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Tumor Necrosis Factor-α Increases Sodium and Chloride Conductance Across the Tight Junction of CACO-2 BBE, a Human Intestinal Epithelial Cell Line

C.W. Marano[1], **S.A. Lewis**[2], **L.A. Garulacan**[1], **A. Peralta Soler**[1], **J.M. Mullin**[1]

[1]Lankenau Medical Research Center, 100 Lancaster Ave., Wynnewood, PA 19096, USA
[2]University of Texas Medical Branch, Galveston, TX, USA

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Abstract. CACO-2 BBE was used to determine the response of a gastrointestinal epithelium to tumor necrosis factor-α (TNF). Incubation of CACO-2 BBE with TNF did not produce any effect on transepithelial resistance (TER) within the first 6 hr but resulted in a 40–50% reduction in TER and a 30% decrease in I_{sc} (short circuit current) relative to time-matched control at 24 hr. The decrease in TER was sustained up to 1 week following treatment with TNF and was not associated with a significant increase in the transepithelial flux of [^{14}C]-D-mannitol or the penetration of ruthenium red into the lateral intercellular space. Dilution potential and transepithelial $^{22}Na^+$ flux studies demonstrated that TNF-treatment of CACO-2 BBE cell sheets increased the paracellular permeability of the epithelium to Na^+ and Cl^-. The increased transepithelial permeability did not associate with an increase in the incidence of apoptosis. However, there was a TNF-dependent increase in [^{3}H]-thymidine labeling that was not accompanied by a change in DNA content of the cell sheet. The increase in transepithelial permeability was concluded to be across the tight junction because: (i) 1 mM apical amiloride reduced the basolateral to apical flux of $^{22}Na^+$, and (ii) dilution potential studies revealed a bidirectionally increased permeability to both Na^+ and Cl^-. These data suggest that the increase in transepithelial permeability across TNF-treated CACO-2 BBE cell sheets arises from an alteration in the charge selectivity of the paracellular conductive pathway that is not accompanied by a change in its size selectivity.

Key words: Tumor necrosis factor — Cytokine — Intestine — Epithelia — Tight junction — Paracellular

Correspondence to: C.W. Marano

Introduction

Chronic gastrointestinal diseases, such as ulcerative colitis and Crohn's disease, are characterized by increased penetration of inflammatory white blood cells into the lamina propria and submucosa of the small and large bowel (Elson, 1996). Studies have shown evidence for increased mRNA and protein levels for TNF in colonic biopsy specimens from these patients (Murch et al., 1993; Breese et al., 1994) as well as evidence that the intestinal permeability of these patients and their first degree relatives is increased (Hollander et al., 1986; Malin et al., 1996). Furthermore, in vitro studies from other laboratories have demonstrated cytokine-induced alterations in ion transport and immunological properties of gastrointestinal epithelial cells (Colgan et al., 1994*a*,*b;* McKay, Croitoru & Perdue, 1996; Madsen et al., 1996). However, with regard to TNF these studies either concluded that this cytokine was without effect on the barrier properties of the epithelium under study (Madara & Stafford, 1989) or had effects attributable to a cytotoxic response to TNF and not a physiological regulation of transepithelial permeability (McKay et al., 1996).

Earlier studies from our laboratory, using a model epithelium generated by confluent LLC-PK$_1$ renal epithelial cells in culture, have demonstrated that within 2 hr of presentation of TNF to the cell sheet there is a transient and rapidly reversible decrease in transepithelial resistance (Mullin & Snock, 1990). The decrease in TER is accompanied by an increase in transepithelial mannitol flux and includes an increased transepithelial flux to molecules as large as raffinose (MW 360) and polyethylene glycol (4000 MW; Mullin et al., 1997). Furthermore, the decrease in TER associates with a 3-fold increase in the incidence of apoptosis in the cell sheet that requires a functionally intact cytoskeleton for restoration of the barrier integrity of the epithelium fol-

lowing treatment with TNF (Peralta Soler et al., 1996). These observations of TNF's effect on the transepithelial permeability of LLC-PK_1 epithelium as well as the successful induction of remission in patients with Crohn's disease by treatment with an anti-TNF chimeric monoclonal antibody (Van Dullemen et al., 1995), however, suggests a need to reevaluate the role of TNF in inflammatory bowel disease and in particular its effects on mucosal barrier integrity.

CACO-2 BBE epithelial cell sheets represent one potential in vitro model system in which to assess the effect of TNF on gastrointestinal transepithelial permeability. Isolated by Peterson and Mooseker (1992), CACO-2 BBE is a clone derived from the CACO-2 cell line originally isolated from a human colonic adenocarcinoma (Fogh, Fogh & Orfeo, 1977). Both CACO-2 and its CACO-2 BBE clone, form polarized epithelial monolayers with an apical brush border membrane morphologically and immunologically comparable to fetal human small intestine (Pinto et al., 1983; Peterson & Mooseker, 1992). Both cell lines are characterized by features typical of differentiated enterocytes with a well-developed microvillar surface in addition to expression of small intestinal brush border hydrolases, e.g., sucrose isomaltase, alkaline phosphatase, aminopeptidase N and dipeptidylpeptidase V (Pinto et al., 1983; Zweibaum et al., 1983, 1984; Peterson & Mooseker, 1992). CACO-2 has been extensively utilized to study the permeability of intestinal epithelium to nutrient and pharmaceutical molecules (Hidalgo, Raub & Borchardt, 1989; Riley et al., 1991; Noach et al., 1994; Thwaites et al., 1995, 1996). Parental CACO-2 as well as CACO-2 BBE have been studied with regard to permeability changes induced by protein kinase C activation (Stenson et al., 1993), energy depletion (Salzman et al., 1995; Unno et al., 1996) and cytoskeletal disruption (Ma et al., 1995). It was our objective in these studies to determine whether a TNF-induced increase in transepithelial permeability could be measured in this model of gastrointestinal epithelium and if so, to characterize the physiology of the TNF-induced alteration in the epithelial barrier function.

Materials and Methods

Cell Culture

The CACO-2 BBE cell line, originally cloned by Peterson and Mooseker (1992) was obtained from American Type Tissue Culture (CRL-2102). The cells were maintained in Dulbecco's Modified Essential Medium high glucose (DMEM; Gibco) supplemented with 10% fetal bovine serum (Hyclone, Logan, Ut.), 2 mM *L*-glutamine, 1 mM sodium pyruvate and 10 μg/ml human transferrin (Gibco) at 37°C and 95% air:5% CO_2. The cells were routinely passaged upon attaining confluence 7 days after seeding at 1×10^6 per T75 cm^2 flask. For these studies, cells between passages 46 and 65 were seeded at a confluent density of 1×10^6 cells per Millicell PCF (4.2 cm^2; Millipore, MA); Anocell (4.0 cm^2; Anotec Separations, Branbury, UK) or Falcon 3102 (4.5 cm^2; Becton Dickinson Labware, Franklin Lakes, NJ) filter ring assemblies. Cell sheets were refed on days 1, 3 and 6 postseeding. All studies were then begun on confluent epithelial cell sheets at 7 days postseeding. Mycoplasma tests, based on an immunofluorescent assay for mycoplasma DNA using Hoechst dye (Chen, 1977), were performed routinely and were negative.

To initiate the experiments on day 7 postseeding, cell sheets were divided into control and TNF treatment groups. They were then fed medium ± TNF (100 ng/ml; Biosource International, Camarillo, CA) on both the apical and basolateral surfaces of the epithelial cell sheet for a period of 24 hr unless otherwise indicated by the study. Following the incubation ± TNF, all cell sheets were typically refed control medium for an additional 24 hr period at 37°C and 95% air:5% CO_2. At 48 hr (day 9 postseeding) cell sheets were all refed control medium 1 hr prior to the measurement of spontaneous potential difference (PD) and transepithelial resistance (TER) to determine the effects of TNF treatment. Additional refeeds with control medium ± agents dictated by the particular study where then conducted.

Electrophysiological Measurements

These methods have been detailed in earlier publications from our laboratory (Mullin & McGinn, 1988). At 0 hr (48 hr following the initiation of TNF treatment as described above) the PD across the cell sheets was measured using a Fluke 8020B multimeter and 1 M NaCl agar-salt bridges in series with calomel electrodes. The cell sheets were then transferred to a modified Ussing chamber for measurement of the TER. Briefly, a voltage deflection, produced by a 1 sec 40 μA direct current pulse, was measured using a Keithley 197 autoranging digital multimeter, and the TER was calculated using Ohm's law. Short circuit current (I_{sc}) was indirectly determined by dividing the PD at 37°C by the TER. These measurements represent the initial readings. After these determinations the cell sheets were refed as indicated by the particular study. For electrophysiological studies using diuretics, cell sheets were left at room temperature for 2- and 5-min determinations of TER.

Transepithelial [^{14}C]-D-mannitol Flux Measurements

Following a 1 hr refeed with control medium, the PD and TER were measured across both control and TNF-treated CACO-2 BBE cell sheets prior to the start of the mannitol flux. The cell sheets were then refed 2 ml of control medium in the apical compartment (ring) and 15 ml of medium supplemented with 1 μCi/ml of 0.1 mM [^{14}C]-D-mannitol (Dupont NEN, 54.5 mCi/mmol) added to the basolateral compartment. Cell sheets were returned to 37°C and 95% air:5% CO_2 for 2 hr at which time 25 μl samples were withdrawn from the apical compartment for liquid scintillation counting of [^{14}C]-D-mannitol. The net unidirectional flux of [^{14}C]-D-mannitol was calculated from the disintegrations per minute of [^{14}C]-D-mannitol present in the apical fluid compartment as described previously (Mullin, Fluk & Kleinzeller, 1986).

Transepithelial $^{22}Na^+$ Flux Measurements

Following the determination of PD and TER across control and TNF-treated CACO-2 BBE cell sheets, the cell sheets were refed 2 ml of control medium ± 1 mM amiloride to the apical compartment and 15 ml of the same medium supplemented with 5×10^{-4} μCi/ml of $^{22}Na^+$

(Dupont NEN) to the basolateral compartment (±1 mM amiloride). The cell sheets were returned to 37°C and 95% air:5% CO_2 pending the sampling of 50 μl aliquots of apical medium for liquid scintillation counting at 15-min intervals up to 75 min. The rate of transepithelial $^{22}Na^+$ flux in the presence of 1 mM amiloride (amiloride-insensitive) and in the absence of the diuretic (total) was derived from a slope of the graph of counts per minute (cpm) of $^{22}Na^+$ appearing in the apical compartment as a function of time. Amiloride-sensitive flux was calculated as the difference between total flux and the flux remaining in the presence of amiloride.

Dilution Potential Measurements

As described above, measurements of PD and TER at 37°C were made on every control and TNF-treated cell sheet. Following these measurements, the cell sheets were maintained at room temperature for the duration of the dilution potential protocol. Culture medium was aspirated and the cell sheets were rinsed three times in Na^+ saline. At t = 0 min the cell sheets were refed with the appropriate saline with 2 ml added to the apical and 15 ml to the basolateral compartment. The PD was then determined at 5 min intervals up to 15 min as described in a previous paper from this laboratory (Marano et al., 1993). The composition of the Na^+ saline [3-(N-morpholino)propane sulfonic acid (MOPS)-buffered saline has been previously described (Mullin et al., 1980). The ''low Na^+'' saline contained only 87 mM NaCl (plus other salts) and 91 mM mannitol to balance osmolarity. All salines were pH 7.3 and 305 mOsm. At the end of each dilution potential study, the salines were removed, the cell sheets were refed with control medium and then returned to 37°C and 95% air:5% CO_2 for 1 hr. At this time, PD and TER were measured to determine the integrity of the cell sheets following the manipulations described above.

[^{3}H]-Thymidine Labeling and DNA Content

The protocol for thymidine labeling and analysis of DNA content has been described in an earlier publication from our laboratory (Marano et al., 1993). Briefly, CACO-2 BBE cell sheets were seeded at confluent density onto Millicell PCF filter assemblies as described above. To initiate the experiment on day 7 postseeding, cell sheets were pulsed with TNF (100 ng/ml) for 5 min on both their apical and basolateral surfaces at 37°C and 95% air:5% CO_2. The cell sheets were then refed with control medium after the 5-min incubation, at 24 hr and then again at 47 hr following treatment. At 48 hr, the TER and PD of the cell sheets were measured. Cell sheets were then refed medium supplemented with 5 μCi/ml of [methyl-^{3}H-]-thymidine (6.7 Ci/mmol, DuPont NEN). Filters were incubated under these conditions for 4 hr at 37°C and 95% air:5% CO_2. At the incubation's end, the culture medium was removed and cell sheets were rinsed three times in phosphate buffered saline (PBS) and then scrapped into 1.5 ml of PBS. One ml of the cell suspension was transferred to a 15 ml conical tube containing 1 ml of 0.8 M Perchloric acid to lyse the cells, release soluble nucleotide and precipitate the DNA. Samples of the initial supernatant containing soluble nucleotides and the supernatant arising from the hydrolysis of the precipitate were taken for liquid scintillation counting. The latter supernatant was also subjected to the diphenylamine assay for the determination of total DNA (Burton, 1956).

Bromodeoxyuridine Labeling

CACO-2 BBE cell sheets incubated in the presence or absence of TNF (100 ng/ml) for 24 hr were simultaneously labeled with Amersham cell proliferation labeling reagent diluted at 1:1000 in the culture medium for the entire duration of the incubation. At the incubation's end the cell sheets were washed 2 × 5 min with phosphate buffered saline (PBS). The cell sheets were then fixed in Fekete's for 30 min at room temperature. The fixed cell sheets were then rinsed in PBS 3 × 5 min followed by a 30 min incubation with 10% normal goat serum at room temperature. BrdU labeled cells were detected using a monoclonal anti-BrdU from Amersham, added to the cell sheets undiluted for 1 hr at room temperature. This was followed by incubation with CY3 goat anti-mouse antibody (1:100) for 1 hr in the dark at room temperature to detect the anti-BrdU. Cell sheets were then washed 3 × 5 min in PBS followed by a quick rinse in dH_2O. The cell sheets were then mounted in glycerol and viewed with a fluorescent microscope.

Quantitative Morphological Assessment of Apoptosis

Confluent cell sheets, seeded onto Anocell filter ring assemblies, were analyzed 24 and 72 hr after treatment ± TNF. TER was measured in each sample prior to processing for histology as previously described (Peralta Soler et al., 1996). The cell sheet-containing filters were rinsed in phosphate buffered saline (PBS) and fixed in Carnoy's fixative for 1 hr. The cell sheets were then rinsed in PBS, stained in Harris' hematoxylin (Fisher Scientific, Malvern, PA) for 2 min and dehydrated. The filters were detached from their ring assemblies with xylene and mounted on glass slides. Clusters of characteristic apoptotic fragments were counted using a 40× objective as described earlier (Peralta Soler et al., 1996).

Electron Microscopy and Ruthenium Red Penetration

Materials for electron microscopy were purchased from Electron Microscopy Sciences (Fort Washington, PA). Cell sheets grown on Millicell PCF (Millipore, Bedford, MA) filter inserts were fixed in 2.5% glutaraldehyde containing 0.2% of the electron-dense dye, ruthenium red, on the apical surface for 30 min. After rinsing in 0.1 M sodium cacodylate buffer (pH 7.4), the apical surface of the cell sheets was reexposed to osmium-containing ruthenium red for an additional 30 min. After rinsing, the cell sheets were dehydrated, cut into small pieces and embedded in Spurr's resin. Ultrathin sections perpendicular to the support filter were cut in a Reichert Ultracut S ultramicrotome and photographed in a JEOL 1200 EXII electron microscope (Peralta Soler et al., 1996).

Calculations and Statistical Analysis

The ratio of the chloride to sodium permeability (P_{Cl}/P_{Na}) of the CACO-2 BBE epithelial monolayer was calculated using the Hodgkin-Goldman-Katz equation (Goldman, 1943; Hodgkin & Katz, 1949) and the change in the transepithelial voltage measured during the dilution potential experiments. The dilution potential measurements were corrected for junction potentials using the method described by Barry (1989). The transepithelial permeability of the CACO-2 BBE monolayer to Na^+ and Cl^- (P_{Na} and P_{Cl}, respectively) was then calculated using the previously determined P_{Cl}/P_{Na}, the measured transepithelial conductance (G_t which is the inverse of TER) and the Kimizuka-Koketsu equation (Kimizuka & Koketsu, 1964; Kimizuka, 1966; Delamere & Duncan, 1977).

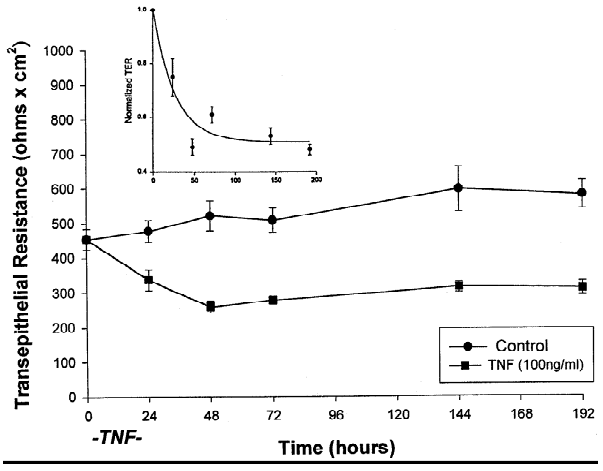

Fig. 1. TNF causes a sustained increase in transepithelial permeability across CACO-2 BBE cell sheets. Data represent the mean ± SE of 4–7 cell sheets per time point measured during two separate experiments. Briefly, cell sheets were cultured at confluent density onto Millicell PCF filter assemblies as described in Materials and Methods. At $t = 0$ hr cell sheets were fed medium ± TNF (100 ng/ml) for a period of 24 hr. All cell sheets were then refed control medium. TER and spontaneous potential difference measurements were taken at the indicated intervals. *Inset:* The time course of the decrease in normalized resistance can be described as the sum of a TNF-sensitive fraction of the TER (which decreases as a single exponential) plus a TNF-insensitive fraction. Best fit values are a TNF-insensitive fraction with a TER of 0.51, a TNF-sensitive fraction of the TER of 0.50 and a time constant for the TNF-sensitive fraction of 26 hr. The time constant represents the time required for the TNF-sensitive fraction of TER to decrease by 63%. Normalized TER was calculated by dividing the mean of the control TER into the TER following TNF treatment at each time point.

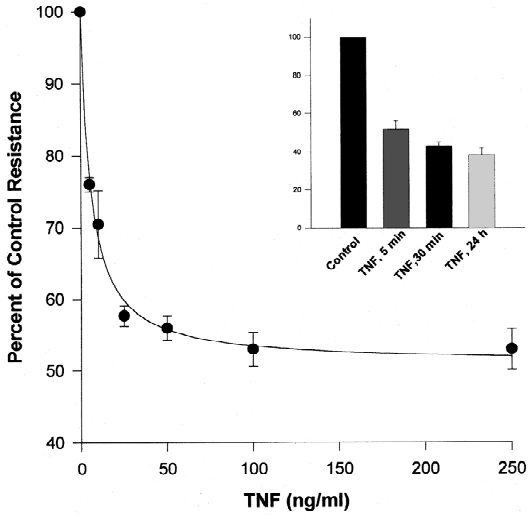

Fig. 2. The dose-dependency of the TNF-induced increase in transepithelial permeability and its independence from the duration of exposure to TNF. Data represent the mean ± SE of up to 8 cell sheets per condition from two separate experiments. Briefly, cell sheets were cultured at confluent density onto Millicell PCF filter assemblies as described in Materials and Methods. At $t = 0$ hr cell sheets were fed medium supplemented with TNF (5–500 ng/ml) for a period of 24 hr. At the end of the incubation with TNF the cell sheets were refed with control medium as described in Materials and Methods. TER and spontaneous potential difference were then measured at 48 hr. The smooth curve is the best fit of Michaelis-Menton equation to the data, giving a best fit dissociation constant of 6 ng/mL. *Inset:* TNF-induced increase transepithelial permeability as a function of exposure time to TNF. Data represent the mean ± SE of 4 cell sheets per condition from an individual experiment. Cell sheets were incubated ± TNF (100 ng/ml) for a period of 5 min, 30 min, or 24 hr. The cell sheets were refed and their TER and PD measured as described above. The data were curve fit by a single exponential and yielded a time constant of 3 min.

All data are expressed as the mean ± SE. Paired and unpaired Students *t*-tests were used to determine significance (GraphPad Instat, San Diego, CA). $P \leq 0.05$ was considered to be significant.

Results

Following a 24 hr incubation with TNF (100 ng/ml), CACO-2 BBE cell sheets showed a decrease in transepithelial resistance (TER) compared to time-matched controls. The decrease in TER was measurable at 24 hr, reached a maximum at 48 hr and was sustained up to 8 days following TNF treatment (Fig. 1). The decrease in TER across the CACO-2 BBE cell sheets was apparent with as little as 5 ng/ml TNF and approached a maximum effect between 50 and 100 ng/ml of the cytokine (Fig. 2). No additional effect on transepithelial permeability was observed with concentrations of TNF up to 500 ng/ml. While the experiments reported herein demonstrate the effect of TNF applied to both the apical and basolateral surfaces of the CACO-2 BBE epithelium, TNF was just as effective at increasing transepithelial permeability when applied to only the apical or basolateral surface (*data not shown*). Furthermore, the increase in transepithelial permeability at 24 hr could be elicited whether TNF exposure of the cell sheet was continuous or for as short a duration as 5 min (Fig. 2). The effect of TNF on transepithelial permeability was unique and not elicited in response to incubation of the cell sheets with IL-6, IL-8, or IFN-γ, cytokines implicated in the alteration of the epithelial phenotype (*data not shown;* Colgan et al., 1994*a*,*b;* McKay et al., 1996).

Unlike the renal epithelial cell sheet generated by LLC-PK_1 in which the TNF-induced decrease in transepithelial resistance establishes within 2 hr and associates with an increased incidence of apoptosis, there was no morphological evidence for an increase in apoptosis in the CACO-2 BBE epithelial cell sheets. Actually following treatment of the cell sheets with TNF for 24 hr there was a significant reduction ($P \leq 0.05$) in the number of apoptotic fragments detectable at the light microscopic level [4.8 ± 0.7 (28 fields/1 filter) *vs.* 3.2 ± 0.3 (27 fields/1 filter) apoptotic fragments, control *vs.* TNF-treated, 24 hr]. This observation remained valid at 72 h [3.8 ± 0.4 (28 fields/1 filter) *vs.* 2.2 ± 0.3 (28 fields/1

Table 1. TNF increases thymidine labeling without altering DNA content in CACO-2 BBE epithelial cell sheets

	TER (ohms xcm^2)	DNA (μg/ml)	Thymidine Labeling (dpm [^{3}H]-thymidine/ μg DNA)
Control	329 ± 7 (28)	73 ± 7 (24)	$1.6 \pm 0.3 \times 10^4$ (12)
TNF (100 ng/ml)	232 ± 6 (28)	78 ± 6 (27)	$2.3 \pm 0.3 \times 10^4$ (11)

Data represent the mean ± SE of (*n*) cell sheets from 5 separate experiments. Cell sheets were cultured and treated with TNF as described in Materials and Methods. At $t = 0$ hr, following measurement of TER and PD, the DNA content and/or the incorporation of [^{3}H]-thymidine into the PCA-insoluble fraction of the cell sheets were determined as detailed in Materials and Methods.

Table 2. Treatment with TNF does not increase the transepithelial flux of [^{14}C]-D-mannitol across CACO-2 BBE cell sheets

	Time (hr) after initial TNF exposure			
	24	48	72	144
Control	1.5 ± 0.3[a]	0.72 ± 0.04	0.65 ± 0.04	0.92 ± 0.03
TNF	1.7 ± 0.4	0.85 ± 0.06	0.85 ± 0.07[b]	0.98 ± 0.03

[a] Data represent the mean ± SE of 6-11 cell sheets from 3 separate experiments presented as 10^{-4} nmole mannitol/hr/cm^2. Cell sheets were cultured and treated with TNF for 24 hr as described in Materials and Methods. At $t = 0$ hr, following measurement of TER and PD across the respective CACO-2 BBE cell sheets, 0.1 mM ^{14}C-[D]-mannitol was added to the basolateral compartment and its appearance in the apical compartment measured after 2 hr.
[b] ($P \leqslant 0.03$) *vs.* time-matched control.

filter) apoptotic fragments ($P \leqslant 0.05$) in control and TNF-treated cell sheets, respectively]. This absence of increased apoptosis in the cell sheet was independently confirmed by Hoechst staining of the nuclei (*data not shown*). Interestingly, though, in the absence of apoptosis in the CACO-2 BBE cell sheet, there was an increase in the incorporation of [^{3}H]-thymidine into the PCA insoluble fraction that was unaccompanied by any significant increase in the total content of DNA (Table 1). The lack of effect on total DNA content was independently confirmed using bromodeoxyuridine labeling of the cell sheet. Counts of total and BrdU labeled nuclei showed no difference between control and TNF-treated CACO-2 BBE cell sheets (136 ± 5 *vs.* 127 ± 5 total nuclei, control *vs.* TNF and 6.2 ± 1.8 *vs.* 7.7 ± 2.1 BrdU-labeled nuclei, control *vs.* TNF).

The reduction in TER across TNF-treated CACO-2 BBE epithelial cell sheets was not associated with an increase in the transepithelial flux of [^{14}C]-D-mannitol, a marker of paracellular permeability, across the epithelium. Despite the sustained decrease in TER there was only a small increase in mannitol accumulation across the CACO-2 BBE epithelium up to 6 days following treatment with TNF (Table 2). However, the increase in transepithelial flux did not routinely achieve statistical significance while the decrease in TER did. This observation was further confirmed at the microscopic level using the electron-dense dye ruthenium red. There was no penetration of this dye into the lateral intercellular spaces of either control or TNF-treated CACO-2 BBE cell sheets (Fig. 3) across the tight junction. Furthermore, there were no ultrastructural alterations in the tight junctions of the TNF-treated cell sheet.

Since a change in transepithelial permeability may mean either a change in solute transport through the cells (transcellular) or between the cells (paracellular), we examined short circuit current (I_{sc}) as well as TER. With TNF treatment of the CACO-2 BBE cell sheet, there was a consistently observed but transient decrease in I_{sc} of about 30% relative to control at 48 hr suggesting a reduction in the movement of charged ionic species *through the cells* of the epithelium. The decrease in I_{sc} was transient and matched control values within 144 hr (Fig. 4). This *transient* TNF-induced *decrease* in I_{sc} makes it unlikely that an increase in transcellular conductance is responsible for the measured reduction in TER (Fig. 4). Similar to the decrease in TER, the reduction in I_{sc} at 48 hr was also independent of the duration of exposure to TNF (*data not shown*).

With the elimination of increased conductance through the transcellular pathway as the cause of the increased transepithelial permeability observed across TNF-treated CACO-2 BBE cell sheets, we went on to further examine the effect of TNF treatment on the conductance through the paracellular pathway. Flow through the paracellular pathway is restricted by the presence of the tight junction, a circumferential band of apically situated proteins that serve to selectively regulate the movement of ions and larger molecular weight solutes between the compartments separated by the epithelium (Farquhar & Palade, 1965; Schneeberger & Lynch, 1992). We had already observed that TNF treatment did not affect the size selectivity across the CACO-2 BBE cell sheet as mannitol flux and ruthenium red penetration were similar between control and TNF treated cells. Since the TER, as a measure of paracellular permeability (for ''leaky'' epithelia), is determined by both the relative charge and size selectivity of the tight junction, we decided to then examine the charge selectivity of the CACO-2 BBE epithelial cell sheet using dilution potential and transepithelial $^{22}Na^+$ flux protocols.

With a 50 mM gradient for NaCl across the CACO-2 BBE cell sheets, the dilution potential protocol revealed an increase in Na^+ and Cl^- conductance (P_{Na} and P_{Cl}, respectively) across the TNF-treated CACO-2 BBE epithelial cell sheets (Table 3). The increased conductance was accompanied by a decrease in the ratio of P_{Cl} to P_{Na}

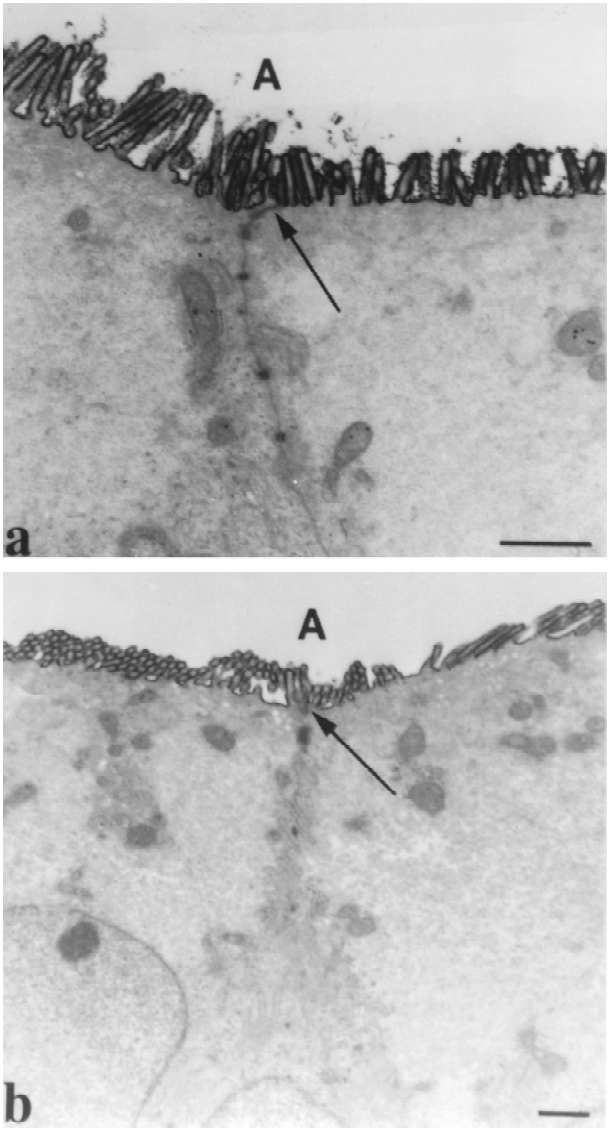

Fig. 3. TNF does not alter the morphology of the tight junction and permeability to ruthenium red between CACO-2 BBE epithelial cells. Cell sheets were cultured at confluent density onto Millicell PCF filter assemblies as described in Materials and Methods. At $t = 0$ hr cell sheets were fed medium ± TNF (100 ng/ml) for a period of 24 hr. All cell sheets were then refed control medium for an additional 24 hr period after which time PD and TER were measured. Cell sheets were then fixed in the presence of ruthenium red applied on the apical surface (A) and processed for electron microscopy as described in Materials and Methods. The arrows indicate the tight junctions. Note the absence of ruthenium red through the tight junctions. The bar equals 1 μM, ''A'' marks the apical surface for *a:* Control (TER: 255 ± 6 ohms × cm^2) and *b:* TNF (100 ng/ml, 24 hr; TER: 137 ± 13 ohms × cm^2)-treated CACO-2 BBE epithelial cell sheets.

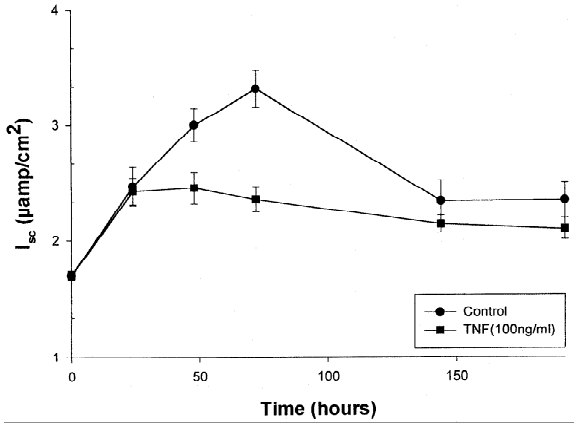

Fig. 4. TNF causes a transient decrease in the short circuit current across CACO-2 BBE cell sheets. Data represent the mean ± SE of 4–7 cell sheets per time point measured during two separate experiments. Briefly, cell sheets were cultured at confluent density onto Millicell PCF filter assemblies as described in Materials and Methods. At $t = 0$ hr cell sheets were fed medium ± TNF (100 ng/ml) for a period of 24 hr. All cell sheets were then refed control medium. TER and spontaneous potential difference (at 37°C) measurements were taken at the indicated intervals. I_{sc} was then derived following Ohm's law in which $V = iR$.

across TNF-treated cell sheets relative to controls ($P \leqslant 0.01$; basolateral to apical) further substantiating an alteration in the ion selectivity of the tight junction. The increase in selective permeability with TNF treatment, which measured as the ratio of P_{C1} to P_{Na}, was symmetrical and independent of the direction of the ion gradient. This was not observed for the control cell sheets.

To further characterize increased transepithelial ion conductance, the flux of $^{22}Na^+$ was measured across control and TNF-treated cell sheets. Relative to control cell sheets, the total transepithelial flux of $^{22}Na^+$ was nearly doubled across the TNF-treated CACO-2 BBE epithelium (Table 4). The TNF-induced increase in transepithelial $^{22}Na^+$ flux was in both the amiloride-sensitive and -insensitive fractions with a proportionally larger increase in the latter. This increase was inhibited by 1 mM amiloride, either in the apical compartment only or in both apical and basolateral compartments. This was consistent with an observed increase in transepithelial conductance measured across TNF-treated CACO-2 BBE epithelial cell sheets, which also demonstrated a proportionally larger increase in the amiloride-insensitive component (Table 5). The increase in transepithelial $^{22}Na^+$ flux was considered to be paracellular based upon the following experimental observations: (i) the increased dilution potential across TNF-treated CACO-2 BBE was independent of the direction of the ion gradient suggesting a single symmetrical barrier to the movement of Na^+ and Cl^- i.e., a paracellular one; (ii) similar to $^{22}Na^+$ flux studies conducted in the presence of amiloride on both the apical and basolateral surfaces, apical amiloride (1 mM) inhibited the basolateral to apical flux of $^{22}Na^+$ (Fig. 5); and (iii) amiloride (1 mM) applied to both the apical and basolateral surfaces did not affect the I_{sc} across CACO-2BBE cell sheets [control 2.3 ± 0.1 (15) *vs.* 2.1 ±

Table 3. TNF alters transepithelial Na^+ and Cl^- conductance across CACO-2 BBE epithelial cell sheets

	P_{Na}	P_{Cl}	P_{Cl}/P_{Na} (apical → basolateral)[a]	P_{Cl}/P_{Na} (basolateral → apical)[a]
Control (13)	1.8 ± 0.2	0.99 ± 0.08	0.52 ± 0.02	0.62 ± 0.02 ($p \leqslant 0.01$)[b]
TNF (13)	3.8 ± 0.4	1.8 ± 0.1	0.48 ± 0.02	0.47 ± 0.02[c,d]

Data are presented as 10^{-6} cm/sec and represent the mean ± SE of 13 separate cell sheets used in three separate dilution potential experiments using a 50 mM NaCl gradient as described in Materials and Methods. The conductance across the cell sheets were 1.4 ± 0.3 *vs.* 2.6 ± 0.4 mS/cm^2 for control and TNF-treated cell sheets, respectively.

[a] Refers to direction of 50 mM NaCl gradient

[b] *vs.* Control P_{Cl}/P_{Na} (apical → basolateral)

[c] not significant *vs.* TNF P_{Cl}/P_{Na} (apical → basolateral)

[d] $P \leqslant 0.01$ *vs.* Control P_{Cl}/P_{Na} (basolateral → apical)

0.1 μA (15), (1 mM amiloride; $P < 0.3$); TNF; 1.3 ± 0.2 (15) *vs.* 1.4 ± 0.2 μA (14), (1 mM amiloride; $P < 0.6$)].

Discussion

Using the renal epithelial cell line, LLC-PK_1, we have demonstrated the ability of TNF to transiently and reversibly increase transepithelial permeability (Mullin & Snock, 1990; Mullin et al., 1992). Similar observations were made using intrahepatic bile duct epithelial cells isolated from rat liver (Mano et al., 1996). It was our objective in undertaking the studies reported herein to determine whether TNF could also influence transepithelial permeability across an epithelial cell line of intestinal origin. The presence of increased mRNA and protein levels for TNF in mucosa of patients with inflammatory bowel disease as well as reports of increased epithelial barrier permeability suggested this to be a reasonable expectation (Murch et al., 1993; Breese et al., 1994; Hollander et al., 1989; Malin et al., 1996). CACO-2 BBE epithelial cells were selected as the model epithelium for this study as they spontaneously manifest the characteristics of differentiated villous enterocytes (Peterson & Mooseker, 1992). Work by other laboratories had already suggested that TNF was without effect on the permeability of the epithelium formed in vitro by T-84 cells (Madara & Stafford, 1989; McKay et al., 1996).

As reported herein, like LLC-PK_1 renal epithelial cells, CACO-2 BBE did respond to TNF with an increase in transepithelial permeability although the characteristics of the response were markedly different. Unlike the

Table 4. TNF increases the transepithelial permeability of CACO-2 BBE cell sheets to $^{22}Na^+$

	(nmoles $^{22}Na^+$/min/cm^2)	
	Control	TNF (100 ng/ml)
Total	26.1 ± 2.6	46.4 ± 3.2
Amiloride-insensitive	11.1 ± 1.0	24.2 ± 2.1
Amiloride-sensitive	15.0 ± 2.1	22.1 ± 3.6

Data represent the mean ± SE of the rate of $^{22}Na^+$ flux across control and TNF-treated CACO-2 BBE epithelial cell sheets during a 75-min period of observation from three separate experiments using four cell sheets per condition. Briefly, cell sheets were cultured at confluent density onto Millicell PCF filter assemblies as described in Materials and Methods. At $t = 0$ hr cell sheets were presented with fresh medium ± TNF (100 ng/ml) for a period of 24 hr. All cell sheets were then refed control medium. TER and spontaneous potential difference (at 37°C) measurements were taken prior to the start of the Na^+ flux study ± 1 mM apical and basolateral amiloride. Following the addition of $^{22}Na^+$ to the basolateral compartment, 50 μl samples were retrieved from the apical compartment at 15-min intervals as described in Materials and Methods. Qualitatively similar results to those shown above were obtained when 1 mM amiloride was applied to only the apical fluid compartment. Mean conductance at the start of the flux study was 2.1 ± 0.1 and 4.6 ± 0.3 mS/cm^2 for control (24) and TNF-treated (24) cell sheets, respectively.

Table 5. TNF increases amiloride-sensitive and -insensitive conductance across CACO-2 BBE epithelial cell sheets

	G_t^o	G_t^{a+bl}	ΔG_t^{a+bl}	$\Delta G_t^{a+b}/G_t^o$
Expt. 1				
Control (4)	2.3 ± 0.2	1.3 ± 0.1	1.1 ± 0.1	0.46 ± 0.01
TNF (4)	4.8 ± 0.1	3.2 ± 0.1	1.7 ± 0.1	0.34 ± 0.01
Expt. 2				
Control (6)	0.66 ± 0.1	0.30 ± 0.04	0.36 ± 0.07	0.54 ± 0.01
TNF (6)	1.3 ± 0.1	0.70 ± 0.05	0.64 ± 0.05	0.48 ± 0.01
Expt. 3				
Control (6)	2.3 ± 0.2	1.0 ± 0.1	1.3 ± 0.2	0.57 ± 0.02
TNF (6)	4.9 ± 0.1	2.6 ± 0.2	2.3 ± 0.2	0.48 ± 0.04

G_t^o: Pre-amiloride conductance corrected for the artifact due to refeeding with fresh medium; G_t^{a+bl}: amiloride-insensitive conductance; ΔG_t^{a+bl}: amiloride sensitive conductance; and $\Delta G_t^{a+b}/G_t^o$: fraction of total amiloride conductance which is sensitive to apical and basolateral amiloride. Data represent the mean ± SE for (''*n*'' filters) from three separate experiments in which 1mM amiloride was applied to both the apical and basolateral surface of the cell sheet.

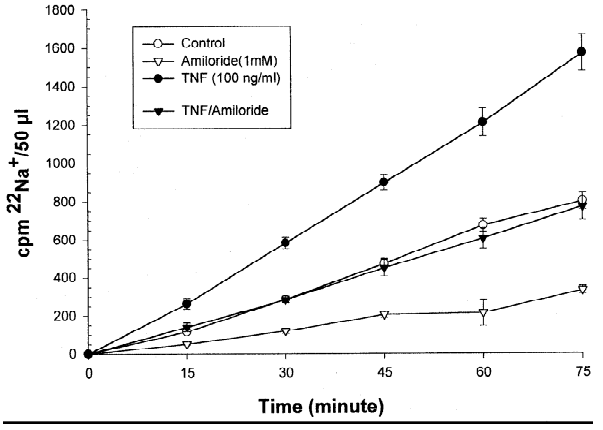

Fig. 5. Apical amiloride (1 mM) decreases the basolateral to apical transepithelial flux of $^{22}Na^+$. Data represent the mean ± SE of 4 cell sheets per time point from a representative experiment. Briefly, cell sheets were cultured at confluent density onto Millicell PCF filter assemblies as described in Materials and Methods. At $t = 0$ hr cell sheets were fed medium ± TNF (100 ng/ml) for a period of 24 hr. All cell sheets were then refed control medium for an additional 24-hr interval. Prior to the addition of $^{22}Na^+$ to the basolateral fluid compartment as described in Materials and Methods, TER and PD were measured and cell sheets were returned to 37°C and 5% CO_2. 50-µl samples were collected from the apical fluid compartment at 15-min intervals for liquid scintillation counting and calculation of the rate of transepithelial $^{22}Na^+$ flux.

rapidly reversible permeability response of LLC-PK_1 epithelium that associated with an increase in transepithelial mannitol flux and incidence of apoptosis, TNF-treated CACO-2 cell sheets sustained the increased transepithelial permeability up to 1 week following treatment without a significant increase in mannitol flux or apoptosis (Mullin et al., 1992; Peralta Soler et al., 1996). Furthermore, the increase in transepithelial permeability was not apparent until 24 hr following treatment in contrast to its onset at 1.5–2 hr in LLC-PK_1 (Mullin & Snock, 1990). The observation of a slow but sustained permeability response is not unique to TNF or to CACO-2 BBE epithelial cells. Using the epithelial cell line, T-84, Colgan and colleagues also demonstrated effects of interferon-γ or II-4 to increase transepithelial permeability that also took a long time (>24–48 hr) to establish (Colgan et al., 1994*a*,*b*). However, like the LLC-PK_1, epithelium, the increase in permeability could be measured upon incubation with TNF for as short an interval as 5 min. The ability of this rather short duration of exposure to TNF to elicit a physiological effect is consistent with its rather rapid activation of transcription factors, NF-KB for instance (Chan & Aggarwal, 1994) and other components of its intracellular signaling cascade (Andrieu, Salvayre & Levade, 1996).

In an ongoing study of TNF levels in patients with inflammatory bowel disease, we have measured mucosal levels of immunoreactive TNF of up to 2.88 pg/µg protein and plasma levels of bioactive TNF (measured in the L929 cytotoxicity assay) of up to 10 ng/ml in patients with ulcerative colitis (*unpublished observations*). These levels are consistent with reports cited in the literature (Sategna-Guidetti et al., 1993; Braegger, 1992) that reported serum levels of bioactive TNF to be 8.17 ± 1.01 ng/ml in active Crohn's disease and 276-5982 pg/g of immunoreactive TNF in stool of patients with active inflammatory bowel disease. These reports are consistent with the dose response data which demonstrates that incubation of the CACO-2 BBE cell sheet with as little as 5 ng/ml TNF is sufficient to evoke an increase in transepithelial permeability and that the maximal increase is achieved between 50 and 100 ng/ml and that no further effect (cytotoxic or otherwise) is observed at greater concentrations of the cytokine. Consideration of these data suggest that the permeability effects of TNF on CACO-2 BBE are occurring at concentrations that might reasonably be achieved in the interstitial space adjacent to and surrounding the enterocyte.

While TNF did not induce apoptosis in the CACO-2 BBE epithelial cell sheet, it did increase [^{3}H]-thymidine incorporation into DNA without affecting total DNA content or BrdU labeling. This observation suggests that TNF may be affecting the turnover of a small population of cells in the epithelium without effecting the total density of the monolayer. Whether or not these cells are responsible for the observed permeability changes, we cannot conclude at this time. It is interesting that only 3% of LLC-PK_1 cells were rendered apoptotic by TNF and this was associated with a 50% reduction in TER (Peralta Soler et al., 1996). Overall these observations strongly suggest that while TNF can affect an increase in transepithelial permeability, such an effect most likely occurs through different mechanisms depending upon the epithelium itself. Unlike LLC-PK_1, the delayed response of CACO-2 BBE to TNF could be dependent upon new protein synthesis as has been discussed by Gorodeski and colleagues (1996) in their studies of nucleotide-mediated permeability changes across cervical epithelium. They postulated that protein synthesis-dependent increases in transepithelial permeability would function to increase the density of pores within the tight junctional strands. They contrasted this with the possibility of a conformational change of existing tight junctional-associated proteins which could lead to an increased probability of the open state of preexisting pores to explain a rapid increase in transepithelial permeability.

The apparent dissociation of changes in TER and transepithelial mannitol flux is not without precedent. We have previously observed a long-term increase in TER that was not associated with a decrease in [^{14}C]-mannitol flux (Marano et al., 1993). Similar observations were made for EGF-induced mitogenesis and the accompanying alterations in transepithelial permeability (Saladik et al., 1995). In CACO-2 Artursson and col-

leagues (1993) also reported a lack of correlation between differences in TER and accompanying mannitol flux. Dissociation of TER from mannitol flux has also been observed by Balda and colleagues in occludin-transfected MDCK cells (1996) and by Lu et al. (1996) in CACO-2 cell lines of increasing passage number. Despite its recognition as the prototypic mediator of apoptosis (Wertz & Hanley, 1996), TNF did not induce an increase in the incidence of apoptosis in the CACO-2 BBE epithelial cell sheet or an increase in transepithelial mannitol flux. This contrasts with LLC-PK_1 in which TNF induces a significant increase in apoptotic events in the cell sheet and an increased transepithelial flux of mannitol (Mullin et al., 1992; Peralta Soler et al., 1996).

The conclusion that TNF is affecting the ion permeability characteristics of the paracellular pathway comes from consideration of the results from the transepithelial $^{22}Na^+$ flux and dilution potential protocols along with documented published findings regarding both the CACO-2 cell line and the pharmacological properties of amiloride. Microelectrode measurements of the electrical properties of CACO-2 cells found the basolateral membrane voltage to be about −63 mV strongly suggesting that the basolateral membrane has little if any conductive Na^+ permeability (Grasset et al., 1984). Thus, the only significant pathway for conductive basolateral to apical $^{22}Na^+$ flux would be paracellular. Furthermore, our studies show apical amiloride reduced the basolateral to apical $^{22}Na^+$ flux without altering the I_{sc}, suggesting that amiloride is acting on the paracellular pathway. Studies conducted by Balaban, Mandel and Benos (1979) demonstrated that amiloride can affect the ion selectivity of the paracellular pathway in addition to its effects on transcellular conductive pathways such as Na^+ channels and the Na^+/H^+ antiporter in frog skin and gallbladder. Taken together with reports from Frizzell and Turnheim (1978), Cremaschi et al. (1979) in rabbit and pig colon, respectively and Grasset et al. (1984) showing the absence of amiloride-sensitive transcellular conductance in CACO-2, these data strongly suggest that altered ionic conductance of the paracellular pathway underlies the TNF-induced decrease in TER. In light of the increase in transepithelial Na^+ and Cl^- conductance, we considered a recent report by Kagan et al. (1992) demonstrating that the addition of TNF-α to the cis compartment of solvent free lipid bilayers resulted in an increase in the bilayer conductance. This TNF-α induced bilayer conductance required that the pH of the cis compartment be less than 6 and that the trans compartment voltage be at least 40 mV positive. Since the pH of the solution bathing the CACO-2 BBE cells was 7.3, and the membrane voltage is cell interior negative (Grassett et al., 1984) then it is highly unlikely that the TNF-α induced increase in transepithelial Na^+ and Cl^- flux was due to the channel forming properties of TNF-α.

Dilution potential measurements were also used to measure transepithelial permeability to Na^+ and Cl^-. Since the calculated selective permeability following TNF treatment is independent of the direction of the ion gradient this suggests a single barrier to the movement of Na^+ and Cl^- i.e., paracellular. The dilution potential data further suggested that the presence of amiloride decreases basolateral to apical Cl^- flux, which recommends a pathway that is paracellular as it is hard to postulate a cellular pathway that allows free movement of Na^+ and Cl^-. Secondly, a large increase in transcellular Na^+ conductance would necessitate a large increase in the conductance across both the apical and basolateral membranes. Increased apical membrane Na^+ conductance would necessarily translate into an increase in I_{sc} which is not observed with TNF treatment. Furthermore, basolateral Na^+ conductance in CACO-2 has been reported to be very low (Grasset et al., 1984). Therefore, it leads us to conclude that the TNF-induced increase in Na^+ and Cl^- flux is paracellular and not transcellular.

It is important to note that in addition to a paracellular effect, TNF certainly has cellular sites of action as evidenced by a transient decrease in I_{sc} across the CACO-2 BBE cell sheet with cytokine treatment. As a Cl^- secreting cell type (Bear, 1991; Bear & Reyes, 1992), a decrease in apical Cl^- secretion or a decrease in the electroneutral basolateral transport of Cl^- via the $Na^+/K^+/2Cl^-$ cotransporter might account for the observed reduction in I_{sc} across CACO-2 BBE cell sheets with TNF treatment. Several of the nutrient transport pathways across CACO-2 have been characterized as Na^+-independent, H^+-coupled thus it is also possible that the effect of TNF on I_{sc} reflects a TNF-induced change in apical and/or basolateral H^+ conductance (Thwaites et al., 1996). TNF has been shown to affect transcellular conductance and membrane potential in skeletal muscle (Tracey et al., 1986), fibroblasts (Lee, 1992) and human colonic enterocytes (Stack et al., 1995) therefore it is not surprising that CACO-2 BBE respond to TNF with a decrease in transcellular ion transport.

The transepithelial permeability response of CACO-2 BBE to TNF stands in contrast to the lack of effect of the cytokine when incubated with epithelial monolayers generated by T-84 cells in culture. The apparent conflict of these results might in fact be explained by a differential effect of TNF on epithelial cells isolated from and manifesting properties of different sections of the crypt-to-villous axis of the gastrointestinal mucosa. The T-84 cell line maintains a phenotype characteristic of crypt epithelial cells (Madara & Dharmasathaphorn, 1985; Madara et al., 1987), whereas CACO-2 BBE were selected for their differentiated properties reminiscent of villous enterocytes (Peterson & Mooseker, 1992). Such differential responses within the intestinal crypt have been reported for intestinal cells of differing topographical and hierarchical status exposed to various cytotoxic

agents and sources of radiation (Ijiri & Potten, 1983; Potten et al., 1992). Therefore, it is possible that epithelial cells along the crypt-to-villous axis will manifest differential sensitivity to endogenous inflammatory mediators as well.

In summary, TNF has effects on both the paracellular conductance and membrane transport properties across the cultured intestinal epithelium, CACO-2 BBE. The paracellular conductance to Na^+ and Cl^- is increased while mannitol permeability is not altered with TNF treatment. The 30–40% drop in transepithelial resistance and associated changes in epithelial ion permeability would make the mucosa more susceptible to disease after the addition of stresses such as ischemia/anoxia, increased concentrations of free radicals or the presence of antigen. The results of these studies recommend CACO-2 BBE as an ideal culture system in which to model the effects of cytokines and other inflammatory mediators on epithelial barrier integrity in IBD as well as disorders of epithelial barrier permeability associated with other clinical situations, such as cardiopulmonary bypass surgery (Riddington et al., 1996), in which systemic elevations of TNF occur and a small percentage of patients manifest increased intestinal permeability (Sinclair et al., 1995). They also suggest that TNF among other cytokines can reduce the barrier integrity of an epithelium as either an initiating response or a secondary phenomenon associated with pathological inflammation. Finally, these results also show that induced increases of paracellular permeability may extend to only one of several classes of solutes, and not necessarily to all solutes irrespective of their size or charge. This therefore implies that tight junctions can increase their permeability to different degrees in response to diverse signal transduction pathways.

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