG uptake in Caco-2 cells. Data are means of six different observations for each data point.

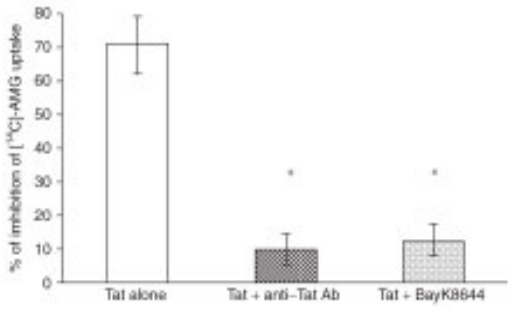

Fig. 3. Modifications of non-metabolizable radiolabelled glucose analogous AMG absorption in human enterocytes induced by Tat in various experimental conditions. Experiments were performed to investigate the involvement of Ca^{2+} (in the presence of Bay K8644), and the specificity (in the presence of anti-Tat antibody) of the effect of Tat in glucose transport. Values are expressed as the maximal percent of glucose transport inhibition in cells exposed for one hour to Tat versus control cells. Data are means ± SE. $^{*}P < 0.01$ versus Tat in standard conditions.

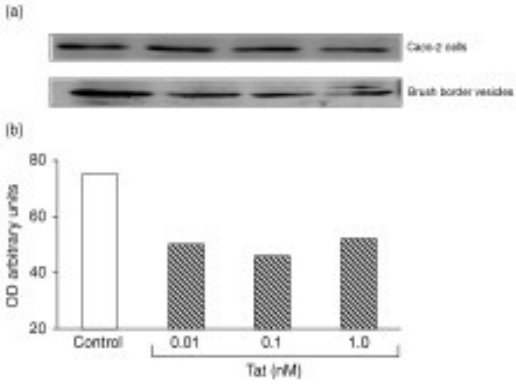

Fig. 4. Na^{+}-D-glucose symporter 1 (SGLT-1) protein expression in Caco-2 cells after 1 h of incubation with increasing concentrations of Tat. (a) The symporter expression is revealed by the appearance of a 75-kDa band that corresponds to human SGLT-1. SGLT-1 protein expression was sought in whole cells and in BBM vesicles to evaluate the delivery of the symporter to the apical surface of the enterocyte. (b) Densitometric quantification of the SGLT-1 band expression in BBM vesicles reported in (a). Results are representative of three repetitive experiments.

Western blotting analysis

To test whether the inhibitory Tat effect on glucose uptake was dependent on SGLT-1 expression, we studied the symporter expression either in whole Caco-2 cells and in BBM vesicle preparations. Total SGLT-1 protein intracellular expression was not affected by incubation with Tat concentrations up to 1.0 nM. On the contrary, incubation of Caco-2 cells with Tat resulted in a dose-dependent inhibition of SGLT-1 expression in BBM (Fig. 4). Maximum inhibition was obtained with 0.1 nM (corresponding to the concentration that induced the maximal effect on glucose uptake). These experiments suggested that Tat induces a missorting of the symporter to apical membrane.

Tat effect on α-tubulin staining

We analysed the effect of Tat on the cytoskeleton organization in Caco-2 cells using α-tubulin as a marker. Following a 1-h exposure to Tat, cells displayed a dramatic decrease in α-tubulin staining, consistent with substantial dysruption in cytoskeleton organization. The maximal effect was observed with 0.1 nM (Fig. 5).

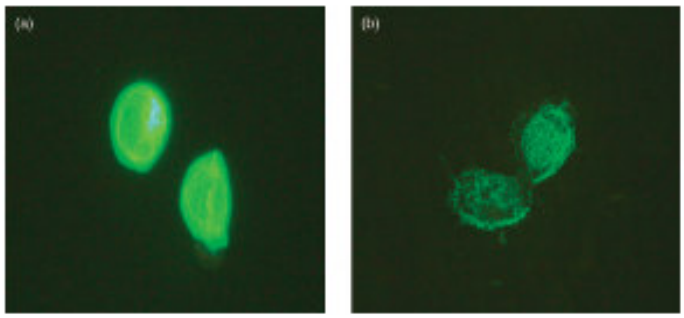

Fig. 5. Effect of Tat protein on α-tubulin staining in Caco-2 cells. Enterocytes were analysed by confocal microscopy at × 100 magnification. In these experiments microtubules appeared in green upon indirect immunofluorescence staining with an anti-α-tubulin monoclonal antibody and secondary fluorescein-conjugated anti-mouse IgG. When compared with controls (a), cells exposed to 0.1 nM Tat for 1 h displayed a marked decrease in α-tubulin labelling (b). Results are representative of three repetitive experiments.

Discussion

Glucose malabsorption is a major feature of the complex picture defined as intestinal dysfunction in HIV-1 infected children [3]. Our data indicate that Tat peptide directly impairs intestinal glucose absorption by inhibiting the SGLT-1 activity on enterocyte brush border. Decreased activity of this symporter may result not only in sugar malabsorption but also in diarrhoea, as SGLT-1 has the properties of a water channel [23]. Regardless of the route of transmission, HIV-1 selects CD4 cells that have surface receptors known as CCR5. The vast majority of CD4/CCR5 cells reside in the gut, which is considered a major target of HIV infection and replication and CD4 T-cell depletion predominantly occurs in the gastrointestinal tract [24]. It has been estimated that in the human small intestine, SGLT-1-mediated active fluid transport can account for as much as 5 l per day [23]. Our results support the concept of a direct etiologic role for Tat in the well-known pathogen-negative AIDS-related diarrhoea. Interestingly, the effects of Tat on SGLT-1 were dose-dependent with a maximal effective dose of 0.1 nM, which is well within the range of what generally measured in the sera of patients with HIV-1 infection [16]. Similar to the Tat effects on ion transport and on cell proliferation [16], also the inhibition of glucose uptake involves L-type Ca^{2+} channels as suggested by the experiments with the specific L-type Ca^{2+} channels agonist Bay K8644. Interestingly, Tat and Bay K8644, compete for binding to dendritic cells, which reinforces the concept that the effects of Tat on glucose uptake are L-type Ca^{2+} channel-dependent [25].

Microtubules are normally present in enterocytes and are important for intracellular transport [8]. Microtubule-disrupting drugs such as colchicine and nocodazole cause acute diarrhoea and missorting of several apical proteins in the enterocytes, including SGLT-1 [12,26]. Enteric microtubule depolymerization occurs in HIV-1 infected individuals [8]. In addition, α-tubulin staining was dramatically decreased in the intestinal HT29 cell line after exposure for 1 h to HIV-1, probably as a consequence of direct Gp120 action [12]. The decreased α-tubulin staining is consistent with a major change in cytoskeleton organization which, in turn, could lead to SGLT-1 missorting. The total SGLT-1 expression remained stable within the cell, whereas the symporter expression at BBM level was significantly decreased suggesting a functional rather than direct structural damage. The similar dose response profile of the effects exerted by Tat on ion and water transport, sugar absorption and cell structural damage suggest that Tat-induced enterocyte alterations occur via a single pathway. This pathway recalls that induced by the non-structural peptide 4 (NSP4) produced by Rotavirus [27]. Like NSP4, Tat is a protein capable of inducing Ca^{2+} dependent enteropathogenic and enterotoxigenic effects and of inhibiting glucose uptake by causing changes in the enterocyte cytoskeleton [16,27–30]. Such peptides are called 'virotoxins' [27]. Collectively, our previous findings on the Tat–enterocyte interaction [16] together with the results of the present study suggest that glucose malabsorption in AIDS patients results from the following cascade: 1) binding of Tat to plasma membrane of the enterocyte, 2) increase in intracellular Ca^{2+} concentration, 3) depolymerization of microtubules, 4) accumulation of transporting vesicles containing brush border proteins, 5) missorting of SGLT-1, which results in inhibition to glucose uptake. Calcium-dependent pathway is one of the four established intracellular signal transduction mechanisms leading to water and electrolyte secretion, the other three being cAMP, cGMP and Nitric oxide intracellular concentrations [31]. Following the increase in intracellular Ca^{2+} concentration and in parallel with structural damage, Tat also induces net Cl^- secretion [16]. The clinical manifestations of Tat effects are nutrient malabsorption and large volumes of diarrhoea, commonly observed in the advanced stages of HIV-1 infection. With the rapid turnover of intestinal cells, the acute impairment of the symporter by circulating Tat may induce constant damage to maturing enterocytes thereby causing chronic sugar malabsorption that is observed in patients with AIDS. Whether not all patients with high viral load have diarrhoea and/or intestinal malabsorption is not known. However, diarrhoea may not be evident in all patients with high circulating Tat due to the variable role of homeostatic pathway. However the observed restoration of intestinal digestive-absorptive functions, in parallel with the decrease of viral load (4), and the decreased risk of diarrhea in patients undergoing HAART support the direct involvement of HIV in both ion secretion and enterocyte damage [32].

There are at least two therapeutic implications of this work: first, the classic glucose-containing oral rehydration solution may be relatively ineffective for treatment of dehydration in these patients. This is supported by the clinical data reporting a high rate of parenteral rehydration in HIV-1-infected subjects [33]. Second it is of clinical relevance that all Tat effects on the enterocytes are substantially blocked by specific antibodies, which suggests that interdiction of extracellular Tat by active or passive immunization would reduce its pathogenic effects in the intestine.

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Guanylin and *E. coli* Heat-Stable Enterotoxin Induce Chloride Secretion through Direct Interaction with Basolateral Compartment of Rat and Human Colonic Cells

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ABSTRACT

We previously detected specific binding activity of *Escherichia coli* heat-stable enterotoxin (ST), the guanylin exogenous ligand, in rat colonic basolateral membranes. Because guanylin circulates in the bloodstream, we tested the hypothesis that it modulates intestinal ion transport by acting on the serosal side of intestinal cells. The effects of the mucosal and serosal addition of ST and guanylin on ion transport were investigated in the rat proximal colon and in Caco-2 cells in Ussing chambers, by monitoring short-circuit current (Isc). cGMP concentration was measured in Caco-2 cells by RIA. Mucosal ST addition induced an increase in Isc in rat proximal colon consistent with anion secretion. Serosal addition induced the same effects but to a lesser extent. The electrical effects observed in Caco-2 cells paralleled those observed in rat proximal colon. A pattern similar to the electrical response was observed with cGMP concentration. Guanylin addition to either side of Caco-2 cells induced the same effects as ST, although to a lesser extent. In all conditions, the electrical effect disappeared in the absence of chloride. ST directly interacts with basolateral receptors in the large intestine inducing chloride secretion through an increase of cGMP. However, the serosal effects are less pronounced compared with those observed with mucosal addition. Guanylin shows the same pattern, suggesting that it plays a role in the regulation of ion transport in the colon, but the relative importance of serosally mediated secretion remains to be determined. (*Pediatr Res* 58: 159–163, 2005)

Abbreviations
BBM, brush border membrane
BLM, basolateral membranes
CFTR, cystic fibrosis transmembrane regulator
GC-C, guanylyl cyclase C
Isc, short-circuit current
ST, heat-stable enterotoxin

ST elaborated by *Escherichia coli* and other bacteria are structurally related peptides that activate the transmembrane protein GC-C, located on the intestinal BBM of small and large intestinal enterocytes (1–3). ST binding to GC-C results in the generation of cGMP (4,5), which activates type-II cGMP-dependent protein kinase, leading to the phosphorylation of the CFTR and eventually results in chloride and bicarbonate secretion (6–8). The clinical manifestation of ST effects is diarrhea. This is of the secretory type and is particularly severe in younger infants (9).

Because the endogenous ligands of GC-C receptor, guanylin, and uroguanylin (10,11) activate the same pathway and cause an increase in Isc in intestinal epithelial cells, it has been suggested that they play a role in regulating intestinal fluid secretion. In most mammalian species, both peptides are produced predominantly in the intestine, although uroguanylin is also expressed in the kidney (12–14). Proguanylin and prouroguanylin are secreted into the intestinal lumen but they are also detected in the bloodstream as 11-kD prohormones and are each cleaved to the active 15 amino acid carboxy termini that bind GC-C (15–17). Circulating uroguanylin induces natriuresis, kaliuresis, and diuresis in isolated perfused rat kidney. It has been suggested that uroguanylin represents a gut-to-kidney signaling hormone that, upon ingestion of high-salt meals, causes natriuresis in anticipation of increased intestinal salt absorption (18,19). However, guanylin and uroguanylin may have other physiologic roles (20,21), including the acti-

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vation of a cGMP signal transduction pathway that may take part in the regulation of the turnover of epithelial cells by continuous replenishment of the epithelial cells (22,23).

ST is more potent than either guanylin or uroguanylin in stimulating intestinal secretion as judged by the effects on Isc of intestinal epithelium mounted in Ussing chambers. Because of these properties, ST is commonly used to study the GC-C–dependent secretory system in the rat and human intestine (24–28). Receptors for ST are located in the BBM. The addition of ST to the mucosal side of human colonic T84 cell stimulated active chloride secretion and the production of cGMP (26). Furthermore, ST showed similar binding kinetics in human intestinal and in colon-derived Caco-2 cells. Receptors for ST have been detected in the rat colon (28).

We have previously shown that ST binds specifically to highly purified BLM from rat colon (29). Because the endogenous ligands for GC-C, guanylin, and uroguanylin circulate in the blood, we hypothesized that guanylin or ST might be active when applied to the serosal surface of the colon.

To test this, we performed experiments with addition of ST to either side of rat colon mounted in Ussing chambers. We also used cell monolayers from human colon carcinoma cell line Caco-2. The experiments were repeated using guanylin to test our original hypothesis.

MATERIALS AND METHODS

Animals. Sprague-Dawley rats weighing 200–250 g were used. Animals were fed Purina rat chow and fasted 6 h before experiments but were allowed free access to water.

Cell growth and culture. The human intestinal epithelial cell line Caco-2 has been established from a moderately well-differentiated colon carcinoma. Caco-2 cells were grown in Dulbecco's modified Eagle minimum essential medium with high glucose concentration (4.5 g/L) at 37°C in 5% CO_2 atmosphere as previously described (30). The medium was supplemented with 10% FCS, 1% nonessential amino acids, penicillin (50 milliunits/mL), and streptomycin (50 μg/mL). The medium was changed daily.

Cells were grown on uncoated, nontransparent polycarbonate Transwell filters (0.4 μm pore size, 24.5 mm diameter; 2×10^6 cells were plated per filter). Cells were between the 50th and 70th passages and were used for intestinal transport studies 15 d after seeding when they formed a single layer of confluent cells connected by tight junctions and produce transepithelial electrical resistance (TEER) typical of polarized epithelial cells.

Intestinal ion transport studies in animals. Rats were killed by cervical dislocation and a 5-cm segment of proximal colon, 1–2 cm distal to the cecal-colonic junction, was rapidly removed and rinsed with ice-cold Ringer solution with the following composition: 53 mM NaCl, 5 mM KCl, 30.5 mM Na_2SO_4, 25 mM mannitol, 1.69 mM Na_2HPO_4, 0.3 mM NaH_2PO_4, 1.25 mM $CaCl_2$, 1.1 mM $MgCl_2$, and 25 mM $NaHCO_3$. Four paired fragments of unstripped colonic mucosa were mounted in Ussing chambers. In each experiment, one fragment served as control of baseline electrical parameters.

The solution was maintained at 37°C with water-jacketed reservoirs connected to a thermostated circulating pump and constantly gassed with 95% O_2/5% CO_2. Transepithelial potential difference (PD), Isc, and tissue ionic conductance (G) were measured as previously described (30). Isc is expressed as μA/cm^2, G as mS/cm^2, and PD as mV. Electrical parameters were recorded before and at various times after the addition of ST to the mucosal or the serosal side.

All animal experimentation described was approved by the Institutional Animal Care and Use Committee.

Intestinal ion transport studies in Caco-2 cells. Each filter was mounted as a flat sheet between the mucosal and serosal compartment of Ussing chambers. Each compartment contained 10 mL of Ringer's solution with the following composition: 114 mM NaCl, 5 mM KCl, 1.65 mM Na_2HPO_4, 0.3 mM NaH_2PO_4, 1.25 mM $CaCl_2$, 1.1 mM $MgCl_2$, 25.0 mM $NaHCO_3$, and 10.0 mM glucose.

ST or guanylin were added to either the mucosal or serosal side of the filter.

To see whether the electrical effect was consistent with anion secretion rather than cation absorption, experiments were performed, both in cells and rats, in Cl^--free Ringer's solution in which SO_4^- replaced Cl^- ions at equimolar concentration.

Cell and rat colonic fragment viability was assessed at the end of each experiment in paired controls by measuring the electrical response to serosal addition of 5 mmol of theophylline. An Isc increase of at least 3-fold compared with the preaddition value was considered proof of cell viability.

Determination of cGMP concentrations. After completing the Ussing chamber studies, the filter was rapidly removed from each chamber, transferred to ice-cold 5% trichloroacetic acid (TCA) and homogenized. The homogenate was centrifuged at 2000 *g* for 3 min at 4°C, and the supernatant was collected and evaporated to dryness under vacuum (Speed VAC 110, Savant Instruments, Farmingdale, NY). The dried sample was redissolved in 0.5 M acetate buffer, pH 5.8 with 0.01% sodium azide, and cGMP concentration was measured using a RIA commercial kit (cGMP ^{125}I assay system; Amersham Pharmacia Biotech UK, Ltd., Little Chalfont, Buckinghamshire, UK), according to the manufacturer's instructions. Results were calculated as picomoles of cGMP per square centimeter and expressed as fold increase over basal level.

ELISA assay for ST. The presence of ST in the fluid bathing the mucosal surface of epithelium after ST serosal addition was tested by an ELISA test with MAb raised against pure 18-amino acid *E. coli* ST as described elsewhere (31).

Chemicals. All chemicals, including ST and guanylin, were of reagent grade and were obtained from Sigma-Aldrich Italy (Milan, Italy); culture media were from Invitrogen (Milan, Italy). Transwell filters were from Costar (Costar Italia, Milan, Italy).

Statistics. Each experiment was run in duplicate and repeated at least four times. Results are expressed as means ± SD. Two-tailed, unpaired *t* test was applied to evaluate statistical significance. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Electrical effects of ST in rat colon. The addition of ST (10^{-6} M) to the mucosal side of rat colon induced a prompt increase of Isc that reached a maximum 2 min after addition and then slowly decreased toward baseline (Fig. 1). Isc increase was entirely related to a modification of PD inasmuch as no variations of G were observed.

The addition of ST to the serosal side also induced a rise of Isc that was very similar to that observed with mucosal addition but with a reduced Isc peak (Fig. 1). Both the mucosal and

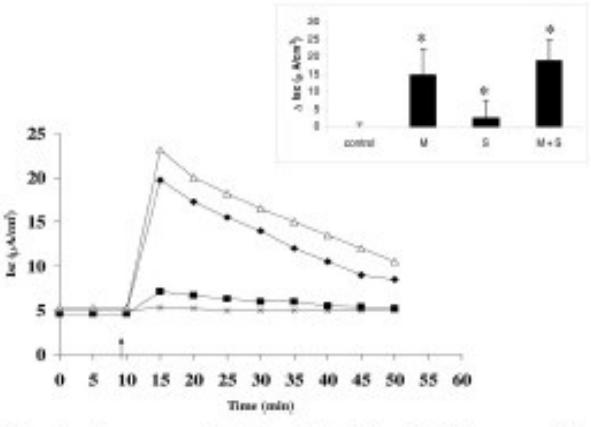

Figure 1. Time course of the effect of the addition of ST to the mucosal (◆) or serosal (■) compartments on Isc of rat colon mounted in an Ussing chamber. The addition of ST (10^{-6}M) to each side of colonic specimens induced a rapid increase in Isc. The simultaneous addition of ST to the mucosal and serosal (△) compartments of rat proximal colon in a concentration capable of inducing a maximal response induced no further increase in Isc. No increase in Isc was observed, in the absence of Cl^-, upon either the mucosal (×) or serosal (data not shown) addition of ST. Mean and SD of the peak effect of ST in at least four experiments is shown in the inset (*$p < 0.05$ *vs* controls).

serosal effects were dose dependent, and on both sides the maximal effect was observed with ST concentration of 10^{-6} M (data not shown). After determining the dose-response, ST was simultaneously added to both the mucosal and serosal compartments in the concentration capable of eliciting the maximal effect, and it did not correspond to the sum of those observed with ST addition to either side. A further Isc increase compared with the mucosal effect was consistently observed (Fig. 1). However, this increase did not reach the level of statistical significance.

All electrical modifications were inhibited in chloride-free buffer (Fig. 1).

Electrical effects of ST in Caco-2 cells. The addition of ST (10^{-6} M) to the mucosal side of Caco-2 cells induced an electrical response that was similar to that observed in rat proximal colon (Fig. 2). Half maximal effect was observed at a concentration of 5×10^{-7} (Fig. 3*A*). The Isc response was maximal at a ST concentration of 10^{-6} M. Higher concentrations of ST did not result in any further increase in Isc, indicating a saturation pattern of the effect (Fig. 3*A*).

The concentration of ST stimulating a maximal response in Isc was the same for both mucosal and serosal addition (Fig. 3*A*). The addition of ST to the serosal side induced a rise in Isc that was very similar to that observed with mucosal addition with regard to the time course. However, the potency of maximal electrical effect was approximately 25% compared with that observed in response to mucosal addition (Fig. 2). Also in this model, the simultaneous addition of ST to both the mucosal and serosal compartments induced an Isc peak response that was not significantly increased compared with that observed with mucosal addition. All electrical modifications were inhibited in chloride-free buffer (Fig. 2) .

Electrical effects of Guanylin in Caco-2 cells. The same experiments performed with ST were repeated using guanylin. The addition of guanylin (10^{-6} M) to the mucosal side of

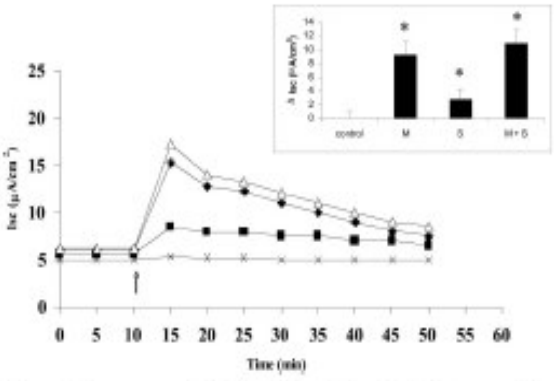

Figure 2. Time course of the effect of the addition of ST to the mucosal (◆) or serosal (■) compartments on Isc of Caco-2 cells mounted in an Ussing chamber. The addition of ST (10^{-6} M) (*arrow*) to each side of Caco-2 cells induced an increase in Isc that was similar to that observed in the rat model. The simultaneous addition of ST to the mucosal and serosal (△) compartments induced no further increase in Isc. No increase in Isc was observed, in the absence of Cl^-, upon either the mucosal (×) or serosal (data not shown) addition of ST. Mean and SD of the peak effect of ST in at least four experiments is shown in the inset (*$p < 0.05$ *vs* controls).

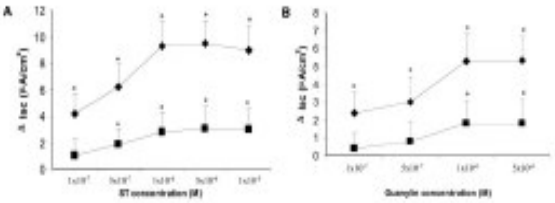

Figure 3. (*A*) Effect of increasing concentrations of ST on Isc of Caco-2 cells. The addition of ST to the mucosal (◆) or serosal (■) side resulted in a dose-dependent increase of Isc. There were four experiments per dose. *Statistically significant *vs* controls ($p < 0.05$). (*B*) Effect of increasing concentrations of guanylin on Isc of Caco-2 cells. The addition of ST to the mucosal (◆) or serosal (■) side resulted in a dose-dependent increase of Isc. There were four experiments per dose. *Statistically significant *vs* controls ($p < 0.05$).

Caco-2 cells induced an increase of Isc (data not shown) closely resembling that observed with ST. However, the potency of guanylin was approximately 50% of that observed with ST at equimolar concentrations.

Guanylin addition to the serosal side also induced a rise in Isc, whose magnitude was approximately 30% of mucosal effect. The concentration of guanylin stimulating the maximal response in Isc was the same for both mucosal and serosal addition and corresponded to 10^{-6} M (Fig. 3*B*). When guanylin at a concentration of 10^{-6} M was simultaneously added to both the mucosal and serosal compartments, the magnitude of the Isc response was greater than that observed with addition to the mucosal side. However, the difference did not reach statistical significance. Therefore, all the features of guanylin-induced secretion were similar to that of ST, although the potency of the secretory effect was reduced.

Effects of mucosal or serosal addition of ST or guanylin on cGMP concentration. As shown in Figure 4, ST and guanylin stimulated the production of cGMP in a dose-dependent manner in Caco-2 cells. Maximal ST-stimulated cGMP production was observed with an ST concentration of 10^{-6} M added to the mucosal compartment, resulting in a 14-fold increase in cGMP levels. Comparatively, the maximal cGMP increase upon ST serosal addition was 9-fold (Fig. 4*A*). When guanylin was added, the maximal increase of cGMP was reduced compared with the effect obtained with *E. coli* ST (Fig. 4).

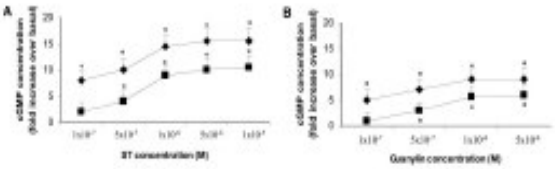

Figure 4. (*A*) Effect on cGMP concentration of the addition of ST to the mucosal (◆) or serosal (■) side of Caco-2 cells monolayers. ST induced an increase of cGMP concentration in a dose-dependent manner. The maximal increase was observed with 10^{-6} M of ST added to the mucosal or serosal compartment. There were four experiments per dose. *Statistically significant *vs* controls ($p < 0.05$). (*B*) The effects of guanylin showed the same pattern observed with ST. The magnitude of toxin effect was higher than that of its endogenous ligand. There were four experiments per dose. *Statistically significant *vs* controls ($p < 0.05$).

The simultaneous addition of ST or guanylin in a concentration of 10^{-6} M to both sides of the Caco-2 cells induced a slight further increase in cGMP, not statistically different from that observed upon mucosal addition (Fig. 5).

Therefore, the mucosal or serosal addition of ST or guanylin was associated with an increase of cGMP concentration that showed a dose-response pattern that was similar to that observed in Ussing chamber experiments.

ELISA Assay for ST. The fluid bathing the mucosal surface of epithelium mounted in Ussing chambers was collected after ST addition on the serosal side. It produced an ELISA negative ST, indicating that there was no transepithelial flux of the toxin (data not shown).

DISCUSSION

Our data show that ST is active when added to the serosal side of rat colon or Caco-2 cells and that its effect is similar to that induced by mucosal addition, although less potent. We used the rat proximal colon to perform our initial experiments because we had previously detected ^{125}I-ST binding activity in basolateral membranes purified from this intestinal segment (29). The time and kinetic features of the electrical effects observed in the present work were similar to those detected with mucosal ST addition and consistent with anion secretion. The complete loss of the effect in the absence of chloride supports this interpretation.

ST effects and its mechanisms were further investigated in Caco-2 cells, a well-established model to study intestinal secretion. The electrical response was similar to that observed in the animal model, suggesting that serosally applied ST works by a similar pathway compared with that stimulated by apical addition. We also investigated its mechanisms and comparatively examined the effects of mucosal and serosal ST addition. The dose of ST capable of inducing the maximal increase in Isc on each side was identical. However, when maximal concentrations of ST were simultaneously added to the apical and basolateral compartments, the observed increase in Isc was not different from that observed with ST mucosal addition alone. This indicates that the pathway of ST is the same, independent of the route of ST access to the enterocyte. This is strongly supported by a cGMP determination that showed a pattern identical to electrical data. Together with our data on the specific binding activity in BLM of rat proximal colon (29), this suggests that the same secretory pathway involved in mucosal ST response is activated by a basolateral stimulus. However, the magnitude of both the electrical response and the increase in cGMP upon serosal addition were reduced compared with that induced by mucosal addition. This is consistent with our finding that the specific binding in BBM of rat proximal colon was higher than in BLM, suggesting a decreased receptor density in the latter (29). The similarity in the dose-response effect to mucosal or serosal ST addition does not fit with this interpretation. However, a broad range of ST concentrations was used and it is possible that minor kinetic differences, such as a different response with double ST concentrations, were not identified. Alternatively, because the maximal ST concentration was the same on either side but the effect was different, the possibility of a different receptor-effector coupling in the BLM may explain the observed results.

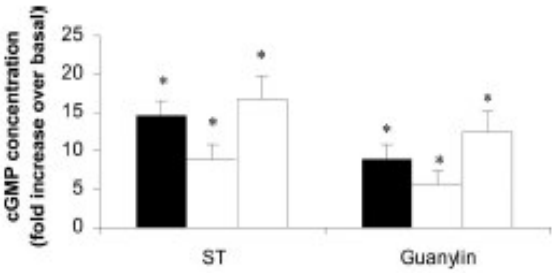

Figure 5. Effects on cGMP concentration of the addition of ST (*left side*) and guanylin (*right side*) to the mucosal (■) and serosal (□) side and to both sides (⊡) of Caco-2 cell monolayers. The simultaneous addition of ST or guanylin at concentration of 10^{-6} M to both sides of the Caco-2 cells induced a further increase in cGMP concentration that did not reach the level of statistical significance compared with that observed upon mucosal addition. *Statistically significant *vs* basal ($p < 0.05$).

Our finding of a response to serosal ST addition differs from that reported by others (27,32–34). We have performed experiments to look for ST in the fluid bathing the mucosal surface of epithelium, after serosal ST addition. A transepithelial serosa-to-mucosa ST flux might have explained the observed results. We did not find any evidence of transepithelial flux of ST. We cannot explain why our data differ from that published by others other than the fact different cells and animal species were used.

Guanylin induced the same effects of ST, although its potency was reduced. A similar finding of a reduced secretion induced by a mucosal guanylin addition compared with ST has been reported previously (34). We observed the same difference in potency between the endogenous and exogenous GC-C ligands with the serosal application. Overall, our data therefore support the existence of a ST/guanylin-dependent activation pathway *via* the serosal route. The two ST-like endogenous ligands produced in the intestine, guanylin and uroguanylin, are released into the blood and may exert their effects on the serosal side of polarized enterocytes. Guanylin and uroguanylin are, in fact, secreted into the intestinal lumen as well as into the bloodstream in response to sodium chloride administration and may regulate ion and water transport in the intestine and kidney by luminocrine and endocrine actions (12–14).

An active role of the enterocyte in ion transport has been hypothesized (35). We have reported that the enterocyte responds to an enterotoxin-induced secretion through the activation of constitutive nitric oxide synthase functioning as a breaking force of ion secretion. The data in this article add to the concept that enterocytes play a major role in regulating ion transport. Guanylin and uroguanylin may regulate intestinal electrolyte homeostasis by acting on the serosal surface of the colonic mucosa as well as on the apical membrane of small intestinal enterocytes.

ST effector GC-C shows an age-related pattern and peak at the newborn stage (36). It may therefore be hypothesized that the ST/guanylin-receptor system could help prevent excessive growth and proliferation of potentially pathogenic microorgan-

isms through their washing out at an age in which the susceptibility to intestinal infection is highest.

Children with cystic fibrosis (CF) lack the CFTR anion channel and in these patients the colon is unresponsive to ST (37). The CF newborn infant has a high risk of intestinal obstruction due to impaction of meconium. We have previously shown that ST receptors have an age-related pattern, peaking in the first 3 d of life (38). This suggests that guanylin may have the additional specific function of promoting chloride secretion to allow meconium output.

In conclusion, the results of this work show that 1) ST and guanylin induce chloride secretion when added to the serosal side of intestinal epithelium; 2) the effect has similar kinetic features compared with mucosal addition, but is less potent; 3) the effect is not additive to that observed with mucosal addition, although a further minor increase in either Isc and cGMP is consistently observed; and 4) guanylin is less potent than ST as a secretagogue. Overall, the results of this work raise the hypothesis that the colon is a target organ for both the paracrine and the endocrine effects of guanylin, acting on either side of the epithelium to reduce absorption and induce net Cl^- secretion.

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MAJOR ARTICLE

Zinc Inhibits Cholera Toxin–Induced, but Not *Escherichia coli* Heat-Stable Enterotoxin–Induced, Ion Secretion in Human Enterocytes

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Background. Because zinc deficiency in malnourished children is associated with severe diarrhea, use of zinc supplementation has been proposed as an adjunct to oral rehydration. However, the effects of zinc on enterocyte ion transport are largely unknown. The objective of the present study was to investigate the effects of zinc on transepithelial ion transport under basal conditions and under conditions of enterotoxin-induced ion secretion.

Methods. Ion transport was investigated by monitoring electrical parameters in human intestinal Caco-2 cells that were mounted in Ussing chambers and exposed to increasing concentrations of zinc, both in the absence and presence of either cholera toxin (CT) or *Escherichia coli* heat-stable enterotoxin (ST). Intracellular cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) concentrations were also determined.

Results. The addition of zinc to the luminal or basolateral side of enterocytes induced a chloride-dependent, dose-related decrease in short-circuit current, indicating ion absorption. It also resulted in a substantial reduction in CT-induced ion secretion and in cAMP concentration. *E. coli* ST–induced ion secretion and cGMP concentration were not affected. Ion absorption peaked at 35 μmol/L zinc, whereas excess zinc load induced active ion secretion.

Conclusions. By causing a decrease in cAMP concentration, zinc directly promotes ion absorption and substantially reduces CT-induced, but not *E. coli* ST–induced, ion secretion.

Worldwide, infectious diarrhea is still associated with high morbidity and mortality in persons of pediatric ages. The death rate has been estimated to be as high as 2.5 million children every year, with almost all deaths occurring in malnourished children in developing countries. Besides rotavirus, the major causal agents of diarrhea are *Vibrio cholerae* and enterotoxigenic *Escherichia coli* [1]. Cholera toxin (CT) and *E. coli* heat-labile enterotoxin (LT) induce secretory diarrhea by causing an increase in cAMP concentration, whereas *E. coli* heat-stable enterotoxin (ST) does so by activating the guanylate cyclase/cGMP system [2]. The use of oral rehydration solution (ORS) for treatment of diarrhea has become widespread and has resulted in reduced mortality from dehydration, but ORS does not decrease diarrheal duration and stool output. An active search for agents that are capable of inhibiting intestinal fluid losses has been ongoing for >2 decades. Although a number of candidate drugs have emerged, none has found a place in the routine management of acute diarrhea. Several clinical trials in developing countries have indicated that zinc is effective in the prevention and treatment of diarrhea in children [3–7]. A meta-analysis concluded that zinc supplementation given with appropriate fluids and foods during acute diarrhea reduces the duration and severity of illness in children in developing countries [8].

Zinc is an essential trace element in humans; it is a known constituent of important metalloenzymes, is involved in major metabolic pathways and DNA synthesis, helps to maintain the integrity of biological membranes and ion channels, and plays a major role in intestinal physiological processes [9]. Because there are no zinc stores in the body, its bioavailability is determined by a balance among food intake, intestinal absorption, and losses through urine, skin, and the intestinal tract. Intestinal losses of zinc are substantially

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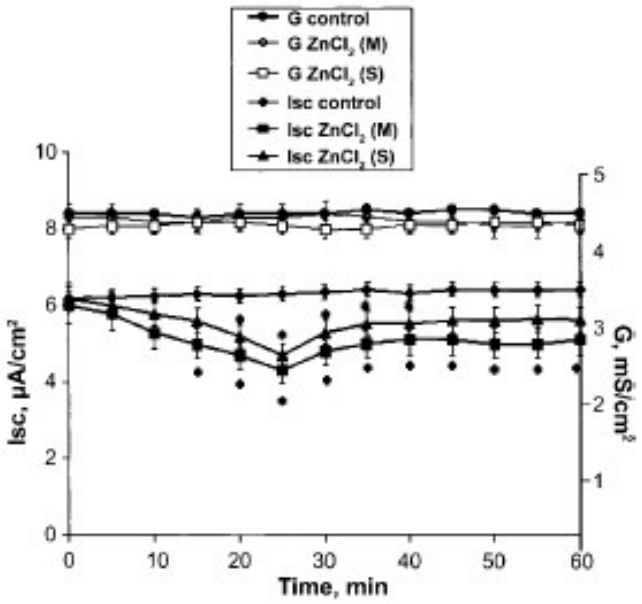

Figure 1. Time course of the effects of the mucosal (M) and serosal (S) addition of $ZnCl_2$ (35 µmol/L) on short-circuit current (Isc) and tissue ionic conductance (G) in Caco-2 cells mounted in Ussing chambers. The decrease in Isc induced by both the M and S addition of zinc indicates ion absorption. No effect on G values was observed. Each experiment was run in duplicate and was repeated at least 3 times. Results are expressed as means ± SD. *$P < .05$, vs. control. mS, millisiemens.

increased during diarrhea [10]. In zinc-deficient animals, CT-induced ion secretion is increased, compared with that in control animals, and secretion is reduced by zinc replenishment [11]. However, the mechanisms that link zinc deficiency with severe diarrhea, as well as the mechanisms that explain the efficacy of zinc in reducing diarrhea, are not clear. We therefore investigated the effects of zinc on transepithelial ion transport under basal conditions and under conditions of CT- and *E. coli* ST–induced ion secretion.

We used a well-established in vitro model that is based on the human epithelial intestinal cell line Caco-2, which is capable of zinc uptake [12]. This model has been validated in recent studies that investigated the effects of enterotoxins and their antagonists [13–15].

MATERIALS AND METHODS

Transepithelial ion transport experiments. Caco-2 cells were grown on uncoated polycarbonate transwell filters and were used in intestinal ion transport experiments 15 days after confluence, as described elsewhere [15]. The filter area was 4.9 cm^2. Each filter was mounted in an Ussing chamber (WPI) as a flat sheet between the mucosal and the serosal compartments. Each compartment contained 5 mL of Ringer's solution with the following composition: NaCl (114 mmol/L), KCl (5 mmol/L), Na_2HPO4 (1.65 mmol/L), NaH_2PO4 (0.3mmol/L), $CaCl_2$ (1.25 mmol/L), $MgCl_2$ (1.1 mmol/L), $NaHCO_3$ (25 mmol/L), and glucose (10 mmol/L); the buffer was constantly gassed with 5% CO_2–95% O_2 and was maintained at 37°C. The following electrical parameters were measured as described elsewhere [16], both before and after mucosal or serosal addition of $ZnCl_2$ and in either the presence or absence of CT or *E. coli* ST: transepithelial potential difference (PD), short-circuit current (Isc), and tissue ionic conductance (G). Isc is expressed as microamperes per square centimeter, and G is expressed as millisiemens (mS) per square centimeter. Cell viability was checked by measurement of the electrical response to the serosal addition of theophylline (5 mmol/L) at the end of each experiment. In experiments performed to investigate the role played by Cl^- in the zinc-induced electrical response, SO_4^- was substituted for Cl^- at an equimolar concentration.

Determination of intracellular concentrations of cyclic nucleotides. After the Ussing chamber experiments were completed, each cell-containing filter was rapidly removed, transferred to ice-cold 5% trichloroacetic acid, and homogenized. The homogenate was centrifuged at 2000 *g* for 3 min at 4°C, and the supernatant was collected and evaporated to dryness under vacuum (Speed VAC 110; Savant Instruments). The dried sample was redisolved in 0.5 mol/L acetate buffer (pH 5.8) with 0.01% sodium azide, and cAMP concentrations were determined by a radioimmunoassay (Biotrak cAMP assay system; Amersham International). cGMP concentrations were measured by use of a commercial radioimmunoassay kit (cGMP ^{125}I assay system; Amersham International), in accordance with

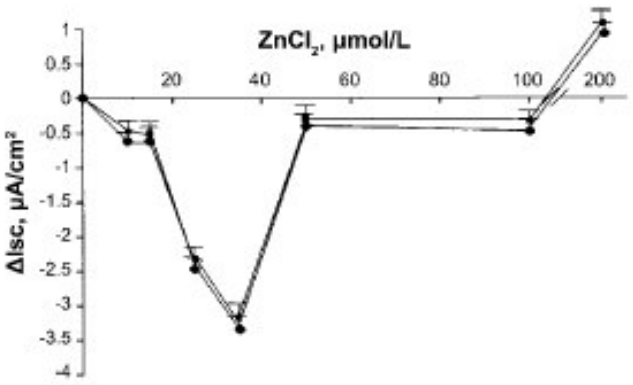

Figure 2. Changes in short-circuit current (Isc) in response to the mucosal (●) or serosal (■) addition of $ZnCl_2$ in increasing concentrations in Caco-2 cells mounted in Ussing chambers. Isc values are expressed as the difference (Δ) between measurements in cells exposed to $ZnCl_2$ for 60 min and measurements in untreated control cells. $ZnCl_2$ induced a dose-dependent decrease in Isc, which peaked at 35 µmol/L. The effect decreased at higher concentrations. A toxic concentration (200 µmol/L) of $ZnCl_2$ induced an increase in Isc to a value above that of the untreated control cells, indicating ion secretion. Each experiment was run in duplicate and was repeated at least 3 times. Results are expressed as means ± SD.

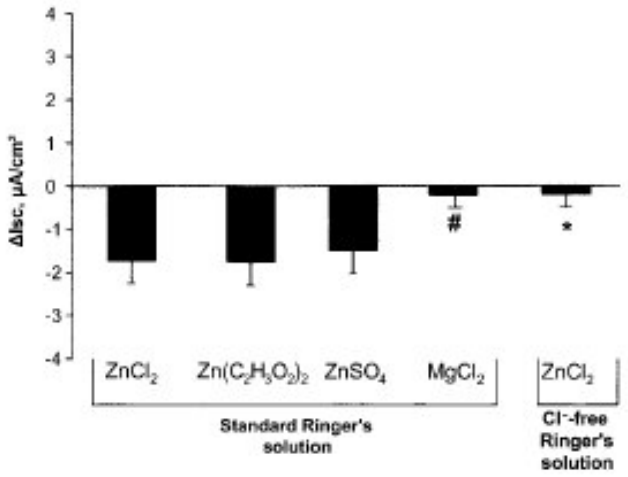

Figure 3. Comparative effects of $ZnCl_2$, $Zn(C_2H_3O_2)_2$ (zinc acetate), $ZnSO_4$ (zinc sulfate), and $MgCl_2$ on short-circuit current (Isc) in Caco-2 cells mounted in Ussing chambers. To test the hypothesis that the proabsorptive effect of $ZnCl_2$ was related to zinc ions, cells were probed in parallel with $ZnCl_2$, zinc acetate, zinc sulfate, and $MgCl_2$. A decrease in Isc was observed with all 3 zinc compounds but not with $MgCl_2$, indicating that zinc was directly responsible for the observed electrical changes. To test the hypothesis that Cl^- transport is the target of zinc, the experiments were performed in Cl^--free Ringer's solution, and no electrical effects were observed. Each experiment was run in duplicate and was repeated at least 3 times. Results are expressed as means ± SD. #$P < .001$, for $MgCl_2$ vs. $ZnCl_2$ and $MgCl_2$ vs. $Zn(C_2H_3O_2)_2$; *$P < .001$, for $ZnCl_2$ in Cl^--free Ringer's solution vs. $ZnCl_2$ in standard Ringer's solution. Δ, difference between measurements in untreated control cells and cells exposed to the substances for 60 min.

the manufacturer's instructions. Results are expressed as picomoles of cGMP per square centimeter.

Reagents and cell culture. Chemicals were obtained from Sigma Chemical. Culture medium was obtained from Life Technologies GIBCO BRL. Transwell filters and supports were obtained from Costar. Caco-2 cells were obtained from the American Type Culture Collection. Cells were grown in Dulbecco's modified Eagle medium that had a high glucose concentration (4.5 g/L) and that was supplemented with 10% fetal calf serum, 1% nonessential amino acids, penicillin (50 mU/mL), and streptomycin (50 mg/mL) and were incubated in 5% CO_2–95% air. The medium was changed daily.

Statistical analysis. Each experiment was run in duplicate and was repeated at least 3 times. Results are expressed as means ± SD. Significance was evaluated by use of the nonparametric 2-tailed Mann-Whitney U test. $P < .05$ was considered to be significant. The SPSS software package for Windows (version 12.0.1; SPSS) was used for statistical analysis.

RESULTS

Effects of Zinc on Transepithelial Ion Transport in Caco-2 Cells

The addition of $ZnCl_2$ at a final concentration of 35 μmol/L to the mucosal side of Caco-2 cell monolayers mounted in Ussing chambers induced a decrease in Isc entirely due to an effect on PD, without affecting G values. The lowest peak was observed 25 min after the addition of $ZnCl_2$ (figure 1). The addition of $ZnCl_2$ to the serosal side induced a decrease in Isc entirely similar to that observed when $ZnCl_2$ was added to the mucosal side, although the magnitude of the response was slightly reduced (figure 1). The effect on Isc was dose dependent; it was detected at a $ZnCl_2$ concentration as low as 10 μmol/L, peaked at 35 μmol/L, and decreased at higher concentrations. To investigate the effects of excess zinc concentrations, cells were loaded with 200 μmol/L $ZnCl_2$. The supraphysiological concentration was based on previous results on the cytotoxic effects of zinc in Caco-2 cells [17]. An ion-secretion pattern was observed in response to zinc overload, as evidenced by the increase in Isc (figure 2).

The same experiments were repeated in Cl^--free buffer. Under these conditions, neither the mucosal nor the serosal addition of $ZnCl_2$ induced changes in Isc. These findings suggest that the changes in Isc observed in the first experiments involved transepithelial Cl^- movement.

To determine whether the proabsorptive effect of $ZnCl_2$ was specifically related to zinc ions, we performed the same experiments in parallel with $ZnCl_2$, zinc acetate, zinc sulfate, and $MgCl_2$. The addition of zinc acetate or zinc sulfate to the mucosal side induced a decrease in Isc entirely similar to that observed when $ZnCl_2$ was added to the mucosal side. In contrast, the addition of $MgCl_2$ at an equimolar concentration had no effect on the electrical parameters, indicating that the proabsorptive effect was selectively related to zinc (figure 3).

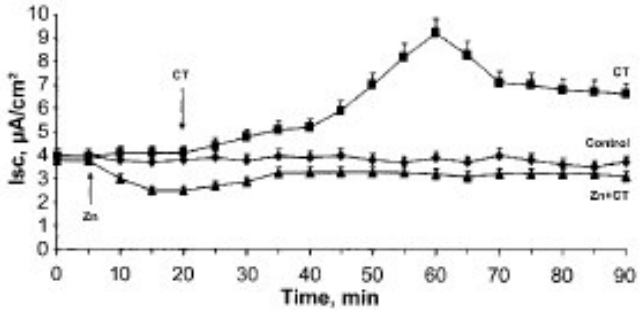

Figure 4. Time course of the effect of cholera toxin (CT), alone or in the presence of $ZnCl_2$, on short-circuit current (Isc) in Caco-2 cells mounted in Ussing chambers. The arrows indicate the time of addition of each agent. CT induced an increase in Isc, consistent with ion secretion. The latter was virtually abolished in the presence of $ZnCl_2$ (35 μmol/L). Preload with $ZnCl_2$ was associated with a decrease in Isc, consistent with ion absorption, which was followed by a modest increase in Isc after addition of CT, reaching the baseline values for the untreated control cells. Each experiment was run in duplicate and was repeated at least 3 times. Results are expressed as means ± SD.

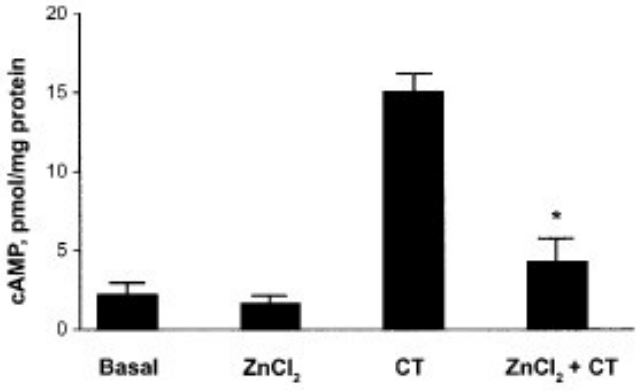

Figure 5. Changes in intracellular cAMP concentration in Caco-2 cells after a 1-h incubation with $ZnCl_2$, cholera toxin (CT), or both. A modest decrease in cAMP concentration was observed in the presence of $ZnCl_2$, whereas CT induced a marked increase in cAMP concentration. The CT-induced increase in cAMP concentration was substantially reduced in the presence of $ZnCl_2$. Data are the means ± SD of 3 different observations. *$P < .001$, for CT alone vs. $ZnCl_2$ + CT.

Effects of Zinc under Conditions of Active Secretion

CT-induced ion secretion. To investigate the effects of zinc under conditions of CT-induced ion secretion, Caco-2 cell monolayers were exposed to the maximal effective dose of CT (6×10^{-8} mol/L), which was added to the mucosal side in the absence or presence of $ZnCl_2$. As shown in figure 4, preincubation with $ZnCl_2$ at its maximal effective concentration resulted in the complete inhibition of CT-induced ion secretion.

Because CT induces intestinal Cl^- secretion by causing an increase in intracellular cAMP concentration [2], we investigated the effect of zinc on this intracellular cyclic nucleotide concentration. To do this, we measured intracellular cAMP concentrations before and after exposure of the cell monolayers to $ZnCl_2$ (35 μmol/L) and CT (6×10^{-8} mol/L), both alone and in combination. Basal cAMP concentration was slightly reduced by the addition of $ZnCl_2$; however, $ZnCl_2$ was effective in substantially inhibiting the increase in intracellular cAMP concentration induced by CT (figure 5). Therefore, similar to what was observed in the ion-transport experiments, the effect of zinc on intracellular cAMP concentration was much more evident under conditions of active secretion than under basal conditions.

E. coli ST–induced ion secretion. To investigate whether zinc is effective in inhibiting *E. coli* ST–induced ion secretion, Caco-2 cells were exposed to 10^{-7} mol/L *E. coli* ST, the maximal effective ST concentration [18]. The addition of $ZnCl_2$ did not modify the increase in Isc induced by *E. coli* ST. In addition, $ZnCl_2$ did not affect either the basal or the *E. coli* ST–induced intracellular cGMP concentration (figure 6).

DISCUSSION

We have obtained evidence that zinc promotes ion absorption through a direct effect on enterocytes. The zinc-induced decrease in Isc is consistent with an increased flux of anions from the mucosal to the serosal side of enterocytes, as a consequence of their increased absorption or decreased secretion. The negation of the Isc response observed in the experiments with Cl^--free Ringer's solution indicates that Cl^- transport is the target of zinc. Because the same response was obtained with different zinc salts but not with $MgCl_2$, the absorptive effect must be entirely zinc specific.

Zinc was able to stimulate ion absorption after addition to either the mucosal or serosal side of epithelial monolayers. Several clinical and experimental data have shown that diarrhea is more severe in zinc-deficient subjects [19]. The absorptive effect induced by the serosal addition of zinc provides an explanation for the mechanism that allows zinc deficiency to be associated with severe diarrhea. The results of the present study are in agreement with previous results that showed decreased net water and electrolyte absorption in the small and large intestines of zinc-deficient rats [20]. The increased volume of stool observed in zinc-deficient children with infectious diarrhea [5–7, 19] may well be the consequence of a reduced intestinal basal absorptive tone and of a limited enterocyte compensatory absorptive capacity due to zinc deficiency.

However, the effects of zinc on intestinal ion transport, al-

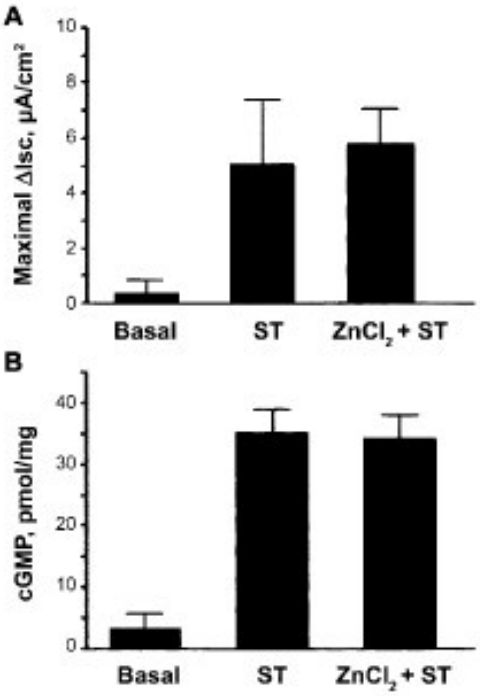

Figure 6. Effect of the addition of *Escherichia coli* heat-stable enterotoxin (ST), alone and in the presence of $ZnCl_2$, on short-circuit current (Isc) *(A)* and cGMP concentration *(B)* in Caco-2 cells. *E. coli* ST induced an increase in both Isc and cGMP concentration, neither of which was changed by the presence of zinc. Data are the means ± SD of 3 different observations. *$P < .001$, for ST alone vs. $ZnCl_2$ + ST.

though observed under basal conditions, were maximal under conditions of active ion secretion induced by CT. At the maximal effective concentration, zinc was effective in preventing virtually all CT-induced ion secretion, and its effect on intestinal ion transport was paralleled by changes in cAMP concentration. These findings are consistent with previous findings from an animal model that showed that zinc supplementation is able to reduce intestinal cAMP-dependent ion secretion induced by theophilline [21]. In addition, zinc-induced inhibition of cAMP production through a reversible inhibition of adenylate cyclase activity has been reported in neuroblastoma cells, suggesting that zinc plays a wider—and previously unidentified—role in the regulation of intracellular cyclic nucleotide concentration [22].

Enterocyte cAMP is the signaling molecule for CT and other heat-labile bacterial enterotoxins [2]. It is 1 of the 3 intracellular mediators of active ion secretion, the other 2 being cGMP and intracellular calcium concentration [2]. We have previously shown that cAMP plays a central role in the regulation of ion secretion in the enterocyte, in concert with NO released by the activation of constitutive NO synthase (cNOS). In the enterocyte, cNOS becomes activated in the presence of CT-induced ion secretion and decreases cAMP concentration [23]. Thus, there is a cNOS/NO/cAMP pathway acting in the enterocyte as a breaking force to limit active ion secretion such as that induced by CT, and cAMP is the target of the breaking force. In the present study, we found evidence that cAMP is also under the control of extracellular zinc through a direct interaction with the enterocyte. In contrast with the observed effect zinc had on the cAMP/CT pathway of intestinal secretion, zinc had no effect on *E. coli* ST–induced secretion and on its effector cGMP. However, we cannot rule out the possibility that *E. coli* ST–induced diarrhea may be more severe in zinc-deficient children or that zinc may exert some beneficial effect during *E. coli* ST–induced diarrhea in children. Nonetheless, it is possible to hypothesize that these zinc-related positive regulatory actions on intestinal fluid transport could be further reinforced in vivo by 1 or more of the previously observed inhibitory effects that zinc has on intestinal permeability, responses to histamine and serotonine, inducible NOS (iNOS) activity, and production of uroguanylin (UG) [24–26]. At least in part, these effects are related to zinc regulation of specific gene expression. Specifically, overexpression of both the UG and iNOS genes has been previously demonstrated in a zinc-deficient–animal model. Interestingly, repletion with zinc reversed up-regulation of the iNOS gene within 1 day, whereas 3–4 days of up-regulation of the UG gene was required to achieve normal concentrations; this suggests that the mechanisms of UG and iNOS gene dysregulation are different [27]. Dysregulation of these genes may contribute to the severity of zinc-responsive diarrheal disease, as well as to the severity of intestinal inflammatory diseases.

It is known that zinc should be used cautiously in children, because of the risk of overdose. Increased mortality has been reported in malnourished children receiving as much as 6 mg/kg/day of zinc [28]. In the present study, an increase in Isc was observed in response to excess zinc load, indicating that further ion secretion may be induced by administration of zinc and providing direct proof of the danger of the administration of excessive amounts of zinc. However, most clinical trials and meta-analyses have shown that, at lower doses (such as 1.5 mg/kg/day), zinc is safe and effective [4–8]. Here, we have demonstrated that zinc does affect basal ion transport when used in concentrations (10–22 μmol/L) that are within normal plasmatic ranges and are very similar to the plasmatic concentrations reported in clinical studies in patients with diarrhea [7, 8, 29]. Furthermore, we have demonstrated that different zinc salts exert the same effects on intestinal ion transport—this suggests that different zinc formulations could be successfully used in clinical practice.

In conclusion, the results of the present study have provided evidence that zinc has direct effects on enterocyte ion transport. Zinc promotes ion absorption and prevents active secretion induced by CT, with a direct effect on cAMP concentration. Although the addition of zinc does not affect cGMP-mediated ion secretion, zinc may still have a protective effect that is associated with its action on basal ion transport.

There is an ongoing debate on the efficacy and risks of the new universal ORS, whose formulation was released by the World Health Organization/UNICEF in 2002. The new ORS has a reduced sodium concentration and is recommended for treatment of adults and children with cholera and noncholera diarrhea. On the one hand, some scientists believe that, because of the reduced sodium concentration, there is an increased risk of hyponatremia in patients with cholera diarrhea [30]. On the other hand, hypoosmolar ORS may substantially reduce childhood deaths by reducing the need for intravenous fluids [31]. We are well aware of the immense benefits of having a universal ORS [32], and we suggest that zinc should be considered as one of its components.

Acknowledgment

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Management of Gastrointestinal Disorders in Children with HIV Infection

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Contents

Abstract

A double scenario characterizes the epidemiology of HIV infection in children. In countries where highly active antiretroviral therapy (HAART) is available, the pattern of HIV infection is evolving into that of a chronic disease, for which control strictly depends on patients' adherence to treatment. In developing countries with no or limited access to HAART, AIDS is rapidly expanding and is loaded with a high fatality ratio, due to the combined effects of malnutrition and opportunistic infections.

The digestive tract is a target of the disease in both settings. Opportunistic infections play a major role in children with severe immune impairment, with *Cryptosporidium parvum* being the leading agent of severe diarrhea. Several therapeutic approaches are effective in reducing fecal output, but the eradication of the parasite is rarely obtained. Other opportunistic infections may induce severe and protracted diarrhea, including atypical mycobacteria and cytomegalovirus. Diagnosis of diarrhea should be individually tailored based on presenting symptoms and risk factors. A stepwise approach is effective in limiting patient discomfort and minimizing the costs of investigations, starting with microbiologic investigation and proceeding with endoscopy and histology. Aggressive treatment of infectious diarrhea is required in severely immunocompromised children. However, antiretroviral therapy prevents the development of severe cryptosporidiosis.

The liver and pancreas are also target organs in HIV infection, although functional failure is rare. The digestive-absorptive functions are impaired, with steatorrhea, nutrient malabsorption, and increased permeability occurring in 20–70% of children. Intestinal dysfunction contributes to growth failure and further immune derangement, leading to wasting, the terminal stage of AIDS. Nutritional management is crucial in HIV-infected children and is based on aggressive nutritional rehabilitation through enteral or parenteral routes and micronutrient supplementation.

HIV may play a direct enteropathogenic role and is implicated in both diarrhea and intestinal dysfunction. This explains the efficacy of antiretroviral therapy in inducing remission of diarrhea and restoring intestinal function.

Gastrointestinal side effects of antiretroviral drugs are increasingly observed; they are often mild and transient. Severe reactions are rare but require the withdrawal of drugs.

In conclusion, severe enteric infections and intestinal dysfunction characterize the intestinal involvement of HIV infection. This is more common in, but not limited to, children who do not receive effective antiretroviral therapy. Diagnostic approaches include microbiologic and morphologic examinations and assessment of digestive processes, but immunologic and virologic data should be also carefully considered. Treatment is based upon specific anti-infectious drugs, antiretroviral therapy, and nutritional rehabilitation.

The main gastrointestinal problems of HIV-infected children are diarrhea, hepatic and/or pancreatic infections, and digestive-absorptive dysfunctions. All of these contribute to nutritional failure, which is the hallmark of the terminal stage of AIDS. In addition, children receiving HAART may experience gastrointestinal, hepatic, and pancreatic adverse effects.

In this article we review the main gastrointestinal problems of children with HIV infection and delineate their consequences on nutritional status. The approach to the child, including diagnosis and treatment of diarrheal diseases, is described. We also include indications for nutritional management, since this is crucial to the outcome of the disease. Finally, the intestinal adverse effects of HAART are discussed.

The content of this review is largely based upon data from pediatric studies, since HIV infection in children has several peculiarities that make this a distinct disease from HIV infection in adult patients. In instances where data from children were not available, adult data were used.

1. Background

1.1 The Current Status of AIDS Epidemics

HIV infection is a progressive viral disease characterized by immune impairment.[1] The so-called highly active antiretroviral therapy (HAART) is effective in inhibiting HIV replication and the associated progression of the disease and has dramatically modified the course of AIDS. HAART has been shown to be effective in reducing the morbidity and mortality associated with AIDS and the incidence and severity of opportunistic infections, prolonging the survival of most infected children.[2,3] Today, the global picture of AIDS presents a striking double face:[4] in rich countries, the incidence of new infections is under relative control, mainly due to the efficacy of preventive measures and the wide availability of HAART; in poor countries, where HAART is not accessible due to the lack of economic resources, the incidence of HIV is increasing at an alarming rate, and is associated with a high rate of death.

The clinical condition of children in different settings reflects this double scenario. Most children in rich countries have a fair clinical condition, under effective HAART, but some do not,

because of therapeutic failure due to viral resistance or poor adherence. In poorly adherent children, drug-resistant strains are rapidly selected causing a progressive course of HIV infection. In poor countries, the combination of HIV-induced immune derangement, enteric infections, and malnutrition produces lethal consequences as shown by the dramatic death rate observed in childhood.

The Centers for Disease Control and Prevention 1994 HIV classification scheme for children under 13 years of age includes clinical and immunologic criteria. There are three clinical classes (from A to C, which corresponds to the so-called full-blown AIDS) and three immunologic classes (from 1 to 3, which corresponds to severe immune impairment).[5] The classification scheme is designed to stage the disease and define the probability of a stable or progressive outcome. In addition, viral load can be quantitatively determined and has a prognostic meaning. Patient classification directs the choice of HAART. The AIDS dogma states that once an infected person has been classified as being in an advanced class, he/she remains in that class or proceeds to a further (worse) class. According to the classification, a child who has suffered from an opportunistic infection, would be classified in class C, even if full recovery was obtained and the recent history indicates immunologic and clinical improvement.

There is no universal agreement on when to start antiretroviral therapy.[6] Most clinicians support an aggressive approach, including the initiation of treatment in all children under 12 months of age and in those who show clinical evidence of the disease. Children without signs or symptoms, but in whom there is moderate or severe immune impairment are also candidates for treatment. Treatment usually consists of a combination of three drugs, including two inhibitors of viral reverse transcriptase and one inhibitor of viral protease.[6] Some clinicians support a more conservative therapeutic strategy and delay the initiation of therapy or start with a less aggressive treatment.

Due to the ability to prolong survival in HIV-infected children, the number of HIV-infected children is increasing, and their routine management is being transferred from reference centers to outpatient family physicians.

1.2 The Digestive Tract as a Target Organ in HIV Infection

The digestive tract is a major target for HIV infection. Children with severe immune impairment have a high rate of severe gastrointestinal problems, mainly due to opportunistic infections. On the other hand, children that are successfully receiving HAART may have intestinal abnormalities such as diarrhea or residual intestinal malabsorption. These may not be evident on clinical grounds, but may be harmful in the long term, particularly when nutritional deficiencies occur as a consequence of abnormal absorptive processes.

Although extremely severe conditions, such as intractable diarrhea and wasting, are no longer commonly seen in children on HAART, understanding the pattern of gastrointestinal diseases associated with full-blown AIDS is essential for the proper management of HIV-infected children. However, as HIV must be regarded as a persistently evolving problem, the reader should be aware that the pattern of disease in children with HIV infection, even in developed countries, is not always that of a young patient whose infection is completely controlled by HAART. Rather, there is a broad disease spectrum.

1.3 The Classical Pattern of Digestive Tract Involvement in Full-Blown AIDS

The major gastrointestinal manifestations of full-blown AIDS are diarrhea and weight loss. Malnutrition is a common condition at this stage of HIV infection and has a multifactorial origin (figure 1). Failure to thrive includes two distinct patterns: wasting and stunting. The former is characterized by low bodyweight for height or length and is an AIDS-defining condition, whereas the latter is characterized by an abnormal linear growth for age. In the initial phases of the disease, bodyweight is decreased. If malnutrition persists for a long time, usually more than 1 year, then linear growth becomes impaired.

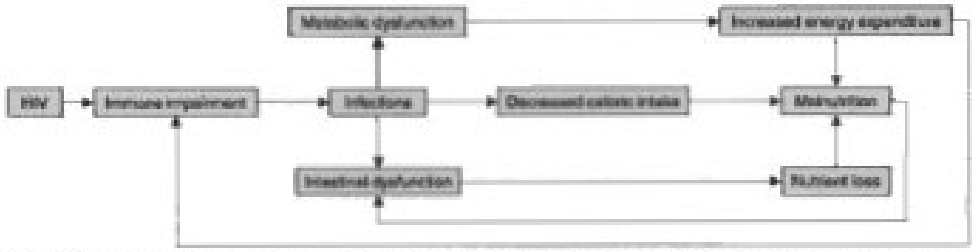

Fig. 1. Pathways of malnutrition in HIV-infected children. Malnutrition is the endpoint of several conditions. A complex interplay exists among these conditions. Severe infections frequently caused by opportunistic agents and intestinal dysfunction play a major role, contributing to wasting, an AIDS-defining condition.

HIV-associated malnutrition is defined by:[7]

- downward crossing of two major percentiles on the standard weight or height for age charts;
- loss of ≥1.4 z-score units;
- failure to follow a parallel growth curve, if the growth pattern is below the 5th percentile;
- loss of >5% of bodyweight;
- growth velocity below the 3rd percentile for at least 6 months;
- weight for height ratio under the 5th percentile for age.

2. Diarrhea

2.1 Epidemiology and Etiology of Infectious Diarrhea

In most HIV-infected children, diarrhea is the result of intestinal infections. These infections are a major problem, both in terms of frequency and severity. An increased incidence of diarrhea is observed in HIV-infected children compared with seronegative control individuals in countries where enteric infections are highly endemic, such as Africa.[8] The risk of diarrhea is also high in children with HIV infection from industrialized countries. The rate of diarrheal episodes was approximately 2-fold greater in HIV-infected children compared with immunocompetent control individuals, before HAART was introduced.[9] HAART has reduced the risk of opportunistic infections in adults.[10] However, the estimated risk of diarrhea in a healthy immunocompetent child living in a country with high hygiene and socioeconomic standards is in the range of 0.5–1.5 episode/child/year in the first 3 years of life.[11] Thus, the probability of intestinal infections is high in infants and younger children, independently of whether they are HIV infected or not.

In addition to frequency, the duration and severity of diarrhea are increased in HIV-infected children. Chronic diarrhea is a well recognized feature of AIDS in Africa and it has been reported in as many as 90% of HIV-positive children; its incidence is 6-fold higher than in HIV-negative control individuals.[12] Similarly, in industrialized countries, diarrhea tends to be more prolonged and severe in children with HIV infection. This is largely due to enteric cryptosporidiosis, although the use of HAART has been shown to substantially reduce this risk.[13]

Classic and opportunistic enteropathogens may induce diarrhea in HIV-infected children. The former may cause severe gastroenteritis that is unusual in immunocompetent children. Opportunistic agents are either not or only slightly harmful to healthy individuals, but may be responsible for severe diarrhea in immunocompromised children. The list of opportunistic enteric agents is rather long, but the role of individual agents largely depends on geographical location. Worldwide, the major role belongs to *Cryptosporidium parvum*.[14] Other frequent opportunistic enteropathogens in Western countries are cytomegalovirus and atypical mycobacteria (*Mycobacterium avium* and *M. intracellulare*). The other most frequent agent of diarrhea in HIV-infected individuals, the parasite *Enterocytozoon byeneusi* or microsporidium, seems to be restricted to the adult population; it has been found in the stools of HIV-infected children with normal stool patterns. In African children, in addition to *C. parvum*, other opportunistic agents are *Isospora belli*, *Cyclospora cayetanensis*, and *Schistosoma duodenalis*. Bacterial agents such as enteroaggregative *Escherichia coli* have occasionally been reported.[15]

2.1.1 Enteric Cryptosporidiosis

In immunocompetent children, *C. parvum* induces mild and self-limiting gastroenteritis. In contrast, in HIV-infected children, it causes severe, dehydrating diarrhea, associated with massive fluid loss. Enteric cryptosporidiosis is a leading cause of death in poor countries. The risk of infection is closely associated with impaired immunity, namely low CD4+ cell counts. The severity of symptoms is associated with the oocyst number.[16] Thus, children with severe immune impairment, labeled as class 3 according to the Centers for Disease Control and Prevention classification scheme,[5] are at the highest risk for severe cryptosporidiosis.

The pathophysiology of cryptosporidiosis involves multiple mechanisms.[17] An enterotoxic activity has been detected in the stools of HIV-infected children with cryptosporidiosis, which is responsible for intestinal secretion of electrolytes and water.[18]

Diarrhea is often watery and has been described as cholera-like and persists in children who are not eating.[19] Abdominal pain is a frequent feature, but vomiting is rare. Generally, anorexia is associated with enteric cryptosporidiosis, but it is not clear whether the anorexia is specifically associated with the parasite infestation or is dependent on the advanced stage of HIV infection. Fulminant disease leading to death within 5 weeks has been reported in adult patients with profound immune impairment.[17] Enteric cryptosporidiosis may also present with chronic diarrhea and relapsing illness.

2.1.2 Bacterial Diarrhea

Several bacterial agents may induce diarrhea, which tends to be more protracted and severe in both adults and children than in immunocompetent individuals.[20] There is no specific hallmark of a bacterial etiology of diarrhea, nor is it easy to suspect a specific bacterial agent based on history and clinical findings.

In children with persistent diarrhea, small bowel bacterial overgrowth should be considered. This has been observed in adult patients and was associated with gastric hypochlorhydria.[20] However, there are no similar data in children. We performed glucose

breath tests in 15 HIV-infected children with pathogen-negative diarrhea; there was no evidence of bacterial overgrowth.

2.1.3 Rotavirus

Rotavirus is the most frequent cause of acute gastroenteritis in children worldwide and one of the most frequent causes of death of children in developing countries.[21] In children with HIV infection, rotavirus can be more severe than in immunocompetent children. However, a study in Malawian children showed that there were no differences in the acute phase of diarrhea between HIV-positive and HIV-negative children admitted for acute rotavirus infection, but the former had an increased risk of death during follow-up and virus shedding tended to be protracted, although it was not associated with diarrhea.[22] We have observed life-threatening rotavirus-associated diarrhea in three HIV-infected children, which is highly unusual in immunocompetent children.[17]

2.1.4 Cytomegalovirus

Cytomegalovirus may act as an opportunistic enteric agent inducing severe colitis or enterocolitis or even an intractable diarrhea syndrome in severely immunocompromised children. In six of eight children with HIV infection, cytomegalovirus has been associated with an increased risk of massive gastrointestinal bleeding and bowel perforation with a high fatality ratio.[24]

2.1.5 Atypical Mycobacteria

Atypical mycobacteria (particularly *M. avium* and *M. intracellulare*) have been implicated in diarrhea in HIV-infected patients, including children,[25,26] but they may be found in children with solid stools.

2.1.6 HIV-Induced Diarrhea

In a substantial number of cases, ≥50% depending on the diagnostic approach, an etiologic agent is not detected in children with diarrhea.[9,27] The hypothesis that HIV is directly capable of causing diarrhea has often been raised. Sequences of retrovirus have been detected in the stools of pathogen-negative children with diarrhea; however, a close relationship with diarrhea was not detected in case-controlled studies.[28]

We have recently reported the results of *in vitro* studies, showing that the transactivating transfer factor (TAT), produced by HIV-1, directly interacts with the enterocyte, inducing chloride secretion through a calcium-mediated mechanism, a finding consistent with secretory diarrhea.[29] Interestingly, TAT levels that induced the maximal diarrheogenic effect corresponded to levels detected in the sera of AIDS patients. Electrolyte secretion was inhibited in the presence of specific anti-TAT antibodies. These results provide strong support to the enteropathogenic role of HIV and may explain the origin of pathogen-negative, watery diarrhea that is often seen in patients with full-blown AIDS.

2.2 Diagnosis of Acute-Onset Diarrhea

In HIV-infected children with mild-to-moderate immune suppression, acute-onset diarrhea is often self-limited and does not require specific diagnostic evaluation or treatment. In such cases, management may be that recommended for immunocompetent children with mild-to-moderate diarrhea, which is based on oral rehydration and early refeeding with full-strength milk or formula.[11] In contrast, in children with severe immune impairment or at risk due to other conditions (such as malnutrition), investigations should be always initiated at an early phase with microbiologic examination of stool samples. The American Gastroenterological Association has produced a technical review, recommending a stepwise approach to managing adult HIV-infected patients with diarrhea.[30] The basic principles of this approach include the high rate of opportunistic infections in individuals with severe immune impairment, the substantial overlapping of the diagnostic yield by flexible sigmoidoscopy compared with the more invasive and expensive colonoscopy, and the superiority of endoscopic over radiographic techniques. The advantage of such an approach is its cost/efficacy and limited discomfort to the patient. The stepwise approach designed for adults may be applied to HIV-infected children, with some modifications that take into account the specific pattern of agents causing diarrhea in children and the low risk of intestinal neoplastic diseases compared with adults.

The immune status of the child should be considered when planning the diagnostic approach, and diagnosis should be more rapid and aggressive in severely immunocompromised children. Initial microbiologic investigation of stool samples should always include a specific search for salmonella, shigella, campylobacter, rotavirus, enteric adenoviruses, and *C. parvum*. Blood culture may increase the yield of bacterial agents (table I).

C. parvum should be specifically investigated for by immunofluorescence, as a screening test, and positive samples should be confirmed by Giemsa and finally by modified Ziehl Neelsen or auramine stain.[14,17] At least three different stool samples should be analyzed.

Antibiotic associated-diarrhea should be considered as children with HIV infection are often receiving a heavy antibiotic load. Toxin-producing *Clostridium difficile* is generally the cause of antibiotic-induced diarrhea.

Small round viruses, astroviruses, picobirnaviruses, and other enteroviruses have been associated with diarrhea in HIV-infected patients. The number of enteric viruses detected by electron microscopy in fecal samples obtained from a population of 47 HIV-

Table I. Diagnostic workup for children with diarrhea[36]

Step 1	Stool tests	Bacterial culture and viral investigation
		Ova and parasite examination
		Clostridium difficile toxin assay
		Modified acid-fast stain and immunofluorescence for cryptosporidia
Step 2	Flexible sigmoidoscopy with mucosal biopsies	Light microscopy (mycobacteria, CMV, cryptosporidia)
		Mycobacterial culture (mycobacteria)
Step 3	Upper endoscopy with duodenal biopsies/total colonoscopy	Light microscopy (CMV, mycobacteria, cryptosporidia)
		Mycobacterial culture (mycobacteria)
		± electron microscopy (enteric viruses and parasites)
		± molecular diagnostics of intracellular agents

CMV = cytomegalovirus.

infected children without diarrhea was greater than that in immunocompetent matched control individuals and similar to that found in immunocompetent children with acute enteritis.[31] However, most enteric viruses have been detected in children with and without diarrhea and their pathogenic role is uncertain. In addition, there is no specific treatment and generally diarrhea is mild and self-limiting. Thus, electron microscopy of stools is of limited significance in HIV-infected children, and the results may be confusing.

If microbiologic investigation is inconclusive, the option is to treat the patient with empiric therapy or to obtain an intestinal biopsy as a second step (table I). Intestinal biopsy is also needed for additional microbiologic information, namely to detect intracellular enteric agents. For selected opportunistic agents, analysis of tissue specimens rather than stools is essential in order to prove a cause-effect relationship with diarrhea.

Identification of cytomegalovirus in intestinal epithelium is required for diagnosis. Upper intestinal endoscopy or sigmoidoscopy may be used to obtain appropriate specimens by biopsy and may provide further macroscopic information (e.g. damaged epithelium). Cytomegalovirus may be detected in intestinal cells using light microscopy (which shows large inclusion bodies in infected cells), electron microscopy, or through molecular techniques. Direct demonstration of cytomegalovirus in intestinal tissue is essential to establish specific treatment, in the light of the potentially severe side effects of treatment.[32]

Mycobacteria are also exclusively identified through microscopy of intestinal cells or molecular testing of tissue specimens.

Endoscopy may be useful in children with protracted diarrhea, although it increases the costs of patient management. Upper endoscopy or sigmoidoscopy may be performed depending on whether the diarrhea is profuse and watery or if it is characterized by an increased frequency of low volume mucoid or bloody stools associated with abdominal pain, suggesting colonic involvement, respectively.[32]

In patients with nonresolving colitis, pancolonoscopy is the third step (table I). Total colonoscopy often yields information similar to that from sigmoidoscopy; the latter is more expensive and invasive. Thus, it has been suggested to reserve pancolonoscopy for patients who are in more severe conditions or are not responding to treatment. Intestinal specimens should be cultured, examined by light microscopy and, subsequently, by electron microscopy. If the clinical picture suggests small bowel involvement, then esophagogastroduodenoscopy is indicated with the same processing of mucosal specimens.[33] Upper endoscopy has been shown to be useful in directing therapy against intestinal infections in children yielding positive results in a substantial number of individuals whose pathogens were otherwise undiagnosed. In a study set up to determine the prevalence of gastrointestinal lesions, 35% had an opportunistic pathogen identified endoscopically.[34]

The indications summarized in table I are intended as a general algorithm of investigations for approaching a child with diarrhea. Baseline immunologic features and clinical judgement should however guide the selection of sequence and aims of investigations. The level of investigations can range from a minimal workup, only including stool examinations, to an intensive and more expensive work-up, including upper and lower endoscopy with multiple biopsies, and to microbiologic and morphologic investigations to optimize diagnostic results.

Among noninfectious causes of diarrhea, AIDS-associated neoplastic diseases, such as intestinal lymphoma and Kaposi sarcoma, are extremely rare in children compared with adults.[35] Celiac disease should be considered in the differential diagnosis of persistent diarrhea. At present, the incidence of celiac disease is estimated to be in the range of 1/250 to 1/5000 in both children and adults, depending on geographic location. Children with symptomatic HIV infection share surprising similarities with those with celiac disease. They not only have intestinal malabsorption (see section 3.1), but also often show increased antibody titers to gliadin, as a

result of nonspecific, HIV-related, polyclonal activation.[78] Antibodies to food antigens are also frequently detected in HIV-infected children.[80] However, HIV-infected children do not generally respond to an exclusion diet.

2.3 Treatment of Diarrhea

Treatment is based on both the baseline parameters of HIV infection and the actual intestinal symptoms. It should be specifically directed against the etiologic agent so that treatment is prompt and effective and the administration of unnecessary medications is prevented; the treatment schedule of a typical HIV-infected child is already complex.

2.3.1 Enteric Cryptosporidiosis

Treatment of enteric cryptosporidiosis includes strong supportive therapy and the use of anti-infectious drugs. Intravenous rehydration is generally needed and often clinical nutritional support is required to balance the rapid weight loss, which may trigger a vicious circle of nutritional and immune derangement (figure 1). Continuous enteral or parenteral nutrition may be considered for nutritional support.

Several anti-infectious drugs have been used in patients infected with *C. parvum* (table II), including the macrolides spiramycin and paromomycin. These agents have had some success in improving symptoms, but have not been able to eradicate the parasite. Albendazole has been successful in adult African patients with persistent diarrhea.[77] Albendazole also promotes intestinal mucosal recovery in association with complete clinical response. However, data on the use of this drug in children are lacking. A new broad-spectrum antiparasitic drug, nitazoxanide, has been shown to be partially effective in the treatment of enteric cryptosporidiosis, but its effect was negligible in HIV-positive children.[78] Oral administration of immunoglobulins or colostrum from immunized cows have been proposed in selected cases.[78] However, eradication of *C. parvum* is not generally achieved, although symptoms may be reduced by lowering the oocyst number.

When cryptosporidial diarrhea causes massive fluid loss, a trial with the long-acting somatostatin analog, octreotide, may be considered. Octreotide has a potent antisecretory effect, which counteracts electrolyte secretion induced by the parasite. In an observational study, two children were treated with octreotide with a substantial reduction of fecal output and one was able to recover completely.[40]

HAART provides protection against enteric cryptosporidiosis. Children started on HAART undergo a restoration of immune function that is associated with prevention of severe cryptosporidiosis.[43] HAART has also been shown to be effective in symptomatic adult patients with cryptosporidial diarrhea.[27]

2.3.2 Bacterial Diarrhea

Bacterial diarrhea may be severe in HIV-infected children and should be treated aggressively. Guidelines on intestinal infections call for aggressive antimicrobial treatment in immunocompromised patients,[11] but randomized controlled studies to establish the efficacy of antibiotics are not feasible for ethical reasons. The use of specific antibiotics (table II) should be carefully considered even in children who show a mild course of the disease, particularly in those who have moderate-to-severe immune impairment (classes 2 and 3 of the Centers for Disease Control and Prevention classification system of pediatric HIV infection). Specific anti-infectious drugs can eradicate the agent. For example, metronidazole is effective for small intestinal bacterial overgrowth. Recovery from antibiotic-induced diarrhea is generally achieved with withdrawal of the responsible antibiotic. In selected cases, vancomycin or metronidazole may be effective for treating persistent diarrhea.

Table II. Specific therapy against intestinal pathogens in HIV-infected children

Pathogen	Treatment
Salmonella	Ampicillin, amoxicillin, trimethoprim/sulfamethoxazole, cefotaxime, ceftriaxone
Shigella	Ampicillin, trimethoprim/sulfamethoxazole, cefixime, ceftriaxone, cefotaxime, ciprofloxacin, ofloxacin
Campylobacter	Erythromycin, ciprofloxacin
Giardia lamblia	Metronidazole, tinidazole, furazolidone, paromomycin
Clostridium difficile	Spiramycin, metronidazole, vancomycin
Cryptosporidium parvum	Paromomycin, azithromycin, nitazoxanide, hyperimmune bovine colostrum (investigational), octreotide
Mycobacterium avium and *M. intracellulare*	Clarithromycin or azithromycin plus ethambutol; additionally rifabutin, rifampin (rifampicin), clofazimine, ciprofloxacin, or amikacin
Rotavirus	Human serum immunoglobulin given orally
Cytomegalovirus	Ganciclovir, foscarnet

2.3.3 Rotavirus

There is no specific drug treatment for rotavirus. However, the administration of human serum immunoglobulin via the oral route, in a single dose of 300 mg/kg of bodyweight, is a valid therapeutic option.[23] We have successfully used passive immunotherapy in immunocompetent children with severe rotavirus diarrhea and also in three children with HIV infection and rotavirus diarrhea.[42] Its mechanism of action is related to high specific neutralizing titers that are consistently detected in all human immunoglobulin preparations.

A vaccine against rotavirus was introduced but rapidly withdrawn, because of a suspected (but never conclusively proven) association with intussusception.[43] While awaiting the development of safer vaccines, administration of immunoglobulin, which is largely available in pediatric hospitals, may be lifesaving for children with severe rotavirus infection.

2.3.4 Cytomegalovirus

Cytomegalovirus may be treated with ganciclovir or with foscarnet.[44] Both drugs produce clinical and histologic improvement in both adults and children although the rate of relapse is high. Treatment should be considered in the light of major toxicity and should be reserved for severe cases.

2.3.5 Atypical Mycobacteria

Atypical mycobacteria may be effectively treated with clarithromycin, but conventional quadruple therapy is often needed.[45] Prophylaxis against atypical mycobacteria with azithromycin was routinely performed in the pre-HAART era. This is no longer considered necessary for children receiving HAART, unless they have a severe immune impairment (class 3 of the Centers for Disease Control and Prevention classification scheme).

2.3.6 Empiric Treatment for Diarrhea

Metronidazole may be used for the empiric treatment of diarrhea. In a substantial number of children with diarrhea of suspected infectious origin, no etiologic agent is detected, at least in the initial diagnostic work-up. Empiric treatment may be considered when symptoms are insufficient to justify more invasive diagnostic approaches, such as endoscopy.[27,30] In these cases, metronidazole alone or the so-called 'bowel cocktail' may be considered, which consists of metronidazole 10–20 mg/kg/day, high-dose oral gentamicin 50 mg/kg/day in three divided doses, and colestyramine. Somatostatin analogs have also been considered for pathogen-negative diarrhea, and may be particularly effective if diarrhea has a secretory rather than osmotic mechanism.[46] They may also be effective for chronic refractory diarrhea due to their effect on intestinal motility.[46] Other agents active on motility have also been proposed, including loperamide, but their use in children may be loaded with severe adverse effects.[47]

When diarrhea is persistent or severe and no enteric etiologic agent is detected, HAART should be re-evaluated. Persistent diarrhea may be associated with an increased viral load and be either the result of HIV itself or involve an as yet unidentified opportunistic agent. In those cases, changing antiretroviral therapy may be an effective option.

3. Dysfunction of the Digestive-Absorptive Processes

Malnutrition and its terminal stage, AIDS-associated wasting, are common in developing countries, but also in developed countries where HAART is widely available. In the US, HIV-associated wasting has increased in relative frequency from the fifth to the second most frequent AIDS-related complication.[4] Malnutrition can be an early manifestation of HIV infection and is associated with a rapid decrease in the CD4+ cell count and an increased rate of opportunistic infections.[48]

A specific HIV-associated disease is AIDS enteropathy. This is characterized by villous atrophy without compensatory crypt hypertrophy and has been detected in severely HIV-infected adults.[30,31] It is not clear whether AIDS enteropathy exists in children. Although the typical morphologic picture of villous atrophy is rarely detected, major dysfunctions of the digestive-absorptive processes are frequently observed in children with HIV infection and involve multiple organs, such as the intestine, pancreas, and liver.

3.1 Intestinal Dysfunction

Intestinal dysfunction is a specific HIV-related syndrome in children. The clinical manifestations of intestinal dysfunction may be limited or absent; it is not consistently associated with diarrhea. Its most prominent features are steatorrhea, reduction of the intestinal absorptive surface, and increased permeability (table III).[31] The prevalence of intestinal dysfunction was as high as 60–80% in

Table III. Prevalence of features of intestinal/pancreatic dysfunction in children naive to highly active antiretroviral therapy[31,49-51]

Features	Frequency (%)
Iron malabsorption	45
Steatorrhea	30–40
Pancreatic insufficiency	30
Lactose malabsorption	25–35
Reduced absorptive intestinal surface	25
Protein loss	15
Increased permeability	15

HIV-infected children in the pre-HAART era,[49] with iron malabsorption being the most common feature (table III). Despite the limited or absent clinical manifestations of intestinal dysfunction, nutrient malabsorption certainly contributes to weight loss, and its clinical consequences may be expected in the long term and associated with progressive failure to thrive.

One of the most frequent features of impaired digestive processes in children with HIV infection is lactose malabsorption.[50] This finding is in keeping with a reduction of the functioning intestinal surface, which has been demonstrated by a high rate of pathologic responses to oral xylose load.[51] Lactose malabsorption is important in that it may induce diarrhea and/or failure to thrive in younger children, whose primary caloric source is milk.

The pathophysiology of intestinal dysfunction is complex and involves multiple abnormalities of various organs and functions (figure 2). There is some evidence of a role of specific enteric pathogens in intestinal dysfunction. The hypothesis exists that *C. parvum* may induce inflammatory changes and secondary digestive abnormalities.[19] However, intestinal function tests have been shown to be unchanged in children before and during enteric cryptosporidiosis.[8] In an observation study including a population of approximately 50 HIV-infected children, intestinal dysfunction was not associated with enteric viruses.[11] A role by HIV itself has been hypothesized based on the presence of HIV-RNA sequences in the stools and in the intestinal cells of children with chronic diarrhea,[28] but evidence of HIV in intestinal epithelium is inconsistent. However, recent data implicate the HIV virus in intestinal dysfunction. The TAT viral protein has been shown to inhibit cell proliferation through a direct interaction with the enterocyte, thereby reducing the functioning surface.[29] Indirect but strong support for the enteropathogenic role of HIV comes from the efficacy of combination therapy in restoring intestinal function processes.[9,12] Data obtained in children switched from a single antiretroviral drug to HAART, showed a rapid normalization of intestinal function tests (figure 3), in parallel with a decrease in viral load and an increase in the CD4+ cell count. This suggests that if children receiving HAART present with malnutrition, careful monitoring of antiretroviral therapeutic efficacy is indicated, as malnutrition may be an early marker of treatment failure.

3.2 Management of Intestinal Dysfunction

Assessment of the digestive-absorptive function is indicated in children with persistent diarrhea and in those without diarrhea, but who are not growing or are losing weight. The diagnostic approach includes noninvasive intestinal tests (table IV). In selected cases, upper intestinal endoscopy and biopsy should be considered. Evidence of dysfunction requires investigation to determine its primary cause. Besides intestinal infections, other causes of malabsorption should be investigated, including celiac disease and other food intolerance.

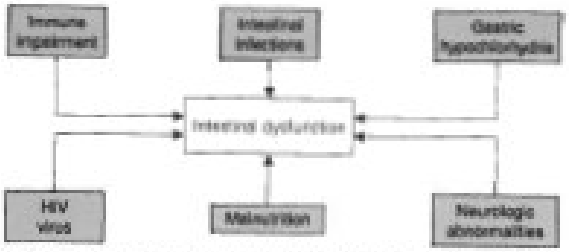

Fig. 2. Pathophysiology of intestinal dysfunction in HIV-infected children. Several conditions may contribute to intestinal dysfunction. Malnutrition and immune impairment are associated with abnormalities of the digestive-absorptive processes, independent of HIV infection. Selected intestinal infections may induce intestinal damage. Gastric hypochlorhydria and neurologic abnormalities have been associated with small bowel bacterial colonization and motility disorders, respectively. Finally, HIV-produced transactivating transfer factor induces electrolyte secretion and inhibits enterocyte growth, suggesting a direct role of HIV.

Treatment of intestinal dysfunction depends on its cause. Children with lactose malabsorption should receive lactose-free milk. In selected children, malnutrition *per se* may be the primary cause of intestinal malabsorption, leading to a self-maintaining condition. This should be approached with aggressive nutritional intervention. Clinical nutrition should be considered in children with severe functional abnormalities and in those not responding to specific therapeutic approaches. A change in antiretroviral therapy may be considered in children with immunologic or virologic failure.

3.3 Liver Dysfunction

Hepatic dysfunction is not a common manifestation of HIV disease in children, although >90% of HIV-infected children will have liver enlargement during the course of their illness.[44] Liver failure is rare, although it may be a rapidly developing complication of terminal HIV infection. Hepatomegaly without other symptoms of systemic disease is most likely associated with nutritional deficiency. However, classic and opportunistic agents may induce liver damage and should be considered in the HIV-infected child.

Hepatitis B and C virus infections are more severe in HIV-infected patients than in otherwise healthy individuals. Hepatitis C virus (HCV) may be vertically transmitted by HIV-HCV coinfected mothers.[53] Hepatitis C induces a chronic progressive disease in HIV-infected adults. The combined treatment may be worth considering in the light of increased survival of HIV-infected children. In these patients, combined treatment with pegy-

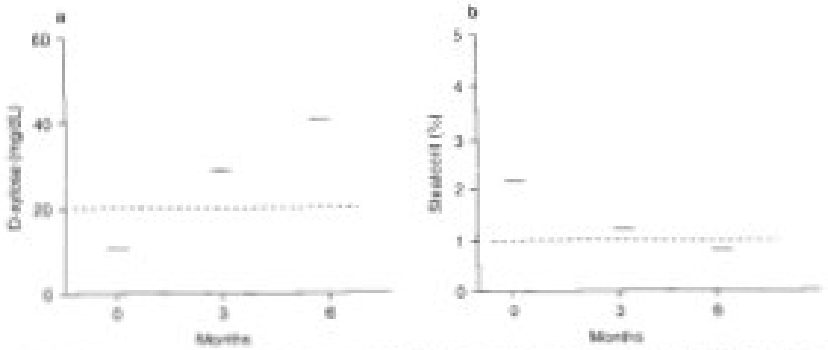

Fig. 3. Effects of ritonavir combination therapy on intestinal function tests in HIV-infected children. A progressive rise of xylose levels (a) and a decrease of steatocrit (b) are observed, reaching normal levels within 6 months of the onset of highly active antiretroviral therapy (HAART) in ten children. HAART was started at time 0. Values above and below the dashed line are normal for xylose and steatocrit, respectively. These data indicate that HAART is effective in inducing full restoration of intestinal function.[80]

luted interferon-α-2a and ribavirin may provide a significant clinical benefit.[54] However, long-term studies are not yet available in children and it is not clear, at present, whether active treatment is indicated. The current recommendation is to treat HIV infection while monitoring HCV and hepatotoxicity induced by antiretroviral drugs.[55]

Coinfection with hepatitis B virus (HBV) is more common than coinfection with HCV and may be associated with significant morbidity in HIV patients.[56] HBV infection is associated with an increased risk of liver disease-associated deaths. The Centers for Disease Control and Prevention recommend that all HIV-infected patients that are hepatitis B seronegative should receive specific vaccination.[57] However, an increasing number of therapeutic options for HBV infection are available for treatment. Several antiretroviral drugs are effective, including lamivudine, which inhibits HBV-DNA polymerase, but chronic treatment with lamivudine has been associated with an increase in resistant strains. Tenofovir disoproxil fumarate and adefovir dipivoxil have been effective in adult HIV-infected patients with concomitant HBV infection but there is no information on their use in children.[56]

While HBV, HCV, and cytomegalovirus may infect the hepatocyte, they have also been associated with biliary tract disease. *C. parvum*, *M. avium*, and *M. intracellulare* also have been found to cause AIDS-related cholangiopathy.[58]

Chronic/progressive liver disease is rare in HIV-infected children. If hepatic transaminase levels are increased for more than 6 months, liver biopsy should be considered and specific pathogens should be investigated for.

Most of the medications used to treat HIV disease are toxic to the liver. Nucleoside reverse transcriptase inhibitors have been associated with a potentially fatal syndrome of lactic acidosis with hepatic steatosis.[59] Toxic effects induced by antiretroviral drugs are described in section 5.

3.4 Pancreatic Dysfunction

Pancreatic dysfunction may be considered as a component of HIV-associated digestive dysfunction. A reduction of fecal levels of pancreatic elastase and/or chimotrypsin has been found in 30% of HIV-infected children.[51] In addition, there is a significant negative correlation between the degree of steatorrhea and fecal levels of pancreatic enzymes. The clinical manifestations of pancreatic involvement may not be evident, because exocrine, rather than endocrine pancreatic function, is involved. Indeed, vomiting or abdominal pain of pancreatic origin are not common in children with HIV infection nor are sensitive hallmarks of pancreatic dysfunction. Pancreatitis may be an unusual but serious adverse effect of selected nucleoside analog reverse transcriptase inhibitors.[60]

In children with steatorrhea, pancreatic function should be assessed by measuring elastase levels in stool samples. Pancreatic enzyme supplementation has been shown to be effective in treat-

Table IV. Noninvasive tests for investigating intestinal function

Test	Interpretation/Indication
Steatocrit	Fecal fat loss
α1-Antitrypsin fecal level	Intestinal permeability
Fecal-reducing substances	Carbohydrate malabsorption
Fecal leukocytes	Colonic inflammation
Fecal calprotectin	Colonic inflammation
Fecal elastase	Exocrine pancreatic function
Xylose oral load	Functional absorptive surface
Dual lactose/mannitol load	Intestinal permeability/functional surface

ing patients with fat malabsorption in a prospective study involving 24 children; complete disappearance of steatorrhea occurred in more than one-third of treated children and significant improvement in half, whereas in only 20% of children pancreatic enzyme supplementation failed to modify fat fecal loss.[56]

4. Nutritional Failure

Independently of its etiology, non-HIV-related malnutrition causes widespread atrophy of lymphoid tissue and malnourished children show a decrease of the T-helper/suppressor ratio, diminished CD4+ cell count, impaired delayed hypersensitivity, and an increase in serum immunoglobulin levels.[57] These features strongly resemble the effects of HIV infection but are reversible with nutritional rehabilitation. When these abnormalities are found in HIV-infected children, one cannot determine if they are related to HIV or to malnutrition. Consequently, assessment of nutritional status and nutritional rehabilitation are essential parts of AIDS treatment.

4.1 Assessment of Nutritional Condition

Nutritional status should be assessed every 3–6 months in all children with HIV infection, in order to identify nutritional problems at an early stage.[5,7] A child with growth failure should be investigated as soon as possible. Assessment should include anthropometric measurements, medical and dietary history, current symptoms, biochemical nutritional markers, assessment of body composition, and intestinal function tests. Caloric intake should be quantitatively determined.

A child who refuses to eat may have a number of heterogeneous conditions: esophageal candidiasis and agevusia are typical AIDS-related conditions, which are not easy to detect in younger children. Many antiretroviral drugs are associated with anorexia, including zidovudine, stavudine, ritonavir, nelfinavir, and indinavir.[5] Other antivirals, such as acyclovir (aciclovir) and ganciclovir, and antifungal drugs, such as dapsone, are also associated with anorexia.[58]

Increased energy expenditure may contribute to weight loss. However, no evidence has been found for an association between hypermetabolic states and impaired growth in children with HIV infection.[54,55] Routine laboratory studies should include complete blood counts and an assessment of protein status. The latter includes an assessment of albumin (half-life 20 days), pre-albumin (half-life 2 days), retinol binding protein (half-life 12 hours), and transferrin (half-life 8 days). The half-life of individual serum proteins may help to distinguish between short- and long-term malnutrition.

Micronutrient deficiencies are frequently observed in patients with AIDS. Iron deficiency is a common problem in HIV infection. It may result from an increased infection rate, or from low intake, but in most cases it is the consequence of intestinal malabsorption. Serum iron and iron binding capacity should be assessed. If low, oral iron loading should be performed as described elsewhere.[59] Iron malabsorption is the most frequent feature of intestinal dysfunction and leads to iron deficiency and ultimately to severe anemia. Folate and B_{12} levels should also be measured, particularly in patients receiving zidovudine. Vitamin A and zinc play a crucial role in maintaining a good immune response and serum levels should be checked. In patients with fat malabsorption, vitamin A and E levels may be decreased.[7]

Assessment of body composition may be helpful. Lean body mass is lost early in the course of HIV disease and may be assessed either by mid-arm circumference and triceps skinfold thickness or, more accurately, by bioelectrical impedance analysis or dual emission x-ray absorptiometry scans.[60] Intestinal function should be thoroughly investigated and this can be performed in a noninvasive manner (table IV).

Psychologic disorders may well play a role in reducing caloric intake. A progressively increasing number of children with HIV

Table V. Nutritional management in pediatric AIDS

Problem	Intervention
Infections	Increase calories and protein; treat the specific pathogenic agent
Anorexia	Increase nutrient density of foods
	Small frequent feedings
	Nutritional supplements
	Appetite stimulants (megestrol)
	Vitamin/mineral supplements
	Enteral/parenteral nutrition
	Psychologic support
Oral/esophageal lesions	Soft, nonirritating foods served cold
	Topical medications prior to feeding
	Treat candidiasis/CMV/other agents
Early satiety	Small frequent feedings
	Gastrointestinal motility-enhancing agents
Diarrhea/malabsorption	Small frequent feedings
	Lactose-free milk or formula
	Cow's milk protein-free formula
	Continuous enteral nutrition/parenteral nutrition
Steatorrhea	Pancreatic enzyme replacements
	Medium chain triglycerides

CMV = cytomegalovirus.

Table VI. Gastrointestinal toxicity of antiretroviral drugs[a]

Drug	More common (mild and transient)	Less common (but more severe)	Rare
Nucleoside reverse transcriptase inhibitors			
Abacavir	Nausea, vomiting, diarrhea, anorexia	Severe hepatomegaly with steatosis	Pancreatitis, increased liver enzyme levels
Didanosine	Diarrhea, abdominal pain, nausea, vomiting	Severe hepatomegaly with steatosis	Pancreatitis, increased liver enzyme levels
Lamivudine	Diarrhea, abdominal pain, nausea,	Pancreatitis, severe hepatomegaly with steatosis	NR
Stavudine	Diarrhea, abdominal pain, nausea, vomiting	Pancreatitis, severe hepatomegaly with steatosis	Increased liver enzyme levels
Zidovudine	None	Liver toxicity	Severe hepatomegaly with steatosis
Non-nucleoside reverse transcriptase inhibitors			
Delavirdine	Diarrhea, abdominal pain, nausea, vomiting	NR	NR
Efavirenz	None	Severe hepatic injury (8%)	NR
Nevirapine	Nausea, abnormal liver function	Severe hepatic injury (5.6%)	
Protease inhibitors			
Amprenavir	Vomiting, diarrhea, nausea	NR	NR
Indinavir	Nausea, vomiting, abdominal pain (33–40%), asymptomatic hyperbilirubinemia (10%)	Exacerbation of chronic gastrointestinal disease	NR
Lopinavir ritonavir	Diarrhea, nausea, vomiting	NR	Pancreatitis, hepatitis
Nelfinavir	Diarrhea (25%)	Abdominal pain, exacerbation of chronic disease	NR
Ritonavir	Nausea, vomiting, diarrhea, abdominal pain (98%), anorexia	Increase in liver enzyme levels (9%)	Pancreatitis, hepatitis
Saquinavir	Nausea, diarrhea (36–45%), abdominal pain (16%), mild increase of liver enzyme levels (55%)	Exacerbation of chronic liver disease	NR
Fusion inhibitors			
Enfuvirtide	NR	NR	Nausea, vomiting, increased liver enzyme levels

a The rates of toxicity presented refer to data available for pediatric patients.[81]

NR = not reported.

infection reach adolescence and have an increased rate of behavioral disorders. Psychologic evaluation may be helpful in the child who is losing weight with no obvious reason.

4.2 Nutritional Management

HAART has a positive effect on several growth parameters, including lean body mass.[56,57] However, nutritional management is also needed to prevent or correct deficiencies. Depending on individual problems, a number of specific interventions may be effective (table V).

HIV-infected children may have an increased energy and protein requirement, particularly during and after infections, and supplemental administration of macro- and micronutrients is indicated. Caloric intake may be increased to 50% above the recommended dietary allowance for age and sex, if the child is able to tolerate it. In younger children with lactose malabsorption, lactose-free formula may effectively replace cow's milk. In children who are not willing to eat a sufficient amount of calories, high caloric density formula may be effective. Calories may also be increased by adding fat or carbohydrate. However, the efficacy of these interventions has not been proved. A child can refuse to eat if she/he has oral lesions, early satiety, or nervous anorexia. In selected cases, appetite stimulants have been used in AIDS patients with conflicting results.[58] Preliminary data showed a positive effect of

megestrol on weight, but not on height in HIV-infected children. This effect was reversed upon treatment withdrawal.[66]

In several cases clinical nutrition is indicated; this includes enteral or parenteral nutrition. Enteral nutrition may be administered via a nasogastric or gastrostomy tube and is indicated in a child who cannot eat or who is not willing to eat. Continuous enteral nutrition may also be effective in children with a reduced absorptive function. The rationale of continuous enteral nutrition is based on the modifications of the absorptive surface/time ratio. A reduced surface that functions for an extended time leads to increased nutrient absorption.[66,67]

Nutritional rehabilitation has a general, non-HIV-related, beneficial effect on immune responses. Continuous enteral nutrition has been used in 16 children with intestinal malabsorption and has been shown to be effective in increasing their bodyweight while restoring intestinal absorptive function.[70] Improvement of nutritional condition was associated with an increase in the CD4+ cell count, thus supporting the close link between nutritional condition and immune function.[70]

In children with extreme wasting or in terminal conditions, enteral nutrition may not be sufficient. In such cases, parenteral nutrition may be a life-saving procedure. In a series of 46 children undergoing parenteral nutrition, all in a critical condition, approximately 30% were able to switch back to oral feeding and experienced an improvement in intestinal nutrient absorption.[70] Parenteral nutrition should be undertaken at an early phase, as soon as other less invasive nutritional approaches have been unsuccessfully attempted.

Consistent data support the importance of selected micronutrients in HIV-infected children. Oral administration of iron is generally sufficient to raise hemoglobin levels in children with normal iron absorption, whereas parenteral administration is required for those with intestinal malabsorption to fully correct anemia.[44]

Retinol (vitamin A) administered to 687 Tanzanian HIV-infected children, resulted in an increase in linear growth, decreased risk of stunting associated with persistent diarrhea,[71] and a 50% reduction in diarrhea-associated morbidity.[72] Zinc supplementation has also been associated with substantial reduction of intestinal and respiratory infections in non-HIV infected children.[73] These interventions are easy to perform and highly cost effective and should be implemented on a mass scale in HIV-infected children, particularly in developing countries.

5. Gastrointestinal Adverse Reactions and Side Effects of Antiretroviral Drugs

The toxicity of antiretroviral drugs is becoming an important issue in the management of HIV-infected children. The main gastrointestinal side effects of antiretroviral drugs are diarrhea, vomiting, nausea, abdominal pain, increased liver enzyme levels and hepatitis, hyperbilirubinemia, increased pancreatic enzyme levels, and pancreatitis.[74]

Table VII. Degree of toxicity of gastrointestinal adverse effects of antiretroviral drugs[75]

Parameter	Grade 1 (mild)	Grade 2 (moderate)	Grade 3 (severe)	Grade 4 (life-threatening)
Abdominal pain	Mild	Moderate – no treatment needed	Moderate – treatment needed	Severe – hospitalization required
Diarrhea	Slight change in consistency and/or frequency of stools	Liquid stools	Liquid stools more than four times the amount or number normal for the patient	Liquid stools more than eight times the amount or number normal for the patient
Nausea	Mild	Moderate – decreased oral intake	Severe – little oral intake	Unable to ingest food or fluid for >24 hours
Vomiting	1 episode/day	2–3 episodes/day	4–6 episodes/day	>6 episodes/day or intractable vomiting
Constipation	Slight change in the consistency/frequency of stools	Hard, dry stools with a change in frequency	Abdominal pain	Distention and vomiting
Appetite		Decreased appetite	Appetite very decreased, no solid food taken	No solid food or liquid taken
Bilirubin	1.1–1.9 × ULN	2.0–2.9 × ULN	3.0–7.5 × ULN	>7.5 × ULN
AST/ALT/GGT	1.1–4.9 × ULN	5–9.9 × ULN	10–15 × ULN	>15 × ULN
Uric acid	7.5–9.9 mg/dL	10–12.4 mg/dL	12.5–15 mg/dL	>15 mg/dL
Pancreatic amylase	1.1–1.4 × ULN	1.5–1.9 × ULN	2.0–3.0 × ULN	>3.0 × ULN

GGT = γ-glutamyl transferase; **ULN** = upper limit of normal.

Table VIII. Main gastrointestinal manifestations of HIV infection in children

Manifestation	Main pathogenic agents	Other causes
Dysphagia/anorexia	CMV/*Candida albicans*	Medications
Nausea/vomiting	*C. albicans*/*Helicobacter pylori*	Medications
Diarrhea/rectal bleeding	*Cryptosporidium parvum*/HIV	Medications, lactose intolerance
Hepatomegaly/jaundice	CMV/hepatitis viruses	Medications
Abdominal pain	Enteric bacteria	Medications
Failure to thrive	HIV (directly or indirectly)	Medications, psychological/social factors

CMV = cytomegalovirus.

The gastrointestinal toxic effects of the main antiretroviral drugs used in the treatment of HIV-infected children are listed in table VI. Nausea, vomiting, abdominal pain, and diarrhea often occur early upon new drug administration. Adverse effects are generally mild and transient and decrease within a few weeks.

Hepatic toxicity is more often associated with long-term therapy and is generally mild. However, severe hepatic toxicity may occur, particularly with protease inhibitors, and may require discontinuation of therapy. Recently, the use of HIV-1 specific non-nucleoside reverse transcriptase inhibitors, including nevirapine and efavirenz, has been shown to be associated with severe hepatic injury, particularly among persons with chronic viral hepatitis.[74]

The pancreas is a target for antiretroviral drug-induced adverse effects. The incidence of pancreatitis is high with nucleoside transcriptase inhibitors, particularly didanosine.[75] However, pancreatic toxicity is often limited to an asymptomatic increase of amylase and lipase levels.

The management of adverse effects depends on their severity. It is widely accepted that adverse effects can be classified as mild (grade 1), moderate (grade 2), severe (grade 3), and life-threatening (grade 4) [table VII]. Generally, adverse effects of grade 1 or 2 do not require dose modifications or drug withdrawal. Clinical or biochemical signs of more severe adverse effects necessitate a reduction or a suspension of treatment.

Adverse effects of antiretroviral agents may compromise their efficacy because they are associated with reduced adherence. A multicenter study performed in adults showed that symptoms such as nausea, anorexia, and taste abnormalities were significantly associated with nonadherence. Nonadherent patients had a higher mean overall symptom score and mean medication adverse-effect score when compared with adherent patients.[77]

6. Conclusions

The epidemiology of HIV infection is rapidly changing in countries where patients have access to HAART. Children with HIV infection reach adolescence in a fair clinical condition with effective antiretroviral therapy and the incidence of opportunistic infections is declining. However, a number of children still have severe disease, either because of permanent impairment of the immune system or because of poor treatment adherence. In addition, adverse reactions and side effects of antiretroviral drugs are observed with increasing frequency.

Involvement of the digestive tract in HIV-infected children may present with a broad pattern of symptoms (table VIII), encompassing many of the intestinal manifestations of non-AIDS immunodeficiency,[78] while having several peculiar features. Many of the intestinal problems are related to infections, but they may be also related to antiretroviral drugs.

The pattern of HIV infection is completely different in poor countries, where most children with HIV infection are living, but where the access to antiretroviral drugs is limited. In its second report on the status of the digestive and nutritional health of children in the world,[79] the Federation of the International Societies of Pediatric Gastroenterology, Hepatology and Nutrition has clearly stated that that combination of malnutrition, HIV-induced immune impairment, and opportunistic infections is responsible for the death of hundreds of thousands of children. The report has produced a list of practical interventions to provide access to antiretroviral drugs, foods, and medical facilities through a global integrated approach, which includes international health organizations, drug companies, political institutions, and charitable foundations.

In this rapidly evolving scenario, the care of HIV-infected children is progressively being transferred out of the hospital and reference centers to home physicians. The latter are therefore required to continuously update their knowledge in this field to effectively manage these children.

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Curriculum vitae et studiorum

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Education/Training:

- High School degree
- 1996 “Federico II” University of Naples “Federico II” Degree in Biology, summa cum Lode
- 1996-2000 Residency in Clinical Pathology at the University of Naples “Federico II”.
- 2002-07 PhD program in “Human Reproduction, Development and Growth ” at the University of Naples “Federico II”, Department of Pediatrics

Reasearch activity:

- Intestinal ion transport physiopathology: effects of bacterial and non bacterial enterotoxins
- Effects of drugs and growth factors on intestinal ion transport and their mechanisms
- Enterocyte growth, differentiation and proliferation: biological effects and signal transdution (MAP kinases, intracellular Ca^{2+}, nitric oxide, cyclic nucleotides)

Technologycal skills:

- Main cellular and molecular biology technics known during traning at Department of Biology and Pathology Cellular and Molecular 'L. Califano', University of Naples ”Federico II” in Prof. Enrico Avvedimento group (1993/1997): cell colture, protein extraction and assay, cell component splitting, western blot, cAMP protein kinase assay, ligand blotting, molecular cloning, DNA plasmid extraction, transient and stable transfection with expression vectors, positive clone selection, CRE-CAT assay, cyclic nucleotides assay.
- Brief training at the Citogenetic and Prenatal Diagnosis Unit, University of Naples ”Federico II” (1997): leukocyte and amniocyte colture, preparation of glass slides with methafasic plates, karyotype.
- Genetic and molecular technics known during training at Department of Pediatrics, University of Naples ”Federico II” in Prof. Generoso Andria group (1998): blood DNA extraction, polymerase chain reaction (PCR), bacteric trasformation with amplification vectors, agarose and polyacrilammide electrophoresis, genomic DNA sequencing, SSCP analysis.
- Studies about intestinal physiopathology were performed at Department of Pediatrics, University of Naples ”Federico II” in Prof. Alfredo Guarino group (1999/actually). Obtained knowledge in specific technics are listed below:

- Intestinal models: Caco-2 cell line, intestinal mucosa organ culture
- Intestinal transepithelial ion transport studies: Ussing chamber system
- Intestinal cell growth studies: cell counting in Neubauer chamber, transepithelial resistance measure (TEER), ^{3}H-thymidine and bromodeossiuridine uptake
- Intestinal cell differentiation studies: enzymatic disaccharidase assay (sucrase and lactase), RNA extraction and Real-Time PCR
- Intracellular pathways studies (MAP Kinases, nitric oxide, intracellular calcium pathways): preincubation experiments with specific inhibitors, western blot, Griess reaction, single cell mycrofluorimetry.

Awards:

John Harris Prize at the 2nd World Congress of Pediatric Gastroenterology, Hepatology, and Nutrition Paris 3-7 July 2004 in recognition of the best gastroenterology presentation:

Buccigrossi V., De Marco G., Bruzzese E., Bracale I., Polito G., Guarino A. Lactoferrin stimulates enterocyte growth and differentiation through MAP Kinase-ERK and restores intestinal monolayer damaged by Rotavirus. 2nd World Congress of Pediatric Gastroenterology, Hepatology, and Nutrition Paris 3-7 July 2004 JPGN 39 (1): S54

ESPGHAN

The
European Society for Paediatric Gastroenterology
Hepatology and Nutrition awards the

John Harries Prize 2004

in recognition of the best gastroenterology presentation at the
ESPGHAN meeting / World Congress of Pediatric Gastroenterology,
Hepatology and Nutrition, 3-7 July, 2004 – Paris, France
to

Vittoria Buccigrossi

Peter Milla, Président — Jean-Pierre Olives, Secretary

7 July 2004, Paris, FRANCE

Full paper:

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