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Proteolytic degradation of intestinal mucosal extracellular matrix after lamina propria T cell activation

S L F Pender, P Lionetti, S H Murch, N Wathan, T T MacDonald

Abstract

***Background*—Proteoglycans, consisting of glycosaminoglycan (GAG) side chains covalently linked to a protein core, are a major component of the extracellular matrix of the intestinal lamina propria.**

***Aims*—This study investigated the effects of lamina propria T cell activation on the proteoglycan component of the matrix.**

***Methods*—The high degree of sulphation of GAGs means that they are polyanionic and thus can be visualised in tissue sections by means of colloidal-gold labelled cationic probes.**

***Results*—In human fetal small intestine there is a dense meshwork of anionic residues in the lamina propria and basement membrane. When explants of human fetal small intestine are cultured ex vivo, and resident lamina propria T cells are activated with pokeweed mitogen, mucosal destruction occurs within three days. This is associated with the rapid loss of anionic sites from the lamina propria. Dermatan sulphate proteoglycan is lost from the tissue and is present at increased concentrations in the organ culture supernatants, indicating that T cell activation has led to solubilisation of lamina propria proteoglycans. Tissue destruction and loss of anionic residues are inhibited in a dose dependent fashion by dexamethasone, and by the protease inhibitor, α_2 macroglobulin.**

***Conclusions*—Proteolytic degradation of the lamina propria may therefore be a mechanism by which T cell hypersensitivity injures the intestinal mucosa.**

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Keywords: T cell, lamina propria, matrix, proteinase.

Department of Paediatric Gastroenterology
S L F Pender
P Lionetti
S H Murch
T T MacDonald

and Joint Academic Department of Obstetrics and Gynaecology
N Wathan

The Medical College of St Bartholomew's Hospital, London

Correspondence to:
T T MacDonald,
Department of Paediatric Gastroenterology,
59 Bartholomew Close,
St Bartholomew's Hospital,
London EC1A 7BE.

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There is increasing evidence that CD4+ T cells play a major part in tissue injury in gut diseases in humans. In coeliac disease for example, lamina propria CD4+ T cells are phenotypically activated,[1 2] and when these cells are cloned,[3] they can be shown to be gluten reactive and produce large amounts of pro-inflammatory cytokines.[4] In vivo, lamina propria T cells containing message for interferon γ are increased in coeliac disease,[5] and freshly isolated lamina propria T cells contain a high frequency of interleukin 2 and interferon γ secreting cells.[6] In Crohn's disease, lamina propria CD4+ T cells are also phenotypically and functionally activated,[7–10] although the antigenic stimulus for this is unknown. Anecdotal reports of the success of anti-CD4 immunotherapy in Crohn's disease also support this idea.[11]

Animal studies also strongly support the notion that CD4+ T cells cause gut injury. In graft versus host disease, CD4+ T cells recognising host alloantigens play a major part in gut injury.[12] Transfer of CD45RB^{hi}, CD4+ T cells into SCID mice results in enteritis within a few weeks, which can be ameliorated with immunotherapy,[13] and transfer of small numbers of unfractionated CD4+ T cells into SCID mice also results in enteritis within five to eight weeks.[14] The stimulus for the enteritis in these models is not known but it is likely to be the resident bacterial flora.[15] Despite all of this evidence however, the mechanisms by which CD4+ T cells mediate tissue injury in the mucosa are still not known.

Studies from this laboratory have unequivocally shown that the activation of lamina propria T cells in explant cultures of human small intestine with pokeweed mitogen or the bacterial superantigen *Staphylococcus aureus* enterotoxin B results in tissue injury.[16 17] The type of injury produced in this system can be manipulated by regulating the T cell response. Thus the addition of pokeweed mitogen to tissue from older specimens (15-18 weeks) produces mucosal destruction in most of the explants within three days. If dexamethasone is added to the explant cultures along with pokeweed mitogen, the changes produced consist of villous atrophy and crypt hyperplasia.[18] Another advantage of this model is that any changes seen must be due to resident cells, with no contribution from bloodborne inflammatory cells.

In this study we have focused on the changes and mechanisms that result in mucosal destruction in this model. Mucosal destruction by definition entails injury to the lamina propria, which consists in the fetus of a loose network of mesenchymal cells, nerve cells, T cells, and macrophages, embedded in extracellular matrix. The matrix is made up mainly of collagen fibres (I, III, IV), elastin, laminin, fibronectin, hyaluronic acid, and proteoglycans.[19 20] Proteoglycans consist of glycosaminoglycan (GAG) side chains covalently linked to a protein core.[21] GAGs exist in four major forms: heparan sulphate, chondroitin sulphate/dermatan sulphate, keratan sulphate, and hyaluronic acid. Sulphated GAGs have a very strong negative charge and can bind and interact with various growth factors,[22] adhesion molecules,[23] and extracellular proteins,[24] and play a major part in maintaining normal

tissue structure and function.[25] Here we show that T cell activation leading to mucosal destruction is associated with the loss of sulphated GACs from the matrix and that this can be prevented by a protease inhibitor, α_2 macroglobulin.

Methods

Organ culture of human fetal small intestine

Human fetal small intestine was obtained within two hours of surgical termination from the Medical Research Council Tissue Bank, The Hammersmith Hospital, and from the Homerton Hospital, London. All the specimens used in this study were aged between 15 and 16 weeks gestation.

A piece of each specimen was snap frozen in liquid nitrogen and the rest was dissected into 1-2 mm square explants. Twenty explants were cultured in 7 ml of serum free CMRL-1066 medium (Sigma Chemical Co, Poole, Dorset) modified according to Autrup *et al*[26] with the omission of cortisone) at 37°C in a 95% O_2 and 5% CO_2 atmosphere. At various time points the explants in each dish were carefully removed, placed on top of one another on a piece of filter paper to absorb excess moisture, snap frozen in liquid nitrogen, and stored at −70°C. Culture supernatants were collected and spun at 1200× *g* for 10 minutes to remove cell debris and stored at −70°C.

Mucosal T cells were activated by adding pokeweed mitogen (10 μg/ml, Sigma Chemical, Poole, Dorset) at the onset of culture. In some experiments dexamethasone (10^{-7} to 10^{-9} M, Sigma), or α_2 macroglobulin (1 to 100 μg/ml, Calbiochem, Beeston, Nottingham) were also added to the cultures.

Detection and quantification of glycosaminoglycans

Sulphated GAGs are highly anionic[27] and so can be identified in frozen sections by immunochemistry using highly cationic charged poly-L-lysine conjugated to colloidal gold.[28] Briefly 6 μm sections were cut and fixed with 4% paraformaldehyde. Sections were incubated with gold conjugated poly-L-lysine probe (British Biocell International, Cardiff) diluted 1 in 100 in phosphate buffered saline, pH 1·2, for one hour. The probe was washed off with deionised water and the reaction developed with a silver enhancing kit (British Biocell International) for about 15 minutes at room temperature. The slides were mounted in Aquamount (BDH/Merck Ltd) without counterstaining. By this technique anionic sites stain dark brown or black. Some sections were pretreated with either chondroitinase ABC (1 U/ml), heparanase II (10 U/ml), hyaluronase (10-100 U/ml), or neuraminidase type X (1 U/ml) at 37°C for four hours before staining as described above. All enzymes were obtained from Sigma.

The density and distribution of gold stained anionic sites in the sections was quantified by computer assisted image analysis (Seescan, Cambridge). A stable light source was used and fixed threshold and light intensity from the light source were used throughout the measurements. The image from the microscope was captured on a video screen and the lamina propria was identified and traced. The optical density of the lamina propria in the area selected was measured electronically and shown as arbitrary units, the greater the optical density, the greater the measurement. Quantification was also verified by scanning sections of different thickness from 4 μm to 8 μm. When this was done, the optical density increased in a linear fashion related to tissue thickness and hence density of charge staining. The same sections were also scanned at different times using the same threshold and light intensity and at each time the optical density obtained was similar, with an inter-observational variance of 1%. At least 10 measurements of different regions of the lamina propria per explant were made and the mean value obtained. Multiple explants (at least eight per group) were analysed, as although each culture contained 20 explants, it was impossible to get all 20 in the plane of the frozen section.

Isolation and chromatography of glycosaminoglycans from fetal gut

GAGs were isolated from tissue and organ culture supernatants using a modification of the method of Klein *et al*.[29] Explants and supernatants were incubated with pronase E (Sigma) in phosphate buffered saline, 50 mg/ml and 25 mg/ml respectively for 20 hours at 37°C to release GAGs from the protein core. The samples were then incubated for five hours with five volumes of alcian blue 8GX solution (alcian blue 0·2%, 50 mM sodium acetate buffer pH 5·8 and 50 mM $MgCl_2$). The GAG/dye complex was isolated by centrifugation at 10 000 × *g*. After removal of the supernatants, alcian blue was dissociated from the GAGs with a mixture of NaCl and methanol (final concentration 2·67M and 33% respectively), and the alcian blue was denatured with 12·5 mM sodium carbonate. The mixture was sonicated in a water bath for 20 minutes and incubated at room temperature overnight with rotating action to make sure the dissociation and denaturation of the dye and GAGs was complete. Alcian blue was removed by centrifugation at 10 000 × *g* and the GAGs from the clear supernatants were precipitated with three volumes of ethanol. After centrifugation at 10 000 × *g* for 30 minutes, the supernatant was removed and the GAGs were left to dry in air overnight. The sample was then redissolved in distilled water, spun at 2000 × *g* to remove any insoluble material and stored at 4°C before paper electrophoresis. For isolation of GAGs from day 0 fetal gut, approximately 1 g of tissue was used. However, insufficient material was available from a single fetus to culture this amount of tissue. Therefore for studies on cultured tissue, GAGs were isolated from 80 explants and their supernatants.

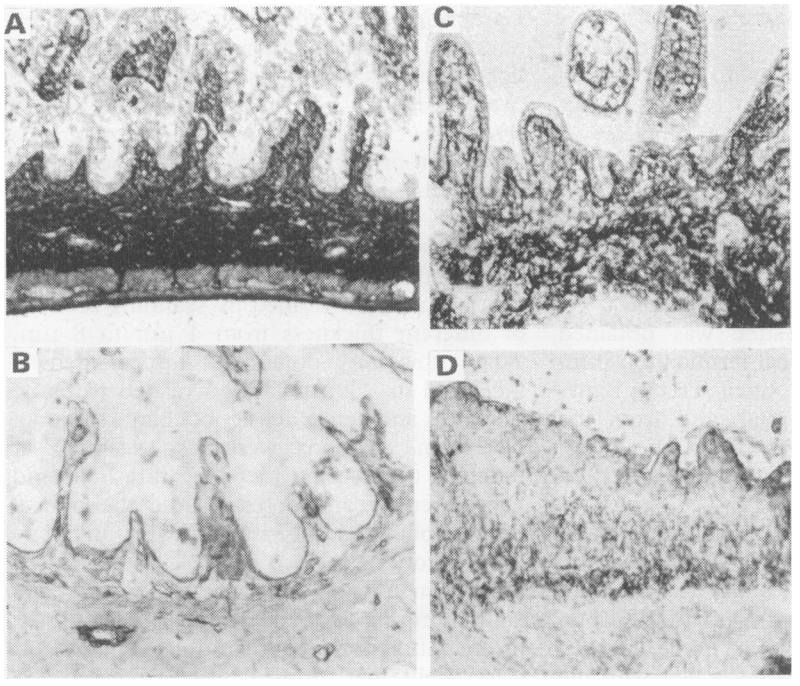

Figure 1: Visualisation of anionic sites corresponding to GAGs in human fetal intestine using poly-L-lysine conjugated to colloidal gold. (A) In day 0 fetal intestine, there is dense staining in the lamina propria, but not in the epithelium. (B) Pre-treating the sections with chondroitinase ABC to digest dermatan and chondroitin sulphate eliminated lamina propria charge staining, but there was still considerable staining on vessels and basement membrane due to heparan sulphate. (C) In day 3 control explants of fetal intestine, there is still considerable GAG staining in the lamina propria, although the overall pattern of staining is less strong than the day 0 sample. Villous morphology is maintained. (D) In day 3 explants stimulated with pokeweed mitogen, there is almost complete loss of GAGs from the lamina propria and only short stumps of villus core remain. A thin interrupted layer of charge staining is visible on the surface, representing the residual basement membrane (Fig 1A-D, original magnification ×200).

One dimensional chromatography was carried out by applying 1 μl of GAGs solution as a 0·7 cm band to a cellulose di-acetate celagram (Shandon, Life Sciences International, Hampshire). After electrophoresis for five hours in 0·1M barium acetate, pH 6·0, at 6·5 V/cm, the celagrams were developed in Genogold and enhanced with silver (British Biocell International). The bands were identified by comparison with the position of standards (heparan sulphate, HS; dermatan sulphate, DS and chondroitinate sulphate, CS) and their identity was confirmed by pretreatment of the samples with the enzymes chondroitinase ABC 0·5 U/ml, neuraminidase 0·5 U/ml or hyaluronidase 50 U/ml before electrophoresis. All enzymes were obtained from Sigma.

Immunohistochemistry

Frozen sections of explants were air dried for 30 minutes at room temperature and fixed in acetone. They were then stained with anti-cytokeratin antibody, anti-CD3, and anti-CD25 antibody (all from Dako Ltd, High Wycombe, Bucks) by the indirect immunoperoxidase technique exactly as described elsewhere.[30] The density of CD3+ and CD25+ cells in the lamina propria was quantified by image analysis exactly as described previously.[31]

Statistical analysis

Differences between groups were compared using either the Mann-Whitney U test, if the data were not normally distributed, or Student's *t* test, if the observations were consistent with a sample from a normally distributed population.

Results

T cell activation and mucosal destruction are associated with the loss of anionic sites from the lamina propria

In day 0 fetal intestine there was a dense network of anionic sites in the lamina propria, on the epithelial basement membrane, and on the lumen of vessels (Fig 1A). Pre-treatment of the sections with chondroitinase ABC eliminated lamina propria charge staining (Fig 1B) while incubation with heparanase II before staining removed only vascular and part of the basement membrane staining (not shown). Pretreatment of sections with neuraminidase or hyaluronidase also had no effect on lamina propria charge staining (not shown). This confirms that chondroitin sulphate and dermatan sulphate are the dominant GAG species in the lamina propria.

In control cultures (day 3) of fetal intestine the same extensive fibrillar network of anionic sites in the extra cellular matrix could be seen (Fig 1C), although due to some oedema, the overall charge staining was less dense than on day 0. None the less villi remained and tissue morphology was normal. In pokeweed mitogen treated explants, associated with mucosal destruction, however, there was almost complete loss of anionic sites from the lamina propria but the subepithelial basement membrane staining remained (Fig 1D). Quantification of charge staining by image analysis showed a steady decrease in the lamina propria of pokeweed mitogen treated explants in the three days after T cell activation (Fig 2).

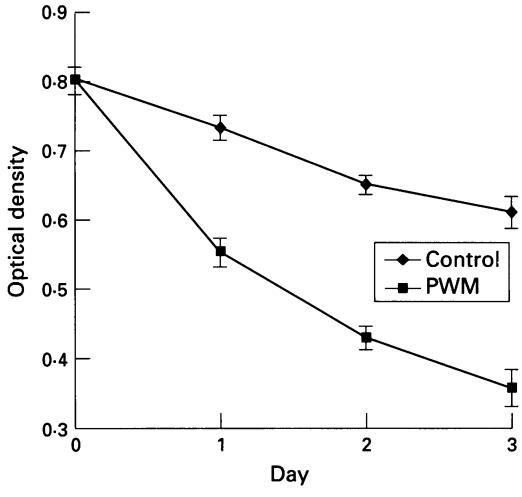

Figure 2: Quantification of anionic sites by image analysis in the lamina propria of human fetal small intestine explants on day 1, day 2, and day 3 of culture. There was a steady decrease in staining in the lamina propria of explants that were cultured with pokeweed mitogen (PWM). Data shown are the mean and SEM from a single experiment in which at least 10 explants per time point was scanned. The loss of charge in pokeweed mitogen stimulated explants was highly significant at all time points ($p<0·01$, Student's t test). Similar results have been obtained in two replicate experiments.

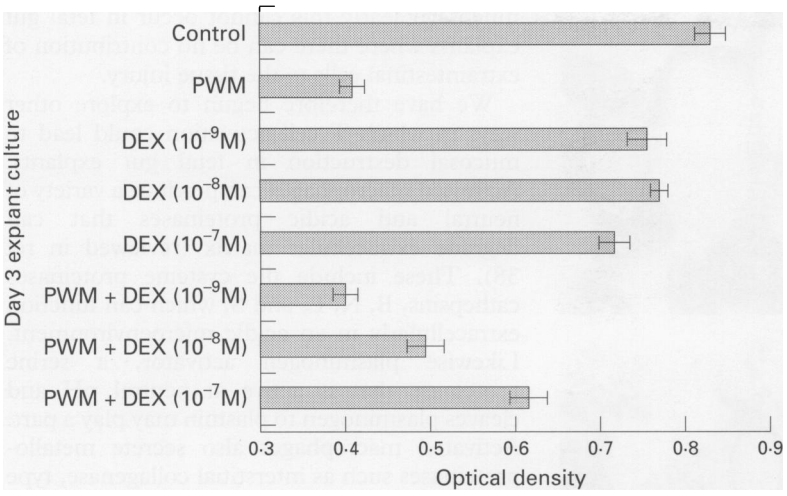

Figure 3: Dexamethasone (DEX) inhibits the loss of lamina propria anionic sites in pokeweed mitogen (PWM) stimulated explants in a dose dependent fashion. All explants were examined on day 3 of culture. The data shown are the mean and SEM of the optical density of at least eight explants per group in a single experiment. Dexamethasone at 10 and 100 nM significantly increased the density of lamina propria GAGs in PWM treated explants compared with the PWM control ($p<0·001$, Student's t test). Similar results were obtained in a second experiment.

The addition of dexamethasone along with the pokeweed mitogen also prevented the loss of anionic sites (Fig 3), and as we have previously shown, also prevented tissue destruction.[18]

By cellulose diacetate electrophoresis, dermatan sulphate and chondroitin sulphate were readily detected in day 0 fetal intestine (Fig 4). Dermatan sulphate was the more abundant species detected. GAGs were isolated from tissues and supernatants after three days in culture (Fig 4). Only trace amounts of heparan sulphate and chondroitin sulphate could be detected in control explants, because of practical limitations in the amounts of tissue that could be cultured from a single fetus, but dermatan sulphate was clearly present. After T cell activation, considerably less dermatan sulphate could be extracted from the explants, even though a similar number of explants were initially cultured as in the control explants (80 explants). In contrast, in the organ culture supernatants, dermatan sulphate was readily detectable in the T cell stimulated cultures but not in the control supernatant, giving direct evidence that T cell activation in the lamina propria leads to solubilisation of lamina propria GAGs. The identity of the bands was further confirmed by pre-treating GAGs isolated from day 0 tissue with various enzymes prior to electrophoresis. The uppermost band disappeared if the GAGs were treated with neuraminidase confirming it as sialic acid. When chondroitinase ABC was used, the dermatan sulphate and chondroitin sulphate bands disappeared.

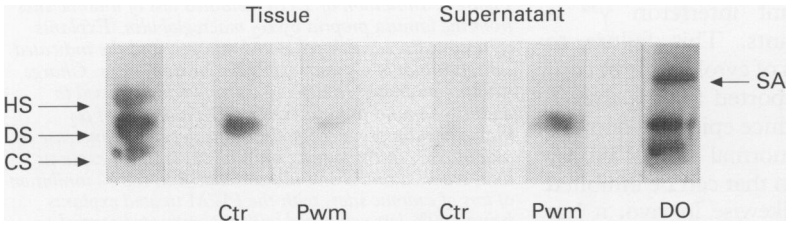

Figure 4: Cellulose diacetate chromatography of GAGs isolated from day 0 and day 3 cultured human fetal small intestine. GAGs standard of 0·3 mg/ml of heparan sulphate (HS), dermatan sulphate (DS) or chondroitin sulphate (CS) were used (lane 1). Although GAGs were isolated from identical numbers of explants (80), on day 3 of culture there was considerably more DS present in control (Ctr) explants than those treated with pokeweed mitogen (PWM). Only trace amounts of HS and CS were present in both samples (lanes 2 and 3). When GAGs were precipitated from the organ culture supernatants, DS was precipitated from PWM treated explant supernatant, but none from control supernatant (lanes 4 and 5). In day 0 fetal intestine, SA, DS, and CS were present.

Inhibition of pokeweed mitogen induced T cell destruction by α_2 macroglobulin

One possible explanation for the loss of anionic sites from the lamina propria after T cell activation could be proteolytic degradation of matrix components. We therefore added the protease inhibitor α_2 macroglobulin at the onset of culture, along with pokeweed mitogen. In control cultures on day 3, morphology was normal, whereas in explants cultured with pokeweed mitogen, there was mucosal destruction and only a thin epithelial layer covered the remnants of the lamina propria (Fig 5A, B). At higher concentrations (10^{-7}M), α_2 macroglobulin completely inhibited mucosal destruction (Fig 5C), although some crypt cell hyperplasia was observed. Analysis of anionic sites in the explants showed that α_2 macroglobulin inhibited the loss of staining induced by pokeweed mitogen (Fig 6).

If proteolytic degradation of matrix is an important end point in tissue injury in this model it would be expected that despite retaining tissue structure, explants cultured with pokeweed mitogen and α_2 macroglobulin would still show evidence of lamina propria T cell activation. We therefore examined this by counting the number of CD3+ cells and CD25+ cells in the lamina propria (Fig 7). Pokeweed mitogen produced an increase in the density of CD3+ cells in the lamina propria, and a huge increase in CD25+ cells. This increase was not significantly inhibited by α_2 macroglobulin (Fig 7).

Discussion

In this paper we show that mucosal destruction after the activation of resident lamina propria CD4+ T cells in explants of human fetal small intestine is associated with the loss of anionic sites in the lamina propria. We assume that this reflects the loss of proteoglycans from the lamina propria matrix because after T cell activation, we can isolate increased amounts of dermatan sulphate GAGs from the organ culture supernatants. There are a number of possible mechanisms by which proteoglycans may be lost from the matrix but inhibition with α_2 macroglobulin strongly suggests proteolysis as the main pathway. We had previously shown that GAGs are lost from the lamina propria in active inflammatory bowel disease[28] and although the present study is not strictly comparable because inflammatory cells of many types are present in inflammatory bowel disease mucosa, it does show that it is possible for cell mediated immune reactions to lead to

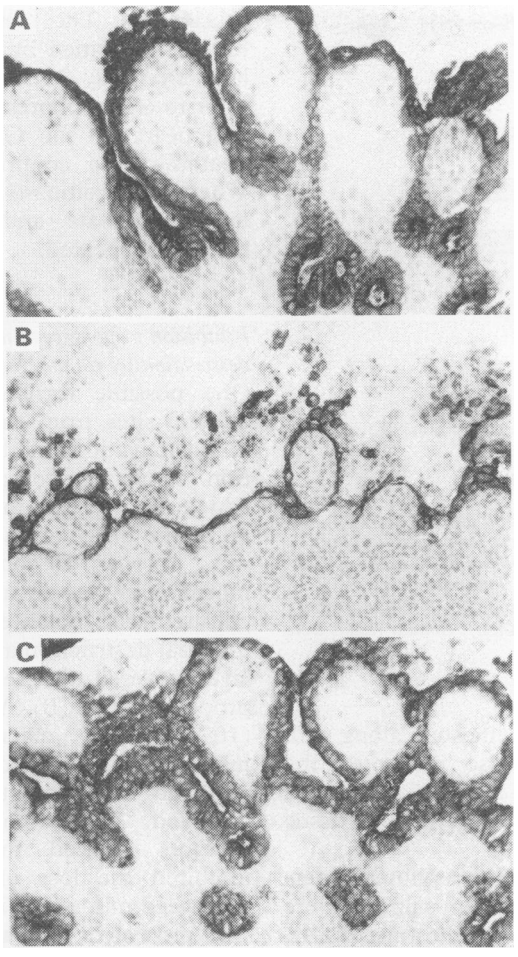

Figure 5: Mucosal destruction after T cell activation with pokeweed mitogen is inhibited by α_2 macroglobulin. In the example shown here, the explants have been stained with anti-cytokeratin to highlight the epithelium. (A) Day 3 control explant. In the pokeweed mitogen stimulated explant (B) there is loss of villi and only a thin epithelium covering residual lamina propria. In the presence of 100 μg/ml α_2 macroglobulin (C) the integrity of the mucosa is retained, there are villi, and the epithelium is columnar (immunoperoxidase with anti-cytokeratin, original magnification ×200).

GAG loss in the absence of bloodborne inflammatory cells.

When lamina propria T cells are activated in fetal gut explants they secrete interleukin 2 and interferon γ.[16 32] Large numbers of non-CD3+ cells in the lamina propria (presumably resident macrophages) become CD25+,[33] and there is an increase in the frequency of tumour necrosis factor α (TNFα) secreting cells.[18] We have however been unable to show any pathological effects when recombinant interferon γ[34] is added directly to explants. This failure to demonstrate direct effects of cytokines is in contrast with the recently reported finding that T cell supernatants can produce epithelial damage when added in vitro to normal jejunal biopsy specimens, a phenomenon that can be inhibited by anti-interferon γ.[35] Likewise in vivo, it has been shown that anti-interferon γ can prevent the villous atrophy seen in murine graft versus host disease,[36] and that villous atrophy can be induced by injection of TNFα.[37] The effects in vivo however could be due to modulation of cytokine induced adhesion molecule expression on gut endothelium, thereby regulating the influx of bloodborne accessory cells into the mucosa. Clearly this cannot occur in fetal gut explants where there can be no contribution of extraintestinal cells to the tissue injury.

We have therefore begun to explore other ways in which T cell activation could lead to mucosal destruction in fetal gut explants. Activated macrophages can produce a variety of neutral and acidic proteinases that can degrade extracellular matrix (reviewed in ref 38). These include the cysteine proteinases cathepsins, B, N, L, and S, which can function extracellularly in an acidic microenvironment. Likewise plasminogen activator, a serine proteinase that is active at neutral pH and cleaves plasminogen to plasmin may play a part. Activated macrophages also secrete metalloproteinases such as interstitial collagenase, type IV collagenases (gelatinases) and stomelysin. These proteinases have activity against proteoglycan core protein, with initial cleavage adjacent to the hyaluronate binding region. Further cleavage of core protein between clusters of GAGs side chains produces small peptides with carbohydrate side chains that can escape from the collagen network.

A crucial part of the evidence however to support a role for proteinases in tissue destruction in this model lies in the fact that we were able to inhibit tissue damage with α_2 macroglobulin. α_2 Macroglobulin is a large glycoprotein present at high concentrations in normal human serum (2 g/l). Native α_2 macroglobulin is a tetramer of four identical subunits joined by disulphide bonds and strong non-covalent interactions. It inactivates

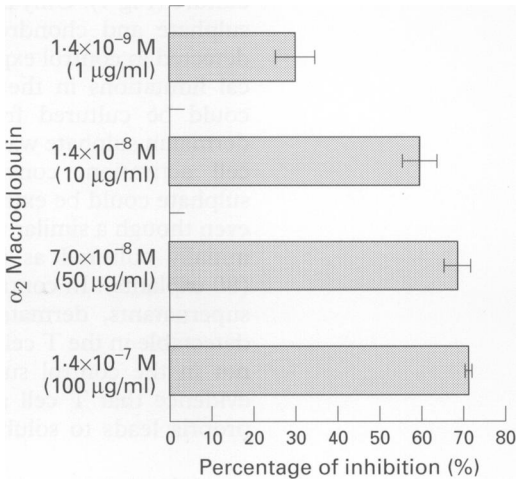

Figure 6: Inhibition of T cell mediated loss of anionic sites from the lamina propria by α_2 macroglobulin. Explants were co-cultured with pokeweed mitogen and the indicated concentration of α_2 macroglobulin for three days. Charge staining on frozen sections of the explants was used to quantify lamina propria GAGs. The addition of α_2 macroglobulin produced a clear dose dependent increase in anionic sites in the lamina propria. At least 10 explants were scanned and the results are shown as the % inhibition of loss of anionic sites, with the PWM treated explants being 100% loss compared with the untreated control explants (0% loss) using the formula

$$\frac{OD_{PWM+\alpha_2M} - OD_{PWM}}{OD_{Control} - OD_{PWM}} \times 100\%$$

Each point represents the mean (SEM) value of three individual experiments. Significant increases ($p<0{\cdot}01$, Student's t test) were seen at α_2 macroglobulin concentrations of 10, 50, and 100 μg/ml.

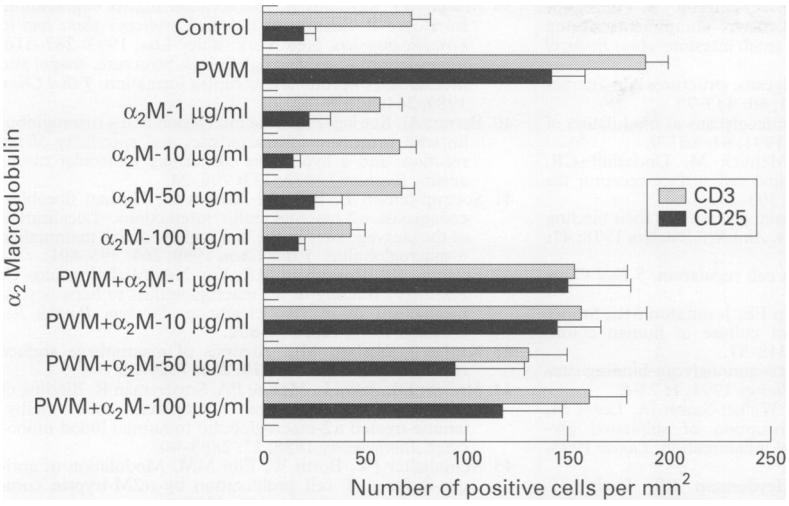

Figure 7: Density of CD3+ and CD25+ cells in the lamina propria after the addition of pokeweed mitogen to explants of fetal gut is not reduced by α_2 macroglobulin. Data shown are from a single experiment and represent the mean (1 SD) of measurements made from at least eight explants at each dose of α_2 macroglobulin on day 3 of culture. Similar results have been obtained in two other experiments.

proteinases from all the major mechanistic subclasses (ref 39, serine, thiol, aspartic, metallo-) using a trap mechanism[40] in which the bait region of the molecule, containing preferred cleavage sites for all classes of endopeptidases is cleaved.[41] The molecule then undergoes a rapid conformational change trapping the proteinase and forming a non-dissociable complex.[39] In vivo, these complexes are rapidly removed from serum by specific α_2 macroglobulin cell membrane receptor-mediated endocytosis by hepatocytes,[42] fibroblasts, and macrophages.[43 44]

The effect of α_2 macroglobulin in inhibiting GAGs loss may however not only be due to its ability to inhibit proteinases, but also through its immunoregulatory functions. Native α_2 macroglobulin does not inhibit T cell proliferation, but if activated by proteinase, it can inhibit antigen specific T cell responses in humans.[45] This is unlikely to be the case in this system because α_2 macroglobulin did not inhibit the increase in lamina propria T cells after pokeweed mitogen activation. The immunoregulatory properties of activated α_2 macroglobulin are also related to its ability to bind cytokines.[46] We consider this unlikely however as cytokine-α_2 macroglobulin complexes probably play a carrier role, with the cytokine in a biologically active form; in addition, the complex may also increase the biological activity of the cytokine by delivering it to cells with α_2 macroglobulin receptors.[46] α_2 Macroglobulin interaction with cytokines may however still play an indirect part in proteolytic degradation of the matrix because both TNFα and interleukin 1β can increase proteinase secretion by fibroblasts.[47 48]

Dexamethasone also inhibited mucosal destruction and loss of anionic sites from the explants, however we feel that this is probably caused by at least two mechanisms. Corticosteroids are potent inhibitors of T cell lymphokine release[49 50] and we have previously shown that in the fetal gut explants they reduce the frequency of interferon γ secreting T cells after pokeweed mitogen stimulation.[18] Addition of corticosteroids in this model also decreases the number of CD25+ cells, probably a direct consequence of the decrease in T cell derived macrophage activating lymphokines.[51] In addition, corticosteroids are also potent inhibitors of proteinase secretion.[52 53]

In summary therefore, we have provided evidence that T cells may produce tissue damage in the gut mucosa via a pathway that entails proteolytic degradation of the extracellular matrix. Ongoing studies are aimed at identifying the classes of proteinases involved, their target proteins, and pharmacological inhibition of their activity.

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