Articles in PresS. Am J Physiol Gastrointest Liver Physiol (September 4, 2003).10.1152/ajpgi.00227.2003

FINAL ACCEPTED VERSION

Prolonged interferon-γ exposure decreases ion transport, NKCC-1 and Na^+, K^+ ATPase expression in human intestinal xenografts *in vivo*

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Short running title: Interferon-gamma decreases intestinal ion transport

Keywords: interferon-gamma, ion transport, human intestinal xenografts, NKCC-1, CFTR, CLCA

ABSTRACT

Interferon-γ (IFN-γ) is elevated in intestinal inflammation and alters barrier and transport functions in human colonic epithelial cell lines, but its effects on normal human small intestinal epithelium *in vivo* are poorly defined. We investigated effects of prolonged IFN-γ exposure on ion transport and expression of transporters using human fetal small intestinal xenografts. Xenograft-bearing mice were injected with IFN-γ, and 24 hours later, xenografts were harvested and mounted in Ussing chambers. Baseline potential difference was not affected by IFN-γ treatment. However, conductance was enhanced, and agonist-stimulated ion transport was decreased. IFN-γ also decreased expression of the $Na^+/K^+/2Cl^-$ co-transporter and the alpha-subunit of Na^+,K^+ ATPase compared to controls, whereas levels of the chloride channels CLCA and CFTR were unaltered. Thus, prolonged exposure to IFN-γ leads to decreased ion secretion, due, in part, to decreased ion transporter levels. These findings demonstrate the implications of elevated IFN-γ levels in human small intestine and validate the human intestinal xenograft as a model to study chronic effects of physiologically relevant stimuli.

INTRODUCTION

The intestinal epithelium transports nutrients, electrolytes and water and provides a selective barrier to luminal contents, which is essential for physiological homeostasis and defense against enteric microbes. A leaky epithelium not only results in excessive loss of fluid from the body, but also increases the uptake of macromolecules from the intestinal lumen with the capacity to activate mucosal immune and inflammatory responses. In chronic inflammatory bowel disease (IBD), where malabsorption and secretory diarrhea are common, abnormalities in barrier and transport functions of epithelial cells have been observed [28,22,23].

Under inflammatory conditions, the epithelium is exposed to a plethora of cytokines and other soluble mediators that are secreted by lymphocytes, macrophages, and epithelial cells themselves. One of these, IFN-γ, a pro-inflammatory cytokine, is secreted by activated T helper-1 (Th1) lymphocytes and natural killer cells and plays a central role in inflammation [5,11], in that it targets and activates macrophages, endothelial, and NK cells, as well as epithelial cells resulting in increases in major histocompability complex (MHC) class I/II expression [7]. In patients with Crohn's disease, which can affect both the small and large intestine, mucosal production of IFN-γ is strongly increased [14,24,27]. The physiologically relevant targets of IFN-γ are not well understood, but this cytokine has been shown to increase paracellular permeability [20,28] and down regulate ion transport functions [28] in colonic epithelial cell lines. Moreover, IFN-γ has recently been identified as a key homeostatic mediator, which may prevent excessive fluid loss by the small intestine in the setting of diarrhea [30].

Transepithelial ion transport is based on the activity of specific ion channels and transporters. Stimulation of colonic epithelial cell lines with IFN-γ has been shown to down regulate expression of the cAMP-dependent chloride channel, cystic fibrosis transmembrane conductance regulator (CFTR) [4] and of the Na^+/H^+ exchangers NHE2 and NHE3 [22], which also was verified in rat intestine [22]. However, intestinal epithelial cell lines, although very useful reductionist models, are limited in that they consist only of cells with a secretory phenotype, and lack a fully differentiated epithelium and other mucosal cells. Furthermore, most available transformed intestinal epithelial cell lines are of colonic origin, which might complicate extrapolation of data to the epithelium of the small intestine, which is frequently affected by Crohn's disease. And, finally, *ex vivo* studies of human intestinal segments are restricted to experiments of short duration, whereas most findings in intestinal cell lines have indicated that many effects of cytokines on epithelial function are only seen after prolonged cytokine exposure.

To overcome these limitations, it is necessary to utilize *in vivo* models of human intestinal epithelium. Thus, in the present study, we sought to develop a defined *in vivo* model of human small intestinal epithelium that could be used to understand persistent cytokine effects on physiological responses of the epithelium. We employed human small intestinal xenografts combined with studies of ion transport in modified Ussing chambers to demonstrate that both calcium- and cAMP-dependent chloride secretion are down-regulated in human small intestinal epithelium by prolonged treatment with IFN-γ, which may be due, at least in part, to decreased levels of NKCC-1 and the α-subunit of Na^+,K^+ ATPase. These data indicate that IFN-γ can exert anti-secretory functions *in vivo*.

MATERIALS AND METHODS

Materials

Blocking reagent for Western Blotting (skim milk), ECL Plus (Amersham Pharmacia Biotech, Piscataway, NJ); BM Chemiluminescence ELISA Reagent (Boehringer Mannheim, Mannheim,Germany); anti-NKCC1 antibody (a gift from Dr. Christian Lytle, University of California, Riverside); anti-calcium-activated chloride channel (CLCA) antibody (a gift from Dr. Cathy Fuller, University of Alabama at Birmingham); anti-CFTR antibody (mouse monoclonal IgG) (R&D Systems, Inc., Minneapolis, MN); anti-Na^+, K^+ ATPase α-1 antibody (mouse monoclonal IgG), anti-Na^+, K^+ ATPase β1 antibody (mouse monoclonal IgG) (Upstate Biotechnology, Lake Placid, NY); carbachol, prostaglandin E_2 (Sigma-Aldrich, St. Louis, MO); TRIzol Reagent; (GIBCO Life Technologies, Grand Island, NY); and ribonuclease-free deoxyribonuclease (Stratagene, La Jolla, CA) were obtained commercially from the sources indicated.

Human fetal intestinal xenografts

Segments of human fetal small intestine (gestational age 16-20 weeks) were transplanted subcutaneously into the rear flanks of mice with severe-combined immunodeficiency (SCID) as previously described [26]. At 10 weeks or more post-transplantation, the intestinal xenografts resembled morphologically mature pediatric intestine, with an epithelium of entirely human origin [26]. Experimental procedures were approved by the UCSD Human and Animal Subjects Committees. SCID mice carrying mature xenografts were injected i.p. with 10 μg human IFN-γ or PBS (controls) 24 h prior to xenograft

harvest. Human IFN-γ has no activity on mouse cells bearing IFN-γ receptors [15], so the direct effects of the cytokine are restricted to human cells in the xenograft.

RNA isolation and detection of mRNA expression

Total cellular RNA was extracted from xenografts using TRIzol Reagent (GIBCO Life Technologies, Grand Island, NY) and treated with ribonuclease-free deoxyribonuclease. For reverse-transcription polymerase chain reaction (RT-PCR), 1 μg of total cellular RNA was reverse transcribed, and complementary DNA was amplified as described previously [13]. The primers for Mig were as described earlier [12]. The primers for Cytokeratin 8, CK8, were sense: 5′-GAT CTC GGA CAC ATC TGT GGT GCT GTC-3′ and antisense: 5′-ATC CCC GTG CTT CCC AGC CAG GCT CTG C-3′ and GAPDH sense: 5′-ATC AAC GAC CCC TTC ATT GAC C-3′ and antisense: 5′-CCA GTA GAC TCC ACG ACA TAC TCA GC-3′. The amplification profile was 25-35 cycles of 45-second denaturation at 95ºC and 2.5 minute annealing and extension at 60ºC. Negative control reactions had no added RNA in the RT and subsequent PCR amplification.

Measurement of mucosal ion transport in human intestinal xenografts

Xenografts were harvested, stripped of seromuscular layers by blunt dissection, and mounted in modified Ussing chambers (aperture = 0.07 cm^2). The mucosal sheets were bathed in circulating Ringer's solution (composition (in mM): 141.0 Na^+, 4.2 K^+, 3.0 Ca^{2+}, 1.2 Mg^{2+}, 125.8 Cl^-, 27.6 HCO_3^-, 3.2 $H_2PO_4^-$, and 10 glucose or mannitol) and gassed with 95% O_2/5% CO_2. The tissues were allowed to equilibrate for 60 min and voltage-clamped to zero potential difference by the continuous application of a short circuit current (I_{sc}). Changes in ion transport across the mucosa in response to agonists

were recorded as changes in I_{sc} (ΔI_{sc}) required to maintain a zero voltage clamp. In some experiments, intermittent measures of changes in open circuit potential differences (PD) were used as a more sensitive surrogate for active ion transport according to Ohm's law.

Immunoprecipitation and Western blotting

Human intestinal xenografts were stripped of seromuscular layers by blunt dissection. The tissue was placed in ice-cold lysis buffer (consisting of 20 mM Tris (pH 7.4), 20 mM NaCl, 10 mM $MgCl_2$, 1 mM $NaVO_4$, 1 μg/ml leupeptin, and 100 μg/ml phenylmethylsulfonyl fluoride), homogenized and incubated at 4°C for 30 min, then centrifuged at 10,000 *rpm* for 10 min and the pellet discarded. An aliquot was removed from each sample to determine protein content, and samples were adjusted so that they contained equal amounts of protein. Samples were boiled for 4 min and proteins separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (DuPont NEN) overnight at 4°C. The membranes were washed in 1% blocking buffer for 30 min, incubated with the appropriate dilution of primary antibody, and followed by washing in Tris buffered saline with 1% Tween (TBST). Following washes, horseradish peroxidase-conjugated secondary antibodies were added to the membrane in 1% blocking buffer, and allowed to incubate for an additional 30 min. This was followed by a further washing in TBST. Immunoreactive proteins were detected using an enhanced chemiluminescense detection kit (Amersham) and exposure of the membrane to X-ray film. The intensity of protein bands was determined by densitometry using NIH Image software.

Histological analysis of xenografts

For immunohistochemical analysis, intestinal xenografts were embedded in OCT compound (TissueTek, Miles Scientific, Naperville, IL, USA) and snap-frozen in methylbutane/dry ice, or fixed in 10% neutral buffered formalin. Formalin-fixed tissues were embedded in paraffin, and 5 μm sections prepared and stained with hematoxylin and eosin. From snap-frozen tissue samples, cryostat sections (5 μm) were prepared, fixed in 10% formalin for 10 min, and blocked (1% BSA, 0.2% blocking reagent, 0.3% Triton-X-100). These sections were incubated with a polyclonal antibody to NKCC1, or a monoclonal antibody to Na^+, K^+ ATPase α-subunit, washed in PBS, and incubated with Cy3-labelled donkey anti-goat IgG or Cy2-labelled anti-mouse IgG secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ).

Statistical analysis

All results are expressed as mean ± SEM for a series of experiments. Student's t-tests were used to compare paired data. One way analysis of variance with a Student Neuman-Keul post-test was used when three or more groups of data were compared. p-values less than 0.05 were considered significant.

RESULTS

Establishment of small intestinal xenografts as a functional model for ion transport studies

To establish human small intestinal xenografts as a model for ion transport studies, we first verified that tissues stayed morphologically intact throughout the time required for experiments. Freshly-isolated human intestinal xenografts (> 10 weeks) resembled normal intestine with fully developed crypts, villi and epithelium, consistent with previous reports [26], and shown in Figure 1A. Figure 1B depicts the histology of a similar tissue stripped of its seromuscular layers. The micrograph indicates the success of the stripping procedure, with only a few residual areas of circular muscle indicated by the arrows. Figure 1C depicts a stripped tissue after being mounted in an Ussing chamber for 2 hours. No morphological changes were seen, and specifically, the tissue retained an intact epithelium.

To study ion transport function in human intestinal xenografts, we mounted the tissue, stripped of seromuscular layers, in modified Ussing chambers (aperture 0.07 cm^2). After equilibration, the tissues were stimulated with the cAMP- dependent agonists, prostaglandin E_2 (PGE_2, 3 μM) or IBMX (1 μM), or the Ca^{2+}- dependent agonist, carbachol (CCh, 300 μM). As seen in Figures 2A and 2B, the tissue responded to the consecutive addition of agonists with an increase in I_{sc}. The cAMP-dependent agonists induced ion transport responses with sustained kinetics, whereas CCh induced transient responses. The functional polarity of the tissue was demonstrated by mucosal versus

serosal additions of CCh, whose receptors are located on the epithelial basolateral aspect. As seen in Figure 2C, the ion transport response obtained with mucosal addition of CCh was significantly less than that obtained with serosal addition.

To investigate whether the observed changes in ion transport were due to chloride secretion, mounted tissues were incubated with the sodium-potassium-chloride co-transporter (NKCC) inhibitor, bumetanide (100 μM), for 20 min prior to stimulation. Further, to determine the involvement of the M_3 muscarinic receptor, known to mediate the effects of CCh on chloride secretion in intestinal epithelial cell lines, an antagonist to this receptor, 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP, 100 nM) was used [10]. As shown in Figure 2C, both bumetanide and 4-DAMP decreased ΔI_{sc} after CCh stimulation by 78.2 ± 13.3 %, n=5, and 81.7 ± 7.6 %, n=4, respectively, indicating the ion transport responses were largely due to electrogenic chloride secretion and evoked by muscarinic M_3 receptor occupancy. These data demonstrate that the voltage clamp technique could reliably detect chloride secretory responses in human intestinal xenografts, which provided the basis for examining the effects of long-term exposure to cytokines, specifically IFN-γ, on ion transport function.

Effect of IFN-γ on xenograft ion transport responses

To examine the effects of IFN-γ on histological appearance of the xenografts, mice were injected with IFN-γ and the morphology of the xenograft tissue was observed. Treatment of intestinal xenografts with IFN-γ for 24 h did not induce any visible morphological changes compared to control tissues (Figure 3), although a slight decrease in the goblet

cell census may occur in IFN-γ-treated tissues. To show that IFN-γ had specific effects on the xenografts, we determined the expression of a known IFN-γ responsive gene, the chemokine, Mig [12]. As can be seen in Figure 3, IFN-γ treatment induced increased levels of mRNA for Mig. In contrast, two controls, cytokeratin 8, CK8, which is an epithelial cell specific gene, and GAPDH, which was assessed as a housekeeping gene that is not regulated by IFN-γ, showed no changes in expression after IFN-γ treatment, indicating that IFN-γ is able to exert a specific effect on small intestinal xenograft tissue *in vivo*.

We next examined whether IFN-γ altered basal epithelial electrophysiological properties. 24 h after i.p. injection of IFN-γ, xenografts were harvested, stripped, and mounted in modified Ussing chambers. No difference was observed in baseline potential difference (PD) between control and IFN-γ-treated tissues (Figure 4). On the other hand, conductance was enhanced in IFN-γ-treated tissues to a small but statistically significant extent compared with controls. Tissue conductance was 154.7 ± 11.8 *vs*. 186.3 ± 9.4 mS/cm^2 in control and IFN-γ-treated tissues, respectively (means ± SEM, $p<0.05$, n=23)

We subsequently studied whether stimulated ion transport responses were altered in xenografts exposed to IFN-γ. The effects of CCh and PGE_2 on ion transport were measured as decreases in PD in these experiments to improve sensitivity. Responses evoked by CCh (300 μM) were significantly reduced in IFN-γ-treated xenografts compared to control tissues (Figure 4A). Figure 4B shows a typical time course of responses to CCh in control and IFN-γ-treated tissues. We also tested whether the

cytokine altered responses to PGE_2. As shown in Figure 4A, PD responses to PGE_2 (3 μM) were also significantly reduced in IFN-γ-treated tissues compared to untreated tissues. Again, Figure 4C illustrates a typical time course of the PD response to PGE_2 in IFN-γ-treated and control xenografts. Note that for both CCh and PGE_2, the initial PD responses were similar in test and control xenografts, but the cytokine-exposed tissue could not sustain normal levels of ion transport over an extended period of time, as illustrated with the prolonged secretory response induced by the cAMP-dependent agonist PGE_2.

Levels of membrane transporters in IFN-γ-treated xenografts

To explore the mechanisms of decreased ion transport responses in IFN-γ-treated xenografts, we assayed protein levels of key membrane ion transporters. Western blotting experiments revealed that IFN-γ-treatment decreased expression of the Na^+, K^+, $2Cl^-$ co-transporter, NKCC1, by 57% compared to controls (Figure 5A). In contrast, levels of the calcium-activated chloride channel, CLCA, and the cAMP-activated Cl^- channel, CFTR, were similar in IFN-γ-treated and control human intestinal xenografts (Figure 5B + C). These data indicate that IFN-γ does not exert a general effect on membrane protein expression.

Having demonstrated that 24 h IFN-γ-treatment in human intestinal xenografts leads to a decrease in NKCC1 levels, but not in chloride channel levels, experiments were also carried out to determine if 24 h IFN-γ-treatment had any effect on Na^+,K^+ ATPase levels in intact human tissues. The levels of the α- as well as β-subunit of Na^+,K^+ ATPase

were determined by Western blotting. As seen in Figure 6A, 24 h IFN-γ-treatment reduced the levels of the catalytic α-subunit of Na^+,K^+ ATPase by 35% compared to control tissues. In contrast, no changes were observed in the levels of the β-subunit of Na^+,K^+ ATPase between IFN-γ-treated and control tissues (Figure 6B).

Immunohistological analysis of xenografts verified the decrease in ion transporters

To investigate if the decrease in ion transporters was apparent by histological analysis and to define the site(s) where protein levels were decreased, snap-frozen tissue was cut and stained for either NKCC1 or Na^+,K^+ATPase α-1 subunit. As illustrated in Figure 7, the decrease in both NKCC1 (A, B) and Na^+,K^+ ATPase α subunit (C, D) could in fact be visualized using immunohistochemical analyses. As illustrated by the Figure, both NKCC1 and Na^+,K^+ ATPase α-1 subunit were strictly limited to the epithelium, with NKCC1 being predominantly in crypt epithelial cells. Immunofluorescent labeling for both transport proteins was appreciably diminished in tissues harvested from mice treated with IFN-γ, verifying that the effects of the cytokine preferentially target the epithelium, at least for these proteins and in this xenograft model.

DISCUSSION

In both acute and chronic inflammation of the intestine, levels of pro-inflammatory cytokines are elevated [8]. Chronic inflammation, as in inflammatory bowel disease (IBD), is often accompanied by diarrhea, but the underlying mechanisms are not fully understood. Diarrhea can result from a leaky epithelium, enhanced chloride secretion (the primary driving force for fluid secretion), and/or decreased re-absorption of fluid [2,30]. Here we have established a model enabling us to investigate human intestinal physiological responses after prolonged exposure to cytokines. Our data show that treatment of human small intestinal xenografts with IFN-γ for 24 h attenuated secretion due to, at least in part, decreased levels of the ion transporters NKCC1 and the α-subunit of Na^+, K^+ ATPase.

The gross morphology of the intestinal xenografts was not altered after 24 h of IFN-γ treatment, as also observed in murine tissues [31], although in IFN-γ-treated tissue a slight decrease in goblet cells may be present. The conductance of IFN-γ-treated tissues was also elevated. Prior studies have yielded conflicting findings on the effect of IFN-γ on barrier function between cell lines and tissues. Barrier function in cell lines has been reported to be markedly reduced by IFN-γ [1,16,20], whereas mucosal resistance in tissues appeared to be unaltered, at least in some studies [8,31]. The findings in intact tissue are, however, in contrast to the findings in this study, where 24 h IFN-γ-treatment induced a small but significant reduction in mucosal resistance (or increase in conductance). It is possible that the time-frame and dose of IFN-γ are both critical to outcome and that longer periods of cytokine exposure might induce larger changes in this

particular epithelial parameter – or that these findings are specific to the small intestine. Notably, the studies of Bode *et al* [8], which utilized human colonic surgical specimens mounted in Ussing chambers, only followed responses to IFN-γ for about 2 hours, likely the longest time that was feasible. Species differences or segmental variation may also explain differences between our findings and those obtained using murine colon in organ culture [31].

In this study, both Ca^{2+}- and cAMP-dependent secretory responses were decreased in IFN-γ-treated xenografts compared to controls. With short term exposure (2 h) IFN-γ had no influence on active ion transport in human distal colon [8]. On the other hand, several reports have implicated IFN-γ as an important factor involved in down-regulation of active anion secretion in intestinal epithelial cell lines after longer exposure times [1,4,9,20,28], where Na^+,K^+-ATPase [9,28], K^+ channels and NKCC1 [9] have been suggested to be involved. The inhibition of I_{sc} by IFN-γ was also suggested [29] to be mediated by NO in experiments conducted in one epithelial cell line (Caco-2BBE), but in murine small intestine, NO played no role in the attenuation of I_{sc} [31]. In our study, elevated levels of inducible nitric oxide synthase (iNOS) protein were found in IFN-γ treated tissues compared to controls (data not shown), but concomitant treatment with iNOS inhibitors for 24 h was not feasible, so the functional significance of this observation for ion transport requires further investigation. Yoo *et al*. [31] have shown that in murine small intestine, IFN-γ down regulates Na^+-coupled glucose absorption as well as cyclic nucleotide-activated Cl^- secretion. Although these findings in mice, where IFN-γ treatment down-regulated Cl^- secretion and Na^+-K^+-ATPase activity, are consistent

with our work reported here, cytokines may in fact exert distinct effects in different species and organs. For example, TNF-α has been shown to stimulate ion secretion in human [8] and porcine [19] intestine, but to attenuate Cl^- secretion in canine tracheal epithelium [25]. Since this might point to species and/or organ differences in the epithelial response to inflammatory mediators, a human derived model system for studying cytokine-induced alterations in intestinal function over prolonged periods of time was greatly needed.

Further, in this study, 24 h treatment of human intestinal xenografts with IFN-γ caused decreased levels of NKCC1, whereas no difference was observed in levels of the chloride channels, CLCA and CFTR. Some authors have shown downregulation of CFTR, at least in cultured epithelial cells treated with IFN-γ, based on either expression levels [4,16,17] or function [9], but this has not been a consistent finding [28]. When effects of IFN-γ on CFTR expression were seen in cell lines [4] they were both time- and concentration-dependent. The precise concentration of IFN-γ in the xenograft is difficult to estimate, but it is conceivable that the i.p. administration of 10 μg to mice may result in concentrations towards the low end of those used in other studies. In addition, the 24 h time point selected here may be too early for alterations in CFTR to occur. In other studies, however, we have observed that effects of various treatments on cultured epithelial cells are actually accelerated in intestinal xenografts, perhaps reflecting the cooperation of subepithelial elements [3]. Moreover, both Western blot and histological studies revealed that the expression levels of NKCC1 and Na^+/K^+ ATPase α-subunit were already reduced after 24 h of IFN-γ-treatment, in contrast to our findings with chloride

channel expression. Others have shown that T cell activation in a mouse model decreased Na^+/K^+ ATPase activity, but not levels of either subunit, after 3 h [21]. This is in accordance with other observations showing that T cell activation induced luminal fluid accumulation by increasing mucosal permeability and reducing Na^+/K^+ ATPase activity [21]. This is also consistent with observations reported in T_{84} cells [28], as well as supporting the hypothesis of Holmgren *et al*. [18] who suggested that the effects of IFN-γ on transport are dependent on altered expression of transporters, and not on changes in second messengers. At the very least, our data show that secretory hyporesponsiveness evoked by IFN-γ in human intestine does not require changes in chloride channel expression. Nevertheless, we cannot exclude the possibility that, in addition to decreased levels of NKCC1 and Na^+/K^+ ATPase α-subunit, reduced expression of CFTR (and perhaps CLCA) could contribute to secretory dysfunction with longer periods of IFN-γ-exposure.

While the ion transport studies in this study were carried out in small intestinal xenografts and are therefore most directly relevant to Crohn's disease, where IFN-γ production from lymphocytes is greatly elevated [6], the similarities of secretory transport mechanisms in the small intestine and colon suggest that our findings may also be relevant to ulcerative colitis where elevated levels of IFN-γ also have been found [6]. Furthermore, our findings are relevant to the recent observation that IFN-γ may be selectively upregulated as an adaptive and homeostatic factor in a model of congenital diarrhea – the NHE3 knockout mouse where levels of CFTR but not NKCC1 or Na^+/K^+ APTase were decreased [30].

Much still remains to be learned about both physiological and pathophysiological effects of IFN-γ and other cytokines on the intestinal epithelium. The seeming paradox that IBD, with increased levels of IFN-γ, is associated with diarrhea while IFN-γ was found to have anti-secretory effects may be explained by the possibility that IFN-γ actually induces diarrhea by impairing absorption. Alternatively, the ability of this cytokine to reduce intestinal secretion could represent an adaptive response to injury, as also suggested by Shull and co-workers [30] in mice lacking NHE3. Since it allows for the study of cytokine effects over prolonged periods of time in human tissue, the model presented here enables studies that can provide novel insights into cytokine effects on intestinal epithelial transport and barrier function. Because of its ability to support chronic exposure, and the existence of species differences, the xenograft model may be the optimal one of those currently available to permit observations that are clinically relevant. Our findings from the current study suggest that elevated mucosal levels of IFN-γ affect the small intestine in an antisecretory fashion, and thus treatments that might reduce elevated levels of IFN-γ could in fact worsen diarrheal disease.

FOOTNOTES

1) *This work was presented, in part, at the 2000 and 2001 meetings of the American Gastroenterological Association and has been published in abstract form (Gastroenterology 118:A3118, 2000, Gastroenterology 120:A1014, 2001)*

2) *This work was supported by grants to KEB and LE from the National Institutes of Health (DK28305, DK35108 unit 5, and DK35108 core B)*

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4) *Abbreviations used: CLCA, calcium activated chloride channel; CCh, carbachol; CFTR, cystic fibrosis transmembrane conductance regulator; CK8, Cytokeratin 8; 4-DAMP, 4-diphenylacetoxy-N-methyl piperidine methiodide; IBD, inflammatory bowel disease; IBMX,3-Isobutyl-1-methylxanthine;* I_{sc}*, short circuit current;* ΔI_{sc}*, changes in short circuit current; IFN-γ, interferon gamma; NKCC1,* Na^+*,* K^+*,* $2Cl^-$ *co-transporter-1; PD, potential difference;* PGE_2*, prostaglandin* E_2*; RT-PCR, reverse transcription-polymerase chain reaction; SCID mice, severe-combined immunodeficient mice; TBST, Tris buffered saline with 1% tween.*

ACKNOWLEDGEMENT

We thank Jennifer R. Smith for expert technical support; Dr. Christian Lytle for the antibody to NKCC1; Dr. Cathy Fuller for the antibody to CLCA; and Ms. Glenda Wheeler for assistance with manuscript preparation.

Figure 1. Morphology of harvested xenografts. Human intestinal xenografts were fixed in 10% neutral buffered formalin before (A) or after (B) stripping off the seromuscular layers, as well as after mounting in Ussing chambers for 2 h (C). Histological analysis of xenografts showed that the morphology of the tissue remained intact through the whole procedure. The arrows in B depict residual circular muscle after stripping. Original magnifications: A; 4x, B; 10x, C; 10x.

Figure 2. Both cAMP- and Ca^{2+}-dependent agonists induce ion transport in xenografts. Intestinal xenografts were stripped of their seromuscular layers, mounted in modified Ussing chambers, and allowed to equilibrate for 1 h prior to stimulation with agonists. A and B depict the effects of calcium- (CCh, 300 μM) and cAMP- (PGE_2, 3 μM, IBMX, 1 μM) dependent agonist on net ion transport assessed as short circuit current (I_{sc}). In C, changes in I_{sc} induced by basolateral addition of CCh (300 μM) are compared with responses to apical addition of CCh, or responses to basolateral CCh in the presence of either the M_3 muscarinic antagonist, 4-DAMP, or the NKCC1 inhibitor, bumetanide. Values are means±SEM for n=4 experiments. Asterisks denote responses that are significant different from those evoked by basolateral CCh, *, $p < 0.05$, **, $p < 0.01$ by ANOVA.

Figure 3. Effect of IFN-γ on histology and gene expression in human intestinal xenografts. The upper panels depict the histological appearance of control and 24 h IFN-γ-treated xenografts stained with H&E. The lower panels show results from studies where

total RNA was isolated from control and IFN-γ-treated xenograft tissues and CK8, Mig, and GAPDH mRNAs were amplified by RT-PCR.

Figure 4. Effect of IFN-γ on transport responses to Ca^{2+}- and cAMP-dependent agonist in human intestinal xenografts. 24 hours after injection, control and IFN-γ-treated xenografts were stripped of their seromuscular layers, mounted in Ussing chambers and stimulated with agonists. A shows ion transport responses, measured as changes in PD at baseline or as evoked by the Ca^{2+}-dependent agonist, carbachol (CCh, 300 μM), and the cAMP-dependent agonist, PGE_2 (3 μM). Values are means±SEM for 11 experiments. Asterisks denote significant responses to secretagogue compared to no addition (*, $p<0.05$; **, $p<0.01$, by ANOVA). ## denotes that IFN-γ significantly reduced secretagogue-induced changes in PD compared to the corresponding value in the absence of cytokine treatment, $p<0.01$ by ANOVA. Panels B and C shows representative time courses of changes in PD evoked by CCh or PGE_2, respectively, in control and IFN-γ-treated tissues.

Figure 5. Effect of IFN-γ on levels of NKCC1 CLCA and CFTR in human intestinal xenografts. Proteins isolated from stripped human intestinal xenografts, either treated with IFN-γ (10 μg/mouse) for 24 h or controls, were analyzed by Western blotting with antibodies specific for NKCC1, CLCA or CFTR. Each panel shows a representative blot on the left, with densitometric analysis for several similar experiments on the right (a.u., arbitrary units). A: NKCC1; B: CLCA; C: CFTR. All values in right hand panels

are means±SEM for eight experiments. Asterisks denote a value that is significantly different from control, $p < 0.01$ by Student's t-test.

Figure 6. Effect of IFN-γ on levels of α- and β-subunits of Na^+,K^+ ATPase in human intestinal xenografts. Proteins isolated from stripped human intestinal xenografts, either treated with IFN-γ (10 μg/mouse) for 24 h or controls, were analyzed by Western blotting with antibodies specific for the α- and β-subunits of Na^+/K^+ ATPase. Each panel shows a representative blot on the left, with densitometric analysis for several similar experiments on the right (a.u., arbitrary units). A: Na^+/K^+ ATPase α-subunit; B: Na^+/K^+ ATPase β-subunit. All values in right hand panels are means±SEM for eight experiments. The asterisk denotes a value that is significantly different from control, $p < 0.05$ by Student's t-test.

Figure 7. Immunohistological analysis of NKCC1 and Na^+/K^+ ATPase α-subunit in xenograft tissues. Frozen sections of human intestinal xenografts, either controls or IFN-γ-treated for 24 h, were stained with antibodies specific for NKCC (red) or Na^+/K^+ ATPase α-subunit (green). The left panels show control tissues stained for NKCC or Na^+/K^+ ATPase α-subunit, respectively. The right Panels show staining for NKCC and Na^+/K^+ ATPase α-subunit, respectively, in tissues treated with IFN-γ for 24 h. Inserts are higher magnification of the boxed areas.

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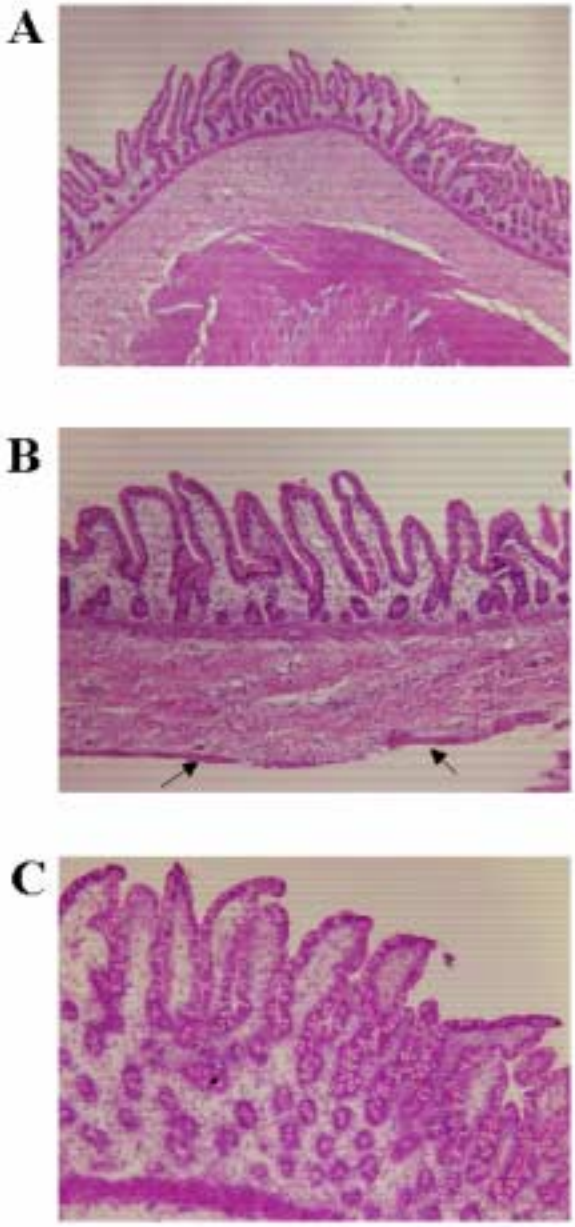

Figure 1

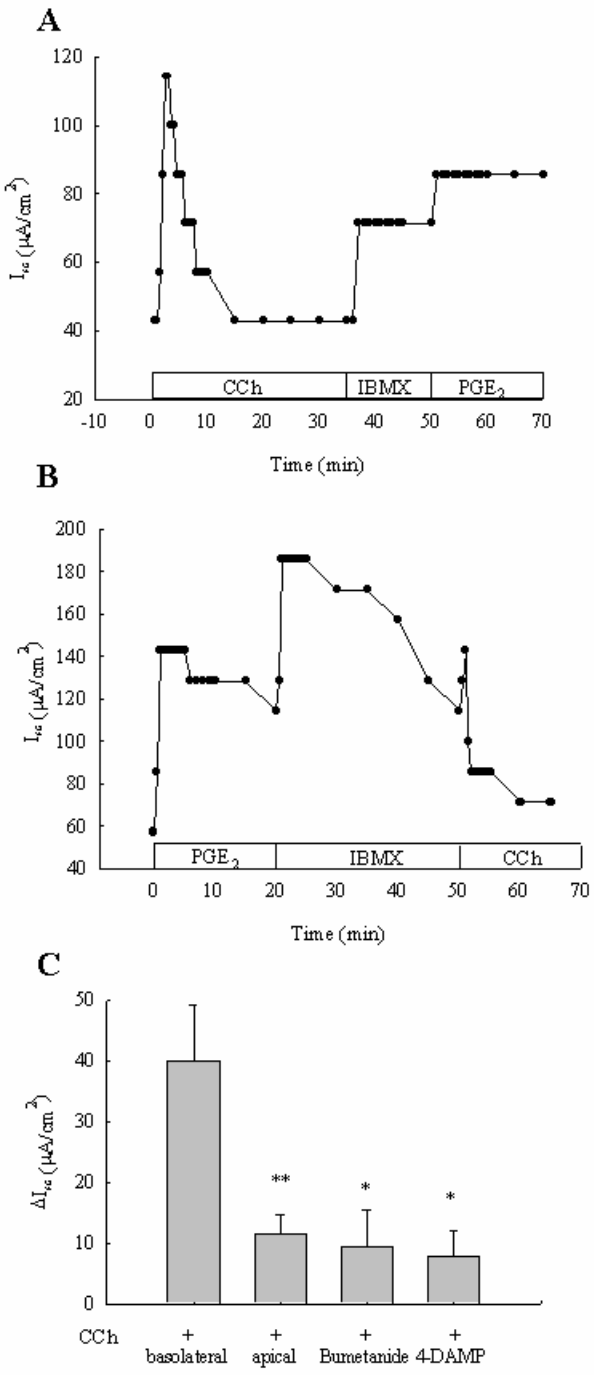
A
120
100
80
60
40
20
I_{sc} (μA/cm^2)
CCh
IBMX
PGE_2
-10
0
10
20
30
40
50
60
70
Time (min)
B
200
180
160
140
120
100
80
60
40
I_{sc} (μA/cm^2)
PGE_2
IBMX
CCh
0
10
20
30
40
50
60
70
Time (min)
C
50
40
30
20
10
0
ΔI_{sc} (μA/cm^2)
**
*
*
CCh
+
basolateral
+
apical
+
Bumetanide
+
4-DAMP

Figure 2

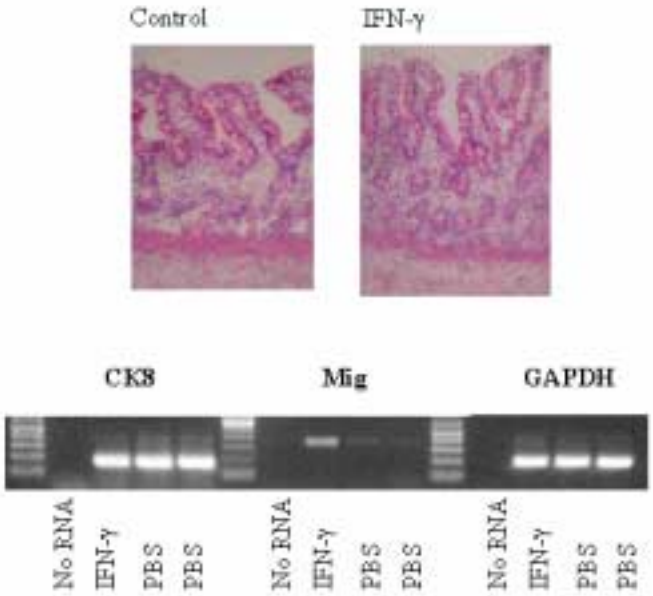
Control
IFN-γ
CK8
Mig
GAPDH
No RNA
IFN-γ
PBS
PBS
No RNA
IFN-γ
PBS
PBS
No RNA
IFN-γ
PBS
PBS

Figure 3

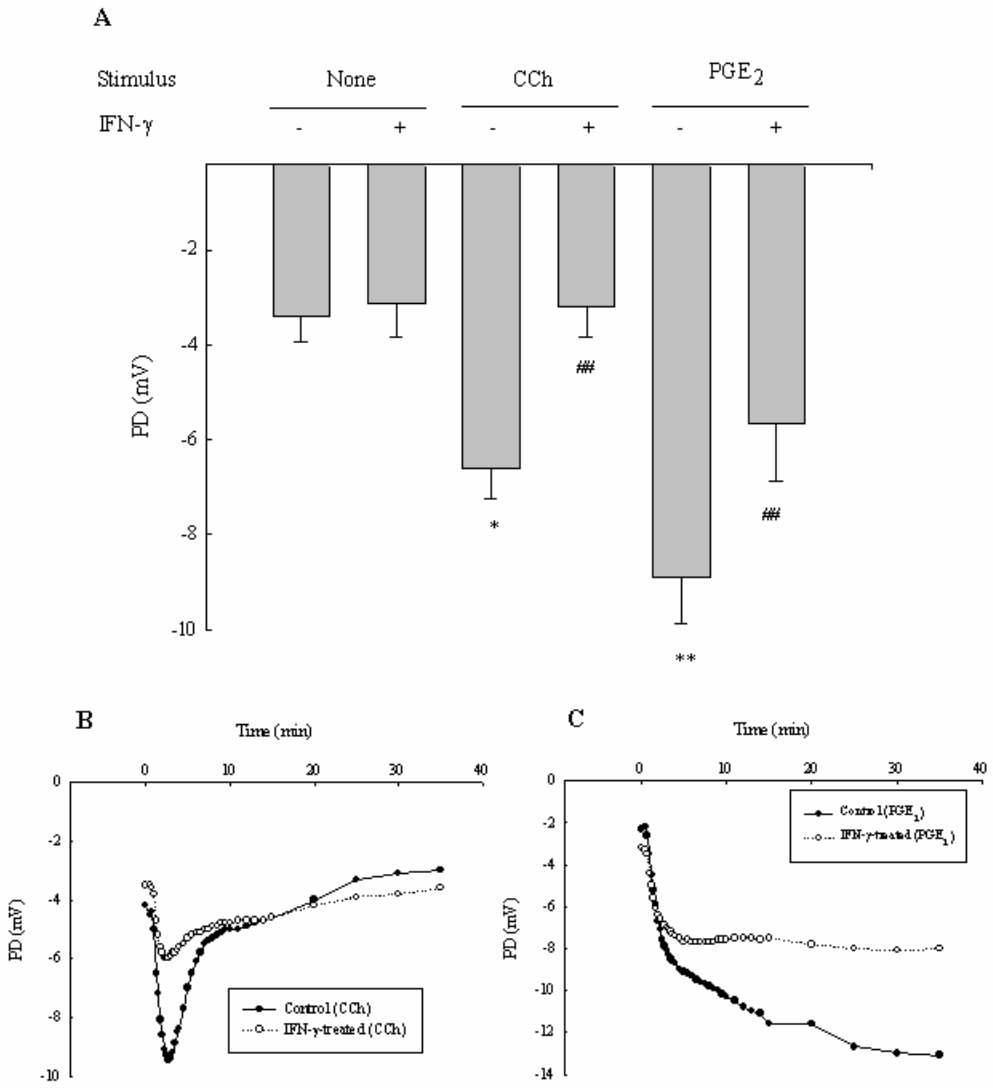
A
Stimulus
None
CCh
PGE2
IFN-γ
- + - + - +
PD (mV)
-2
-4
-6
-8
-10
*
##
**
##
B
Time (min)
0 10 20 30 40
PD (mV)
Control (CCh)
IFN-γ-treated (CCh)
C
Time (min)
0 10 20 30 40
PD (mV)
Control (PGE2)
IFN-γ-treated (PGE2)

Figure 4

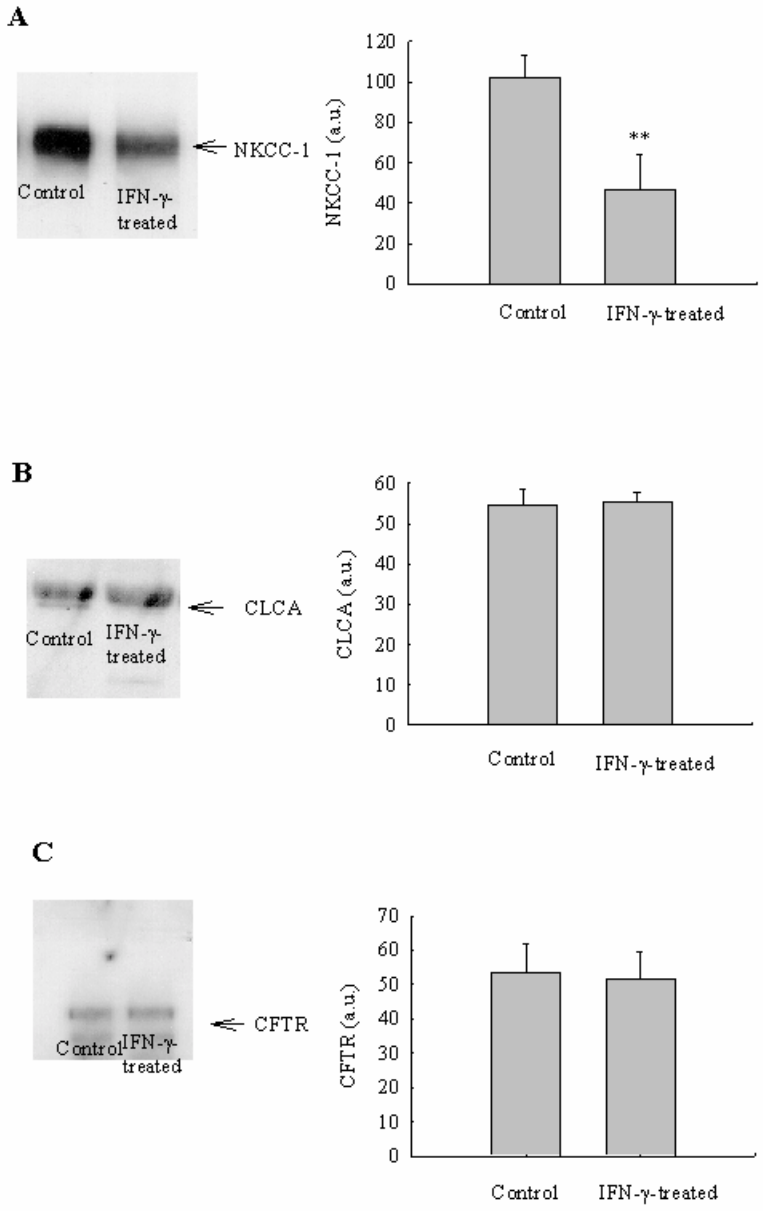
A
NKCC-1
Control
IFN-γ-treated
NKCC-1 (a.u.)
120
100
80
60
40
20
0
**
Control
IFN-γ-treated
B
CLCA
Control
IFN-γ-treated
CLCA (a.u.)
60
50
40
30
20
10
0
Control
IFN-γ-treated
C
CFTR
Control
IFN-γ-treated
CFTR (a.u.)
70
60
50
40
30
20
10
0
Control
IFN-γ-treated

Figure 5

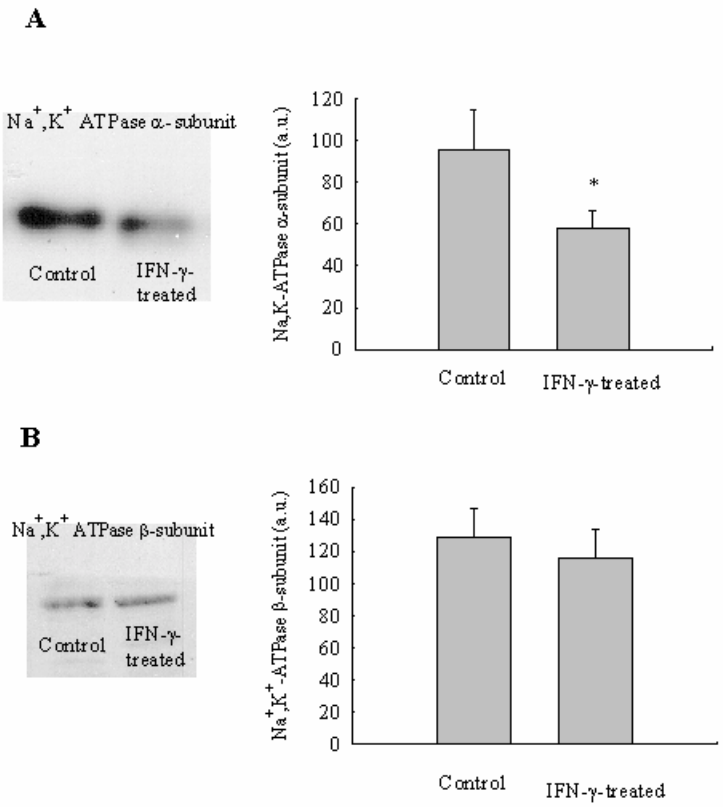
A
Na+,K+ ATPase α- subunit
Control
IFN-γ-treated
Na,K-ATPase α-subunit (a.u.)
120
100
80
60
40
20
0
*
Control
IFN-γ-treated
B
Na+,K+ ATPase β-subunit
Control
IFN-γ-treated
Na+,K+-ATPase β-subunit (a.u.)
160
140
120
100
80
60
40
20
0
Control
IFN-γ-treated

Figure 6

Figure 7