Histological changes in small bowel mucosa induced by gliadin sensitive T lymphocytes can be blocked by anti-interferon γ antibody

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Abstract

The isolation of gliadin specific HLA-DQ2 restricted T lymphocyte clones from the intestinal mucosa of patients with coeliac disease supports a role for cell mediated immunity in the pathogenesis of this condition. Whether supernatants from immune activated T cell clones could produce histological damage to duodenal mucosa in vitro was studied. Biopsy specimens were obtained from 18 patients without coeliac disease or any other demonstrable abnormality. The tissue was maintained in organ culture for 24 hours with organ culture medium alone, with supernatant from gliadin sensitive T cell clones that had (B) or had not (A) been stimulated with gluten, and compared with the effects caused by the addition of interferon γ to the organ culture medium. Both the (B) supernatants (1:100) and interferon γ (100 IU/ml) produced a significant reduction in the enterocyte height (21.5 (3.4) μm and 21.0 (3.2) μm respectively, each $p < 0.001$) compared with specimens grown in organ culture medium alone (27.3 (2.8) μm). The toxic effects of (B) supernatants could be blocked by pre-incubating them with anti-interferon γ antibody. These findings support the role of gliadin sensitive T lymphocytes in the immune pathogenesis of coeliac disease and their secretion of interferon γ may cause the damage to enterocytes observed in this condition.

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Keywords: gliadin sensitive T cells, coeliac disease interferon γ .

There is increasing evidence that cell mediated immunity is important in the pathogenesis of coeliac disease (CD). A recognised feature of this enteropathy is an increase in the number of activated (CD25+) T cells in the jejunal lamina propria, which are predominantly CD4+ (T helper) in origin and whose major function is to secrete cytokines and modulate immune responses.¹ An enteropathy with strikingly similar histological features to those found in untreated CD is a well recognised feature of graft *versus* host disease in both man and experimental animals.^{2,3} This lesion is caused by MHC class II incompatibilities which are recognised by CD4+ T helper cells.⁴ In foetal organ culture it has been shown that the activation of mucosal CD4+ T cells by

anti-CD3 antibodies can produce villous atrophy and crypt hyperplasia accompanied by raised concentrations of interleukin 2 and interferon γ in the growth medium.⁵

Gliadin specific T helper lymphocyte clones have recently been isolated from the small intestinal mucosa of patients with treated CD.⁶ These clones were shown to be immune activated by gliadin when presented by a HLA-DQ2 heterodimer found in 98% of patients with CD in Norway and in other groups of coeliac patients studied.^{7,8} The T cell clones were found to carry diverse combinations of $V\alpha$ and $V\beta$ T cell receptors and the markers CD3+, CD4+, and CD8-.⁶ Immune activation of these T cell clones *in vivo* may result in the secretion of cytokines which could affect cellular biological functions and mediate intestinal mucosal changes such as enterocyte toxicity, villous flattening, and crypt hypertrophy.

This study aimed to determine whether immune activation of gliadin sensitive T cell clones by cereal toxic peptides could produce histological changes in the small intestinal mucosa, and if so which cytokines might be responsible. These experiments would support the role of gliadin sensitive T cell clones in the pathogenesis of CD and help us to understand the role of soluble mediators in the development of the observed histological abnormalities.

Methods

PATIENTS STUDIED

These comprised 18 patients (10 men and eight women, mean age 41.1 years, range 20.3-68.0 years) from whom small intestinal biopsy specimens were obtained as part of their management. The specimens were found to have normal histological appearances in all the patients studied, and after full investigation patients' symptoms were not attributed to any pathological process. Written informed consent was obtained from all the patients and the study was approved by the local ethics committee in accordance with the Declaration of Helsinki.

HANDLING OF BIOPSY SPECIMENS

Biopsy specimens were obtained from the second part of the duodenum with an Olympus IT20 endoscope using 'jumbo' forceps (8 mm). One specimen was immediately fixed in formalin and five were transferred in ice cold

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culture medium and immediately established in organ culture.

ORGAN CULTURE

Small intestinal biopsy specimens were orientated villous side uppermost on a piece of stainless steel gauze resting on the central well of an organ culture dish (Falcon, USA). The tissues were maintained in organ culture for 24 hours using a method modified from that originally described by Browning and Trier.⁹ Growth medium (NCTC 2 ml, Trowell's T8 6 ml, streptomycin/penicillin-1000 IU/ml, 0.2 ml, Hepes buffer 0.1 ml, L-glutamine 0.2 ml, and heat inactivated foetal calf serum 1.5 ml) containing cytokine or supernatant was introduced into the central well until it just touched the undersurface of the tissue. The culture dish was covered and placed in an organ culture chamber (ICN/Flow) which was gassed with 95%O₂/5%CO₂ to 3 psi pressure, sealed and maintained at 37°C for 24 hours.

SUPERNATANTS UNDER INVESTIGATION

Two T cell clones were studied in this experiment and designated TCC 1.27 and TCC 4.32 after Lundin *et al.*⁶ Furthermore, sister clones from TCC 4.32 were also studied. These clones were obtained from the intestinal mucosa of two different patients with treated CD.

The supernatants were derived as follows. T cells were stimulated after seven days in culture. Some 0.5×10^6 T cells were cultured in the presence of 1×10^6 irradiated cells (10 000 rad) from a DR3, DQ2 homozygous Epstein-Barr virus transformed B cell line in 1.5 ml medium (RPMI 1640 with 15% inactivated, pooled human serum and penicillin/streptomycin). Two variants were prepared, one (termed (B) supernatant), where the irradiated B cells had been incubated overnight with 2 mg/ml of a peptic/tryptic digest of gluten (ICN Biochemicals, Cleveland, OH, USA), the other incubated without gluten antigen was termed the (A) supernatant. The cultures were incubated at 37°C in 5%CO₂ for approximately 48 hours. The supernatants were collected after centrifugation and kept frozen at -20°C until used.

PRELIMINARY EXPERIMENTS

Preliminary experiments were performed with the (B) supernatants and individual cytokines to determine their optimal concentration for use in the study. Six groups of patients with three patients in each group were studied. Six biopsy specimens were obtained from each patient. One was fixed immediately and one cultured with organ culture medium (OCM) alone. The remaining four specimens were cultured with increasing concentrations of supernatant B added (1: 1000–1:20) to the OCM. At a concentration of 1:100, all the specimens were morphologically preserved but at higher concentrations the resulting

level of tissue damage made their assessment difficult. Experiments were also performed to study the effects of individual cytokines on small intestinal morphology. Recombinant human cytokines were obtained commercially (Genzyme, Cambridge, MA, USA), their specific activities were supplied by the manufacturer having been determined in bioassays. Four biopsy specimens obtained from each patient were grown with increasing concentrations (10–500 IU/ml of OCM) of each of the cytokines; tumour necrosis factor α (TNF- α), interferon γ , an equal mix of interferon γ /TNF- α , interleukin (IL)-6, and IL1- α .

INITIAL STUDIES OF THE ENTEROTOXICITY OF T CELL SUPERNATANTS

Six biopsy specimens were obtained from each of 18 patients studied:

- One was immediately fixed in formalin and used as a preculture control.
- One was grown in OCM alone (nil, 24 hours) to ensure that the organ culture method was not in itself toxic to intestinal tissue.
- One was grown with (A) supernatant added to the OCM (A, 24 hours) to ensure that a toxic factor was not transferred from the resting T cell or B cell growth medium.
- One specimen was grown with (B) supernatant added to the OCM (B, 24 hours).
- A clear positive control was not available, although in preliminary experiments both the cytokines, TNF- α and interferon γ , were shown to cause toxic changes to small intestinal enterocytes. Therefore the effects of the T cell supernatants were compared with interferon γ , a cytokine secreted predominantly by activated T lymphocytes. One biopsy specimen was grown with 100 IU/ml (IFN 100 IU/ml, 24 hours) and another at a concentration of 200 IU/ml (IFN 200 IU/ml, 24 hours) of OCM.

BLOCKING EXPERIMENTS USING ANTI-CYTOKINE ANTIBODIES

A fixed concentration of (B) supernatant (1:100 in OCM) was pre-incubated at 37°C for 45 minutes with increasing concentrations of each anti-cytokine antibody. For each of the anti-cytokine antibodies studied, six specimens were obtained from three patients. The antibodies used were murine anti-human cytokine monoclonals – TNF- α and IL-2 (from Serotec, Oxford, UK) and IL-1 α , IL-6, and interferon γ (Genzyme, Cambridge, MA, USA). Data obtained from the manufacturers showed that each antibody specifically blocked the effects of the corresponding recombinant and natural human cytokine in several bioassays. We felt, therefore, that they were suitable for neutralising cytokine bioactivity within the T cell supernatants under investigation. The amount of antibody added to OCM was calculated as the amount which would neutralise 50 IU, 100 IU, 200 IU, or 400 IU of cytokine-mediated activity per ml of OCM.

Figure 1: The graph shows the effects of increasing concentrations of cytokines on intestinal epithelial cell morphology. Interferon γ (INF γ) and tumour necrosis factor α (TNF- α) act to produce a significant reduction in enterocyte height. IL=interleukin; OCM=organ culture medium.

ADDITIONAL CONTROLS

The effect of a peptic tryptic digest of gluten (Frazer's fraction III) on the morphological appearances of small intestinal biopsy specimens was also studied. This peptic/tryptic digest was not identical to that used for T cell clones but prepared using a similar protocol. Increasing concentrations (0.5–4 mg/ml) were mixed thoroughly with organ culture medium and the effect on enterocyte cell height was assessed after 24 hours.

TREATMENT OF BIOPSY SPECIMENS AFTER CULTURE

Biopsy specimens were immediately removed, fixed in formalin, and embedded in wax. The sections were cut to 5 μ m and the slides were coded.

COUNTING OF ENTEROCYTE HEIGHT

The sections were assessed by two independent observers who had been blinded to the

culture conditions used. A Leitz Westlar microscope with a calibrated eyepiece graticule (Graticules Ltd, Tunbridge Wells, UK) was used at $\times 125$ magnification to measure the mean enterocyte height. This was assessed using linear measurements from the basement membrane of the enterocyte to its tip.¹⁰ In each biopsy specimen at least five villi were counted and the mean of 50 enterocyte counts calculated in μ m (standard deviation). Cells with basally placed nuclei were selected and counted one third of the distance from the tip of the villus. Areas at the edge of the tissue section or with morphological distortion were not assessed. The correlation coefficient of measurements made by the two observers was 0.90 ($p < 0.001$, Spearman rank correlation).

STATISTICAL METHODS

The differences between groups were assessed by using the Student's *t* test and a difference was considered significant if a *p* value of less than 0.01 was obtained.

Results

After 24 hours in organ culture the small intestinal morphology was reasonably well preserved. The villi tended to become shorter and broader, however, with a slight reduction in cellularity within the lamina propria. A reduction in the mean enterocyte height after organ culture was observed and was found to be of similar magnitude to that documented in previous studies using this method.^{10 11}

Using concentrations of (B) supernatant (TCC 4:32) ranging from 1:100 to 1:20 there was a dose-response reduction in enterocyte height in the small intestinal biopsy specimens after organ culture. At concentrations of greater than 1:100, small intestinal morphology became significantly disrupted. We decided therefore to use supernatant concentrations of 1:100 for further studies. The addition of increasing concentrations of the peptic/tryptic digest of gluten alone did not produce any toxic effects in enterocytes.

The preliminary experiments with cytokines showed that interferon γ caused toxicity to enterocytes at concentrations of 100 IU/ml and 200 IU/ml, but at 500 IU/ml two of three specimens studied were grossly damaged. TNF- α caused a similar reduction in enterocyte height. None of the other cytokines studied had an effect on enterocyte height in organ culture (see Fig 1). We used interferon γ at concentrations of 100 IU/ml and 200 IU/ml to compare its effects on enterocyte morphology with that of T cell supernatants from gliadin specific clones.

In the initial experiments with T cell supernatants (see Table), there was a reduction in the mean (SD) enterocyte height measured in biopsy specimens fixed immediately after excision (nil, 0 hours) compared with those maintained in OCM alone for 24 hours (nil, 24 hours) (28.3 (2.6) μ m *v* 27.3 (2.8) μ m, $p = 0.045$). The mean enterocyte height in tissues grown with supernatants and cytokines

Enterocyte cell height (μ m) (mean (SD)) in small intestinal biopsy specimens tested with T cell clone (TCC) 1-27 supernatants (2) and sister clones of TCC 4-32 (2)

	Preculture	Nil, 24 h	A, 24 h	B, 24 h	IFN 100 IU	IFN 200 IU
(1) TCC:						
1-27	27.4 (1.3)	27.2 (0.7)	27.3 (1.1)	25.4 (0.5)	22.2 (1.1)	20.4 (0.8)
1-27	26.6 (0.8)	25.5 (0.9)	24.0 (0.7)	20.5 (1.0)	20.7 (0.8)	17.7 (0.7)
1-27	24.0 (0.9)	23.7 (0.8)	23.8 (0.7)	20.2 (0.7)	18.5 (1.6)	17.6 (0.8)
1-27	28.0 (1.0)	27.4 (0.8)	27.2 (0.8)	25.5 (1.3)	21.1 (1.1)	18.8 (0.7)
1-27	27.9 (0.9)	25.1 (0.8)	26.2 (0.5)	24.2 (1.2)	18.3 (1.5)	20.7 (1.2)
1-27	29.9 (0.8)	24.9 (1.1)	23.8 (1.1)	23.0 (0.8)	18.1 (0.7)	19.5 (0.9)
1-27	25.1 (0.7)	23.6 (0.8)	24.4 (0.7)	23.8 (1.0)	15.7 (0.6)	16.9 (1.2)
1-27	26.5 (0.5)	26.2 (0.7)	26.3 (0.7)	25.8 (0.6)	19.0 (0.7)	14.8 (0.9)
Mean	26.9	25.5	25.4	23.6 Δ	19.2 Δ	18.3 Δ
(2) TCC:						
4-84	26.1 (1.8)	25.7 (0.8)	25.2 (0.8)	15.6 (0.8)	16.9 (0.9)	13.8 (0.9)
4-32	29.5 (1.4)	28.9 (0.9)	27.2 (0.8)	21.8 (0.9)	24.7 (0.8)	21.2 (1.1)
4-32	26.6 (0.9)	25.9 (0.6)	26.3 (0.6)	20.2 (1.0)	17.8 (1.1)	18.8 (0.8)
4-84	27.4 (1.3)	26.9 (0.9)	26.8 (1.0)	16.8 (0.8)	21.2 (0.8)	16.5 (0.9)
4-67	30.5 (0.9)	29.8 (1.2)	28.8 (1.1)	15.4 (1.0)	23.4 (0.7)	14.9 (0.4)
4-19	32.8 (0.5)	32.2 (1.0)	32.0 (1.1)	24.3 (0.7)	26.2 (0.6)	24.2 (1.0)
4-67	30.8 (0.7)	30.3 (0.8)	29.8 (0.8)	20.8 (0.4)	21.7 (1.1)	20.0 (0.9)
4-19	26.9 (1.5)	26.1 (0.8)	24.4 (1.2)	17.7 (1.0)	20.4 (0.6)	17.7 (0.8)
4-32	29.6 (1.2)	29.2 (0.9)	28.8 (1.0)	21.0 (0.7)	25.0 (0.3)	20.4 (0.7)
4-84	33.9 (1.7)	33.7 (1.0)	32.5 (0.5)	24.8 (0.8)	26.2 (1.1)	24.2 (0.6)
Mean	29.4	28.9	28.2	19.8 Δ	22.4 Δ	19.2 Δ

Tissues from 18 patients were maintained in organ culture alone (Nil, 24 h) with T cell supernatants (A, 24 h and B, 24 h), and with interferon γ (IFN 100 IU and IFN 200 IU). Δ signifies a difference of $p < 0.001$ compared with biopsy specimens grown with organ culture medium alone (Nil, 24 h).

Figure 2: The ability of anti-interferon γ antibodies (anti-IFN- γ) to block the toxic effects of the post gliadin activated T cell supernatants (B) is shown. Anti-cytokine antibodies were added to a fixed concentration of (B) supernatants (sister clones of line T cell clone TCC 4-32) in increasing amounts. The addition to supernatant (B) of antibody in an amount which could neutralise 100 IU of cytokine blocked its toxic effects on enterocytes. No other anti-cytokine antibody produced such an effect. IL=interleukin; TNF- α =tumour necrosis factor α .

was compared with that in tissue grown in OCM alone. There was no significant reduction in specimens grown with (A) supernatants (26.9 (2.6) μm) showing that the resting T cell or B cell culture supernatants alone did not cause a significant reduction in enterocyte cell height. Specimens grown with the gliadin activated (B) supernatants exhibited a significant reduction in enterocyte height (21.5 (3.4)

μm , $p<0.001$). This reduction was greater with the sister clones of TCC 4-32 (19.8 (3.4) μm , $p<0.001$) than TCC 1-27 (23.5 (2.2) μm , $p<0.01$) clones but was significant in both cases. The 1:100 diluted supernatants of both clones tested caused enterocyte changes approximately equivalent to that produced by 100 IU/ml interferon γ alone (21.0 (3.2) μm , $p<0.001$) but not as great as that observed with 200 IU/ml (18.8 (2.9) μm , $p<0.0001$) of this cytokine.

The toxic effects of the (B) supernatants could be blocked by pre-incubating them before their use in organ culture with anti-interferon γ antibody at a concentration equivalent to that which would neutralise 100 IU/ml of human cytokine activity (100 ng/ml or greater). No other anti-cytokine antibody at any of the neutralising concentrations used blocked the damage produced to enterocytes by (B) supernatants (see Fig 2). The changes in tissue morphology are illustrated in Figure 3.

Discussion

CD is one of a growing number of enteropathies in which an abnormal mucosal immune response is thought to be involved in the pathogenesis. It is unique among these enteropathies in that the luminal agent which exacerbates the condition is known (gluten) and susceptibility to the disease is strongly associated with the HLA-DQ (α 1*0501, β 1*0201) (ie DQ2) heterodimer.

The isolation of T lymphocyte clones which respond to coeliac toxic cereal peptides presented by HLA-DQ2 molecules, supports

Figure 3: The photomicrographs show the typical appearances of small intestinal tissue after 24 hours in organ culture with: (a) organ culture medium alone; (b) (A) supernatant; and (c) (B) supernatant (both derived from T cell clone 4-32). There is good preservation of morphology after organ culture and with the addition of non-gluten activated T cell (and B cell) culture (A) supernatant. At a concentration of 1:100 of supernatant (B) and 100 IU/ml of interferon γ significant changes in epithelial morphology are observed which can be prevented by >100 ng/ml of interferon γ antibody (d) ($\times 50$ magnification, haematoxylin and eosin).

earlier work implicating an abnormal mucosal cell mediated immune response in CD. How these cells mediate the development of histological changes is unclear and has been studied in the present report.

We found that different degrees of epithelial cell damage were produced by supernatants from the two gliadin specific T cell clones studied. It is likely that this was the result of differences in the in vitro responsiveness of the T cell clones, causing them to secrete varying amounts of cytokine. In addition, the effects of each supernatant may have differed depending on tissue characteristics, which varied from patient to patient. The results showed that T cell immune activation occurred only after gliadin toxic epitope(s) had been presented with HLA-DQ2 molecules on the surface of B cells. As a result the cells produced soluble mediators, contained within the supernatants which caused enterocyte damage.

Antibodies to interferon γ but not to TNF- α or any other anti-cytokine antibody studied, blocked the T cell supernatant induced toxicity. This finding suggests that the gliadin sensitive clones may be TH1 cells. Analysis of the gliadin activated T cell supernatants using sensitive bioassays has shown, however, that they contain variable levels of interferon γ TNF- α IL-5, and IL-6 and do not therefore seem to fit neatly into this TH1 or TH2 classification.¹²

T cells which have been immune activated by anti-CD3 antibodies have been shown to produce supernatants which can kill HT-29 colonic carcinoma cell lines.¹³ Anti-TNF- α and anti-interferon γ antibodies act synergistically to prevent this toxicity as does anti-interferon γ but not anti-TNF- α antibody alone. Other workers have shown that the HT-29 cell line can express HLA class II molecules when treated with interferon γ and IL-2. These cells are recognised by HLA-DQ restricted T lymphocytes, which in turn proliferate and block the growth of the HT-29 cells, probably by cytotoxic mechanisms.¹⁴ Similarly, mutant p21 ras oncogene protein when presented with HLA-DQ on HT-29 cells is recognised by antigen specific T cells, resulting in arrested growth of this cell line.¹⁵ These observations provide strong evidence that soluble mediators released by T cells can induce changes in epithelial cells.

Studies which observe the effects of cytokines on intestinal epithelial cell line monolayers do not take into account changes in cell morphology resulting from the effects of cytokines on the basement membrane and cytoskeleton.¹⁶ Cytokines and inflammatory cell mediators may have effects on these cell contracts directly or indirectly by effects on the microvasculature and the formation of an excess of nitric oxide.¹⁷ How interferon γ produces the changes in intestinal epithelial cell morphology in organ culture is unclear. It is known, however, that intestinal epithelial cells possess surface receptors for interferon γ ¹⁸ and that this cytokine can affect actin distribution in some cell types thereby affecting their morphology and barrier function.¹⁹

Our preliminary results showed that interferon γ and TNF- α could each cause small

intestinal enterocyte toxicity. These cytokines often act synergistically to promote inflammatory responses and in the intestine have been shown to upregulate intracellular adhesion molecule 1 (ICAM-1),²⁰ HLA class II expression^{21 22} and to have toxic effects on intestinal epithelial cells in vitro.²³

In vivo, interferon γ may produce morphological changes by direct action on enterocytes or by stimulating the production of TNF- α from activated macrophages to enhance this effect.²⁴ Analysis of mucosal cytokine mRNA expression by in situ hybridisation and immunohistochemical analysis of cytokine protein shows that in CD the number of cells secreting interferon γ and TNF- α are significantly greater in the lamina propria than in the epithelium, and this corresponds to the site where activated CD4+ T cells and macrophages are found.^{25 26} It is also interesting to note that in experimental animals the induction of graft *versus* host disease by alloreactive T cells can be prevented by the administration of anti-interferon γ and anti-TNF- α antibodies.²⁷

Our findings support a role for activated gliadin sensitive T cell clones in the immune pathogenesis of CD and case reports of the effectiveness of cyclosporin²⁸ and steroids²⁹ in improving the small intestinal lesions of untreated CD may be explained by the inhibition of T cell function and the secretion of cytokines.

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