Apical effect of diosmectite on damage to the intestinal barrier induced by basal tumour necrosis factor- α

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Abstract

Background—In many digestive diseases the intestinal barrier is weakened by the release of proinflammatory cytokines, including tumour necrosis factor- α (TNF α).

Aim—To investigate the protective effect of apical diosmectite on the intestinal dysfunction induced by the proinflammatory cytokine TNF α .

Methods—Filter grown monolayers of the intestinal cell line HT29-19A were incubated for 48 hours in basal medium containing 10 ng/ml TNF α and 5 U/ml interferon- γ (IFN γ). Next, 1, 10, or 100 mg/ml diosmectite was placed in the apical medium for one hour. Intestinal function was then assessed in Ussing chambers by measuring ionic conductance (G) and apicobasal fluxes of 14 C-mannitol (Jman), and intact horseradish peroxidase. In control intestinal monolayers, diosmectite did not significantly modify G, Jman, or intact horseradish peroxidase.

Results—After incubation with TNF α and IFN γ , intestinal function altered, as shown by the increases compared with control values for G (22.8 (3.7) v 9.6 (0.5) mS/cm²), Jman (33.8 (7.5) v 7.56 (0.67) μ g/h $\times$ cm²), and intact horseradish peroxidase (1.95 (1.12) v 0.14 (0.04) μ g/h $\times$ cm²). G and Jman were closely correlated, suggesting that the increase in permeability was paracellular. Treatment with diosmectite restored all the variables to control values.

Conclusions—Basal TNF α disrupts the intestinal barrier through the tight junctions, and apical diosmectite counteracts this disruption.

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Keywords: diosmectite, intestinal permeability, HT29-19A cell line, TNF α , Ussing chambers.

In many digestive diseases, including infectious diarrhoea,^{1–3} inflammatory bowel diseases^{4–5} and food allergy,^{6–7} the intestinal barrier is weakened by the release of proinflammatory cytokines due to the abnormal activation of the epithelial cells⁴ and underlying immune system.^{8–9} Of these cytokines, tumour necrosis factor- α (TNF α), a 17 kD protein secreted by several types of immune cells, including monocytes/macrophages, lymphocytes, and eosinophils, is mainly involved in intestinal

alterations.^{10–11} Various in vivo studies have focused on its effect on the intestine,^{11–13} in which dose dependent toxicity has been shown. In vitro studies using cultured cell monolayers have also shown the ability of TNF α , alone or combined with interferon- γ (IFN γ), to alter the capacity of the epithelial barrier. In this respect, TNF α seems to act with IFN γ to exert a synergistic effect^{6–13–17} which is due to the induction of TNF α receptors by IFN γ .^{6–18–19} We recently showed that 48 hour incubation with 10 ng/ml TNF α reduced the transepithelial resistance of intestinal HT29.19A cell monolayers, provided that a small amount of IFN γ (5 U/ml), which was not in itself toxic, was present in the incubation medium.⁶

Barrier disruption by inflammation leads to increased stimulation by luminal antigens such as the food antigens, bacterial toxins, and micro-organisms in the different types of cells in the lamina propria, including immune cells, nerve plexus cells, phagocytes, mast cells, and mesenchymal cells. In this connection, mucosal inflammation can be considered a self-perpetuating process in which the disruption of the epithelial layer plays a central role.⁷ Restoring the epithelial barrier might be achieved by acting on the basal or lamina propria side of the epithelium – for instance, antibodies directed against TNF α were found to prevent the effect of TNF α in vitro⁶ and might be beneficial in vivo.^{20–21} An alternative might be to act on the luminal side of the epithelium by removing the offending antigens or restoring the integrity of the epithelial barrier.

Diocetahedral smectite (diosmectite) is a well defined multilamellar argillaceous compound²² commonly used for several gastrointestinal diseases in adults and children. Animal and human clinical studies indicate that it has a beneficial effect in infectious diarrhoea and food allergy. In acute diarrhoea in children, this beneficial effect was shown by reductions in the duration of diarrhoea, the frequency of liquid stools, and the number of cases of prolonged diarrhoea.^{23–26} Because of the large size of the diosmectite molecule, its effect is probably localised in the intestinal lumen or luminal surface of the epithelium: thus, diosmectite adsorbs toxins,^{27–28} bacteria,²⁹ and rotavirus,³⁰ and has a direct effect on the physical properties of the gastric mucus and mucolysis induced by bacteria^{31–32}; it may also repair mucosal integrity, as suggested by the normalisation of the urinary lactulose/mannitol ratio found in children with acute diarrhoea³³ and by the restoration of luminal surface

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integrity in experimental bacterial infection.^{31 34 35} In addition, diosmectite has been shown to increase the effectiveness of the mucus barrier against mucosal damage by pepsin *in vivo*,³⁶ and to protect against the allergic digestive disturbances induced in guinea pigs sensitised to cow's milk.³⁷

The present work was therefore undertaken to assess the apical effect of diosmectite on the alteration in the intestinal epithelial barrier induced by the proinflammatory cytokine TNF- α . The experimental conditions were chosen to take advantage of the *in vitro* system in which the epithelial layer consisting of human intestinal HT29-19A cells could be monitored for barrier integrity properties while introducing a well defined concentration of TNF- α on the basal side and diosmectite on the apical side. The results obtained suggest that diosmectite restores the epithelial barrier damage induced by TNF α .

Methods

Intestinal cell line HT29-19A

HT29-19A cells cloned from the human colon carcinoma cell line HT2938 were used as a model of human intestinal epithelium. These cells were seeded at a density of 10^6 cells/cm² in Dulbecco's modified Eagle's minimum essential medium (DMEM, Eurobio, Paris, France) supplemented with 10% heat inactivated foetal bovine serum (Boehringer Mannheim, Germany), 1% non-essential amino acids (GIBCO, Glasgow, Scotland), and 4 mM glutamine, and cultured in a humidified atmosphere of 5% CO₂-95% air. For experimental studies, cells were seeded at a density of 8×10^5 cells/cm² and cultured in the presence of 50 mg/ml gentamycin (Sigma Chemical Co, St Louis, MO, USA) on polycarbonate Transwell® filters with a pore diameter of 0.4 μ m and a surface area of 1.13 cm² (Costar, Brumath, France). On reaching confluence, these cells progressively formed an epithelial layer in which the apical and basolateral domains were separated by tight junctions. They were used for experimental purposes after at least three weeks of culture, or later, when the cell layer was fully differentiated and displayed stable electrical resistance.

Treatment of intestinal monolayers with cytokines and/or diosmectite

After 21 days of culture, intestinal cells were incubated on their basal side for 48 hours in the presence of 10 ng/ml human recombinant TNF α (Biosource International, Camarillo, CA, USA) and 5 U/ml IFN γ (Genzyme, Cambridge, MA, USA) or under standard conditions. These concentrations were chosen for their capacity to alter the epithelial barrier of HT29-19A as previously described.⁶ After 48 hours, 1, 10, or 100 mg/ml diosmectite (IPSEN, France) were placed for one hour on the apical side of control and TNF α treated cells.

Measurement of HT29-19A intestinal function in Ussing chambers

Filter grown HT29-19A cell monolayers were removed from the culture plates, gently cut out with a sharp razor blade, and mounted in Ussing chambers. They were bathed on both the apical and basal sides (exposed surface area, 0.15 cm²) with 1.5 ml Ringer solution at a controlled temperature and containing (in mM) 140 Na⁺, 5.2 K⁺, 1.2 Ca²⁺, 1.2 Mg²⁺, 120 Cl⁻, 25 HCO₃⁻, 2.4 HPO₄²⁻ and 0.4 H₂PO₄⁻. The pH was maintained at 7.4 and the cells were oxygenated by bubbling both compartments with 95% O₂/5% CO₂. The spontaneous potential difference (PD) was measured with agar bridges connecting each side of the bathing medium to calomel electrodes. Cell monolayers were short circuited using an automatic voltage clamp system (DVC 1000, WPI, Aston, England) after appropriate correction for fluid and system resistance. The short circuit current (Isc) was constantly recorded and the tissue was pulsed at 0.5 mV every 30 seconds. The Isc deflection was used to calculate ionic conductance (G) according to Ohm's law. After equilibration, 5 mM mannitol was placed in both the apical and basal compartments, and ¹⁴C-mannitol (12.2 kBq/ml) and horseradish peroxidase (Sigma type VI, 0.4 mg/ml) were added to the apical compartment. The appearance of mannitol in the basal compartment was monitored by sampling 800 μ l at 10 and 20 minute intervals. Samples of 500 μ l were assayed for ¹⁴C radioactivity by liquid scintillation photometry. Mannitol fluxes were calculated according to specific activity in the medium and expressed in μ g/hr $\times$ cm². The rate of intact horseradish peroxidase transfer across the cell monolayers was determined by enzymatic assay on 200 μ l basal samples, according to Maehly and Chance.³⁹ Results were expressed in μ g/hr $\times$ cm².

Statistical analysis

Statistical analyses were made using the SAS program.⁴⁰ Variance analysis was performed by the general linear model procedure, and Student's *t* test or non-parametric Wilcoxon tests were used to compare means and ranges. All results are expressed as means (SEM).

Results

Effect of diosmectite on HT29-19A epithelial function, under basal conditions

In HT29-19A cell monolayers mounted in Ussing chambers, neither the electrical parameters Isc, PD, and G, nor mannitol or intact horseradish peroxidase fluxes were modified by apical treatment with diosmectite alone at concentrations of 1, 10, and 100 mg/ml (Table I). The persistent tendency towards a dose dependent increase in intact horseradish peroxidase flux was not significant (*p*>0.5). These results suggest that diosmectite alone does not significantly modify the intestinal epithelial function.

TABLE I Effect of diosmectite (DS) on HT29-19A epithelial cell function under basal conditions

Conditions	n	Isc ($\mu A/cm^2$)	PD (mV)	G ($mS \times cm^2$)	Jman ($\mu g/h \times cm^2$)	JHRP ($\mu g/h \times cm^2$)
Control	9	3.68 (0.68)	0.41 (0.09)	9.57 (0.48)	8.29 (1.09)	0.14 (0.04)
DS (1mg/ml)	3	5.49 (2.89)	0.75 (0.41)	7.93 (0.63)	8.99 (1.26)	0.19 (0.07)
DS (10 mg/ml)	9	3.26 (0.86)	0.33 (0.09)	10.77 (1.12)	16.58 (5.21)	0.68 (0.38)
DS (100 mg/ml)	6	2.67 (0.93)	0.35 (0.13)	7.93 (0.51)	12.61 (3.52)	0.62 (0.30)

n=number of filter-grown cell monolayers, Isc=short-circuit current, PD=potential difference, G=transepithelial ionic conductance, Jman=mannitol flux from apical to basal solutions, JHRP=horseradish peroxidase flux from apical to basal solutions. Using variance analysis, no difference was found in the variables measured under the four above conditions.

TABLE II Effects of TNF α and IFN γ on HT29-19A epithelial cell function

	n	Isc ($\mu A/cm^2$)	PD (mV)	G ($mS \times cm^2$)	Jman ($\mu g/h \times cm^2$)	JHRP ($\mu g/h \times cm^2$)
Control	9	3.68 (0.68)	0.41 (0.09)	9.6 (0.5)	8.29 (1.09)	0.14 (0.04)
TNF α	10	7.09 (2.41)	0.32 (0.08)	22.8 (3.7)	29.5 (6.62)	1.95 (1.12)
p value		0.079	0.51	0.0001	0.0005	0.029

Abbreviations as for table I.
p=probability for a difference between the control and TNF α groups, calculated by variance analysis.

Effect of basal TNF α on HT29-19A epithelial properties

Incubating the basal side of HT29-19A cells for 48 hours with 10 ng/ml TNF α and 5 U/ml IFN γ altered their barrier function (Table II). This was shown by a significant increase in G (p<0.001) combined with a significant rise in both Jman (p<0.01) and intact horseradish peroxidase flux (p<0.001), confirmed a paracellular leakage in the epithelial layer, probably through the tight junctions. This was further confirmed by the close correlation between G and mannitol fluxes (Fig 1), as previously shown.⁴¹

Effect of apical diosmectite after basal treatment by TNF α

One hour of treatment with 1, 10, or 100 mg/ml of diosmectite fully restored the epithelial barrier capacity of HT29.19A cells after TNF α injury (Fig 2). This was shown by the significant decrease in ionic conductance to control values, and by the significant decrease in both mannitol and intact horseradish peroxidase fluxes. The effect of diosmectite was not concentration dependent.

Figure 1: Correlation between ionic conductance (G), an index of paracellular ionic transport, and transepithelial fluxes of mannitol (Jman). Each point represents a pair of values obtained in one filter grown monolayer, under the different experimental conditions used. A significant correlation between G and mannitol fluxes (r=0.824; p<0.01) was found, suggesting that ions and mannitol shared the same paracellular pathway.

Discussion

The present results confirm that when TNF α is present in the basal medium, it disrupts the intestinal HT29-19A epithelial cell barrier, and further suggest that when diosmectite is introduced on the apical side of the epithelial layer, it fully restores this barrier's capacity.

In the present study, we analysed both the functional aspects of the TNF α induced epithelial alterations and the potentially beneficial effect of diosmectite. Intestinal permeability was assessed by ionic conductance, an index of epithelial integrity, and by mannitol and horseradish peroxidase fluxes, as a probe of small and large molecule transport respectively.⁶ After a 48 hour treatment with

Figure 2: Effect of diosmectite on the alteration in HT29-19A cell permeability induced by TNF α . Ionic conductance (G), transepithelial fluxes of ¹⁴C-mannitol, and intact horseradish peroxidase were measured simultaneously on the same differentiated HT29.19A cell monolayers mounted in Ussing chambers under control conditions (white bars, n=9) or after preincubation with TNF α and IFN γ (black bars, n=10). The effect of increasing the amount of diosmectite (1, 10, or 100 mg/ml, n=4, 6, and 6 respectively) on TNF α treated cells is shown (grey bars). Values are means (SEM); *values significantly different from all other conditions.

the proinflammatory cytokines TNF α and IFN γ , all these variables had risen significantly, thus confirming previous results showing that TNF α alters intestinal permeability *in vitro*,⁶ especially at the tight junctions.⁴¹ A recent study indicated that the defective cohesion induced by TNF α was linked to the induction of disordered expression of adhesion molecules, as well as disaggregation of actin filaments.¹⁰

Diosmectite alone had no effect on epithelial barrier function. After its administration, there was a tendency towards increased permeability to mannitol and horseradish peroxidase, but it was never significant, indicating that diosmectite alone did not alter epithelial permeability. In previous experiments concerning the clinical use of diosmectite in the treatment of acute diarrhoea, diosmectite did not have any effect on the intestinal absorption of a glucose-containing electrolyte solution.²⁸

Here, however, diosmectite fully restored the barrier properties of the epithelial layer after disruption of the layer by TNF α , as shown by the significant decrease in ionic conductance and the decreases in mannitol and horseradish peroxidase permeabilities. At concentrations of 1, 10, or 100 mg/ml, diosmectite reduced permeability indexes to values not statistically different from the control values. It is difficult to transfer these results to an *in vivo* situation, because of the absence of measurement of the intestinal concentration of diosmectite in patients with diarrhoea.⁴² However, at the daily recommended dose of 9 g in adults and 3 g in infants, it is conceivable that this malabsorbed compound is in this range of concentration at the surface of the epithelial layer. In addition, as the effect of diosmectite was measured after intestinal permeability had altered, this effect was curative, which is of interest for a therapeutic agent.

The present results suggest two mechanisms by which this effect is obtained. Under control conditions, the macromolecular tracer horseradish peroxidase is absorbed along two functional transcellular pathways⁴³: a main degradative pathway which involves intracellular processing, and a minor pathway which allows the transcytosis of intact horseradish peroxidase; paracellular absorption of horseradish peroxidase is prevented as long as the intercellular junctions remain intact. Under pathological conditions – that is, in the presence of TNF α , horseradish peroxidase is absorbed both along the transcellular route and through the damaged tight junctions.^{6, 41} Therefore, the reduction by diosmectite of the increased horseradish peroxidase absorption in the presence of TNF α might be due either to a direct effect on the tight junctions or to an effect on the transcellular pathway.

One of the most intriguing findings of these experiments is that diosmectite acts on the apical side of the epithelial layer. It is known that the diosmectite alters the physical properties of mucus; however, in this model, mucus was not present; in these experiments we used clone 19 of the HT29 cell line that has been selected for its properties of electrolyte

transport. The current definition of the mechanism of the mucosal damage induced by inflammation⁹ is that the mediators are released by the cells present in the lamina propria and also by the enterocytes,⁴ presumably through the basolateral membrane; thus, most of the mediators of inflammation, with the exception of 5'-adenosine monophosphate,^{44, 45} are thought to act on the antiluminal membrane of the epithelium. The present experiments were also designed on this basis, which is why TNF α was placed on the serosal side of the epithelial layer. Because of the large size of the diosmectite molecule (up to 3 μ m, with an interreticular distance of 1.4 nm),⁴⁶ and because it is an argillaceous compound, significant amounts are unlikely to be present, and active in the cell or in the basal reservoir. We were unable to devise a method of measuring diosmectite transepithelial transport similar to the method used to measure horseradish peroxidase transport, and the data obtained by electron microscopy (not shown) were not informative on this issue. Therefore, it is possible that diosmectite interferes with a message which is present on the luminal side of the epithelium, a message triggered by TNF α on the basolateral side of the membrane. We do not know the nature of this message, but it may be of interest to search for it. In this respect, recent results may provide an interesting clue: in the absence of inflammation, pneumococci did not adhere to endothelial cells, but their activation with thrombin or TNF α caused a 20-fold to 40-fold increase in bacterial entry into these cells; this pathological event was found to be due to an increase in the surface expression of a specific platelet activating factor receptor.⁴⁷

In conclusion, the present results suggest that diosmectite may reduce the consequences of intestinal inflammation by acting from the luminal side of the epithelium.

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