Detection of interferon gamma mRNA in the mucosa of patients with coeliac disease by in situ hybridisation

M Kontakou, R P Sturgess, R T Przemioslo, G A Limb, J M Nelufer, P J Ciclitira

Abstract

In situ hybridisation has been used to study interferon gamma (IFNγ) mRNA expression in the small intestine of patients with coeliac disease. Sections of jejunal biopsies were obtained from five patients with treated and five with untreated coeliac disease and five disease controls. These sections were hybridised with radiolabelled specific DNA oligonucleotide probes. The lamina propria of untreated coeliac disease patients contained a significantly increased number of IFNγ producing cells compared with controls but there was no significant difference between the coeliac patients treated with a gluten free diet and controls. The results suggest that IFNγ may play a part in the immunopathogenesis of coeliac disease.

(*Gut* 1994; 35: 1037–1041)

Coeliac disease is characterised by gluten induced damage to the small intestinal mucosa, which exhibits villous atrophy, crypt cell hyperplasia, and infiltration of the lamina propria with lymphocytes and plasma cells.[1–3] There is also an increased ratio of intraepithelial lymphocytes to small intestinal surface enterocytes.[4 5] Another important feature of coeliac disease is the enhanced expression of class II major histocompatibility complex antigens.[6 7] In most cases these changes revert to normal on removal of gluten from the diet. It has been suggested that coeliac disease represents an abnormal cellular immune response in genetically susceptible people to the ingestion of cereal peptides, which are as yet unidentified. During this immune response many cytokines which are capable of initiating cellular and molecular changes are released.

Interferon gamma (IFNγ) is produced during immune reactions, mainly by antigen, mitogen, or lectin stimulated T lymphocytes and also by large granular leukocytes. IFNγ has the ability to induce expression of major histocompatibility complex by human enterocytes in vitro[8 9] and to activate macrophages and natural killer cells.

Studies of the role of IFNγ in other enteropathies such as Crohn's disease suggest that there is an increase in the number of IFNγ secreting cells compared with controls.[10] Conflicting results have been reported, however, on interleukin 2 and IFNγ expression in the mucosa of patients with Crohn's disease and ulcerative colitis.[11–13] These results were mainly attributed to the insensitivity of the various methods used.[14] In coeliac disease, detection of IFNγ in paraffin embedded sections of normal and disease mucosa was achieved by an immunohistochemical technique.[15] It was reported that a relatively large proportion of both intraepithelial and lamina propria lymphocytes in normal small bowel produce IFNγ. The proportion of lymphocytes that produced IFNγ seemed to decrease in untreated coeliac disease patients and returned towards normal in patients on a gluten free diet. The same study, however, reported increased expression of class II major histocompatibility complex antigens on the surface enterocytes of untreated coeliac mucosa, usually associated with increased levels of IFNγ. It was suggested that although IFNγ is probably produced in increased amounts in coeliac disease, it may also be secreted at a higher rate so that less stainable product is available for detection. To evaluate this theory cytokine production in tissues should be assessed by localising not only protein expression but also mRNA levels.[16 17]

We have used a radioactive in situ hybridisation technique to detect and localise mRNA expression of IFNγ in jejunal biopsies specimens from patients with coeliac disease, in order to study the role of this particular cytokine in the pathology of the disease.

Methods

PATIENTS AND BIOPSY SPECIMENS

Peroral jejunal biopsy specimens were taken from adult patients with coeliac disease on a normal diet (n=5, mean age=45 years, range=34–56), on a gluten free diet (n=5, mean age=49·4, range=30–66), and from controls (n=5, mean age=56, range=31–75) as part of their diagnostic investigations. The controls were subsequently diagnosed as having the irritable bowel syndrome and all had normal jejunal morphology. The diagnosis of coeliac disease was made according to ESPGAN criteria.[18] All treated patients had been on a gluten free diet for at least two years and had normal or near normal villous architecture. The biopsy specimens were obtained using a Quinton multiple biopsy instrument. Tissue specimens were immediately orientated, embedded in optimal cutting temperature compound (Cryo-M-bed; Brights, Huntington, UK) snap frozen in liquid nitrogen cooled isopentane, and stored in liquid nitrogen until processed. Cryostat

The Rayne Institute, St Thomas's Hospital, London
M Kontakou
R P Sturgess
R T Przemioslo
G A Limb
J M Nelufer
P J Ciclitira

Correspondence to:
Professor P J Ciclitira, Gastroenterology Unit, UMDS, The Rayne Institute, St Thomas's Hospital, London SE1 7EH.

Accepted for publication 29 November 1993

TABLE I *Oligonucleotides with DNA sequences complementary to interferon gamma mRNA used in this study*

IFNγ.1	5'AAA GAG TTC CAT TAT CCG CTA CAT TCG AAT3'
IFNγ.2	5'GTA TTG CTT TGC GTT GGA CAT TCA AGT CAG3'
IFNγ.3	5'ACC CAA AAC GAT GCA GAG CTG AAA AGC CAA3'

sections were cut at 5 μm on a Bright open-top cryostat at −20°C and collected onto glass slides that had been washed with distilled water rendered RNase free by diethylpyrocarbonate treatment[19] and precoated with poly-L-lysine. Sections were briefly fixed in acetone for five minutes and stored at −20°C.

PROBES AND LABELLING METHOD

DNA oligonucleotide probes were prepared in-house using a Beckman synthesiser. IFNγ mRNA detection was achieved using a mixture of three probes, each 30 bases long, with cDNA sequences coded by three different exon regions of the IFNγ gene (sequence published by Gray and Goeddel).[20] The probes used in this study and their sequence are listed in Table I. Specificity of the probes was confirmed by northern blotting of mRNA coding for IFNγ, isolated from blood mononuclear cells that had been cultured in the presence of 1 μg/ml phytohaemmaglutinin or 10 μg/ml concanavalin A according to Maniatis *et al.*[19] Computer analysis of the sequences was achieved using the DNASIS program.

Four pmol of each oligonucleotide were labelled by the addition of an α ^{35}S dATP 'tail' to the 3' termini using terminal deoxynucleotidyl transferase, according to the supplier's protocol (Promega). After the labelling reaction the probes were purified by passing them through a prepacked Sephadex G-50 DNA grade column (Pharmacia), and their specific activity was measured using a Rackbeta liquid scintillation counter. The specific activity of the probes was not less than 1×10^8 cpm/μg.

PRETREATMENT AND HYBRIDISATION

The method used was a modification of that previously described by Hamid *et al.*[21] All solutions used were made up with RNase free distilled water. Sections, kept at −20°C, were air dried for at least one hour before fixation with freshly prepared 4% (w/v) paraformaldehyde in phosphate buffered saline pH 7·4 for 15 minutes, followed by two washes in 15% (w/v) sucrose in phosphate buffered saline for 10 minutes each. Pretreatments also involved incubation with 0·3% (v/v) Triton-X-100 in phosphate buffered saline for 10 minutes, followed by incubation at 37°C with 1 μg/ml proteinase K for 30 minutes, postfixation in 0·4% (w/v) paraformaldehyde for 10 minutes and treatment with 0·1 M triethanolamine containing 0·25% (v/v) acetic anhydride for 10 minutes. The sections were prehybridised in a mixture of 2×saline-sodium citrate buffer, 50% (v/v) formamide and 1×Denhardt's solution at 37°C for one hour and then hybridised with 2 ng of labelled probe overnight at 37°C in a solution containing: 2×sodium citrate buffer, 50% (v/v) formamide, 0·4 mg/ml denatured sheared salmon sperm DNA and 10% (w/v) dextran sulphate (MW 500 000). Posthybridisation stringency washes were carried out at 39°C, for 30 minutes each, in 2×sodium citrate buffer, 1×sodium citrate buffer+50% formamide and 0·1×sodium citrate buffer. Sections were dehydrated serially in 60%, 90%, and 95% ethanol and dried at room temperature.

CONTROLS

A sense sequence complementary to the IFNγ.1 probe was used as a negative control. An oligo dT probe was also used for the detection of total mRNA and served as a positive control. To establish whether the hybrids formed were indeed RNA/DNA, some sections were treated with either DNase-free RNase (negative hybridisation signal) or RNase-free DNase (no effect on hybridisation) after the proteinase K treatment. Sections were incubated at 37°C with either of the two enzymes at a concentration of 600 U/ml for 30 minutes.

AUTORADIOGRAPHY

Dried sections were dipped in Ilford K5 photographic gel emulsion that had been diluted 1:1 in prewarmed distilled water, left to dry in a dark room for approximately two hours, and then incubated at 4°C in a light-proof box containing silica gel for five days. Slides were developed in 44 g/l Dektol solution (Kodak) for two minutes, washed briefly in distilled water and fixed in 30% (w/v) sodium thiosulphate at 4°C for 15 minutes. Finally, the slides were thoroughly rinsed in running tap water and counterstained with Mayer's haematoxylin.

QUANTITATION AND STATISTICAL ANALYSIS

The number of positive cells within the lamina propria were counted per unit area by using an eye piece graticule (1 mm^2) under ×400 magnification. At least five consecutive areas of the lamina propria (within and below the villi going to the deeper lamina propria) were counted for each section and for each specific probe a minimum of two sections were prepared and scored blindly for each patient. Each investigation with specific probes was repeated twice for all patients and the mean taken. The results were expressed as the mean number of positive cells per unit area (0·0025 mm^2). The standard deviation of the mean was calculated for each group and the Student's *t* test was used to compare results for different patient groups.

Morphometric measurements were performed by an eyepiece micrometer under ×200 magnification. For each section a minimum of five measurements of villous height to crypt depth ratio (V/C) were

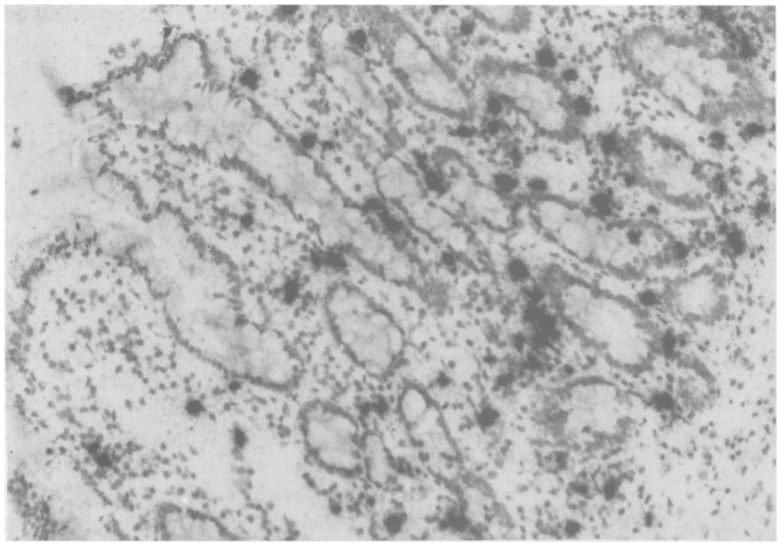

Figure 1: Jejunal biopsy specimen from an untreated coeliac disease patient showing the distribution of cells expressing mRNA for IFNγ. Positive cells are abundant in the lamina propria but not the epithelium (original magnification ×200).

made and the mean and standard deviation calculated.

Results

IFNγ mRNA EXPRESSION

In situ hybridisation, using ^{35}S-labelled antisense oligonucleotide probe for IFNγ, showed that numerous cells infiltrating the jejunal mucosa contained IFNγ mRNA. Positively stained cells were found mainly in the superficial lamina propria, and occasionally in the deeper lamina propria. There was no staining of the intraepithelial lymphocytes or surface enterocytes. The results of one untreated patient and one with normal mucosa, are shown in Figures 1 and 2 respectively. A similar label intensity was obtained in the mucosa of the other patients within the groups. The number of IFNγ expressing cells in the lamina propria of the untreated coeliac disease patients was increased significantly ($p<0{\cdot}001$) compared with treated patients and controls. The difference in the numbers of IFNγ mRNA expressing cells between treated coeliac patients and controls was not significant. These results are summarised in Table II, together with the morphometric analysis for each group. The untreated coeliac patients showed a high degree of villous atrophy (ratio of villous height to crypt depth ≤ 1), while the treated patients had near normal mucosal morphology (normal ratio of villous height to crypt depth between 3–5).

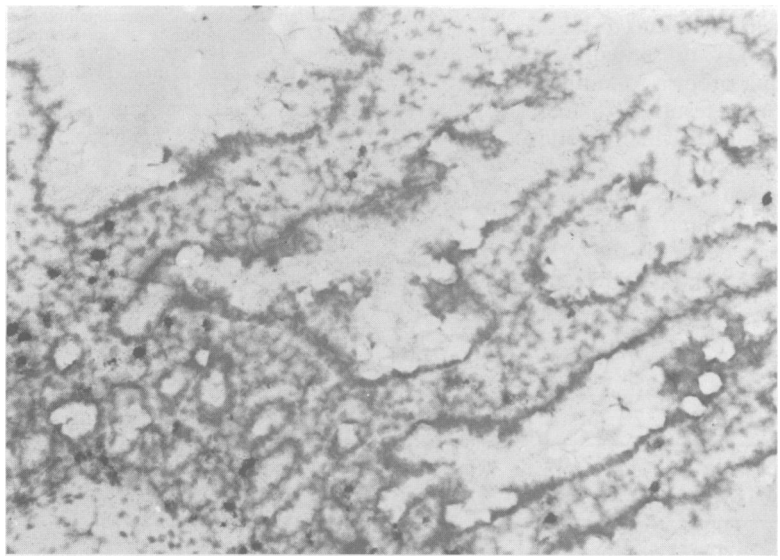

Figure 2: Distribution of IFNγ mRNA expressing cells in normal jejunal mucosa. Fewer positive cells are seen compared with untreated coeliac mucosa (Fig 1), but still very clearly identified in the lamina propria (original magnification ×200).

IN SITU HYBRIDISATION CONTROLS

The absence of hybridisation signal, in all patient groups, with the ^{35}S-labelled sense probe (Fig 3) indicated that the binding of the IFNγ antisense probe was specific. Treatment with RNase, but not DNase, before hybridisation also resulted in failure of hybridisation with labelled sense and antisense probe, indicating that the hybridisation was indeed on mRNA. Hybridisation with oligo dT probes for assessment of total mRNA was detected in all biopsies.

Discussion

In this study we have shown that IFNγ is expressed by lamina propria cells, increasingly so in active coeliac disease, by assessing the presence of cytoplasmic messenger RNA. IFNγ is known to be produced mainly by activated T lymphocytes and most of those found in the lamina propria are of the CD4$^+$ (helper/inducer) subset.[22] Although the phenotype of the positive cells was not identified in this study, the localisation of these cells in the lamina propria indicates that IFNγ is probably expressed by CD4$^+$ T cells. Recent studies on activated T lymphocytes in coeliac disease reported the induction of proliferative activation of CD8$^+$ (suppressor/cytotoxic) intraepithelial lymphocytes but non-proliferative activation of lamina propria CD4$^+$ T cells.[23] It was suggested that gluten specific CD4$^+$ lamina propria T cells probably produce cytokines that induce epithelial crypt cell hyperplasia as well as local proliferation of intraepithelial lymphocytes. This agrees with reports in Crohn's disease that IFNγ and interleukin 2 are produced by activated CD4$^+$ cells.[14] The same report suggested that there is a continuing delayed hypersensitivity response within the mucosa in Crohn's disease which may be responsible for the pathological damage of the mucosa. In mice, IFNγ is synthesised by the TH1 subpopulation of CD4$^+$ cells, which have been shown to cause a delayed hypersensitivity response, partially ascribed to the production of IFNγ, in response to local presentation of antigen.[24]

IFNγ, as well as tumour necrosis factor α, has been shown to be a capable effector molecule in immune destruction of self tissues,[25] and to reduce the epithelial function of the human colon carcinoma cell line HT29 cl 19A.[26] Both cytokines can destroy target tissues of known autoimmune responses,

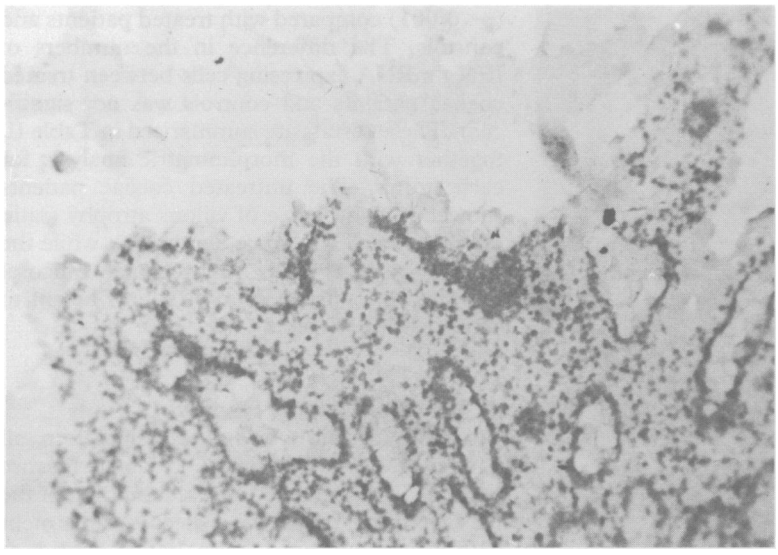

Figure 3: Jejunal biopsy from an untreated coeliac disease patient showing absence of hybridisation signal with the sense IFNγ probe, used as a negative control (original magnification ×200).

individually or in combination, as was shown in an animal model of diabetes where a combination of both cytokines destroyed isolated pancreatic β islet cells in vitro.[27] IFNγ also inhibits the proliferation of rodent intestinal epithelial cell lines[28] and decreases epithelial tight junction permeability.[29 30] In the present study the treated coeliac disease patients had a near normal mucosal morphology, as supported by a normal villous height to crypt depth ratio, and the number of IFNγ producing cells showed no significant difference from the controls. It is therefore possible that in coeliac disease there is an association between the observed damage to the villi and increased IFNγ production. This hypothesis should be confirmed by studying the effect of IFNγ on epithelial differentiation and proliferation in human jejunum organ culture.

A possible cellular immunopathogenic mechanism involving the intestinal mucosa in coeliac disease has been described in a recent review.[7] Short chain toxic peptides produced by the digestion of gluten are absorbed across the small intestinal epithelium, bound to HLA class II molecules, and presented by macrophages in the lamina propria to antigen specific $CD4^+$ T cells. As a result, cytokines such as IFNγ are produced which could lead to increased expression of HLA class II antigens on surface enterocytes[9] and adhesion molecules within the lamina propria.[31] Damage to enterocytes may occur via the local production of toxic mediators, both within the lamina propria and the epithelium, and also by direct cytotoxic mechanisms.

TABLE II *Number of interferon gamma mRNA (IFNγ) expressing cells in the lamina propria/0·0025 mm² and degree of villous atrophy (expressed as villous height to crypt depth ratio) in each of the patient groups studied*

Group	No	IFNγ mRNA expressing cells (mean (SD))	Villous height to crypt depth ratio (mean (SD))
Coeliac disease:			
Untreated	5	18·58 (2·71)	0·88 (0·16)
Treated	5	5·0 (1·83)	3·62 (0·19)
Controls	5	4·1 (2·63)	4·12 (0·33)

The initial event in the action of the interferons is their binding to specific receptors on the cell surface, present on most cells. After the binding, the interferon receptor complexes are internalised by receptor mediated endocytosis.[32] For this reason, the localisation of the IFNγ product by immunohistochemical methods has been very difficult to interpret, because it will also identify target cells as well as any complexes passively uptaken by phagocytes and mucosal epithelial cells.[33] Cytokine production can also be affected by competitive inhibition of cytokine receptor by specific and non-specific receptor antagonists. This mechanism has been well described for interleukin 1.[34] One or more of the mechanisms described above could explain the conflicting results between the present study using in situ hybridisation and a previous report using immunohistochemistry.[15] Although, mRNA levels can also be misleading as some cytokines are regulated post-transcriptionally, in situ hybridisation remains a very powerful technique enabling the localisation of the cellular synthesis of various cytokines. The results of the present study support the hypothesis that the primary events in the immunopathogenesis of coeliac disease occur in the lamina propria and suggest that IFNγ is probably associated with the damage to the villi that is observed in this condition.

The authors wish to thank the St Thomas's Research (Endowments) Committee, the Nutricia Research Fund, and the Jean Shanks Research Foundation.

1 Marsh MN. Mucosal pathology in gluten sensitivity. In: Marsh MN, ed. *Coeliac Disease.* Oxford: Blackwell Scientific Publications, 1992: 136–91.
2 Marsh MN. The morphology and immunopathology of the jejunal lesion in gluten-sensitivity. *Eur J Gastroenterol Hepatol* 1991; **3:** 163–8.
3 Ciclitira P, Ellis H, Wood G, Howdle P, Losowsky M. Secretion of gliadin antibody by coeliac jejunal mucosal biopsies cultured in vitro. *Clin Exp Immunol* 1986; **64:** 119–24.
4 Spencer J, MacDonald T, Walker-Smith J, Ciclitira P, Isaacson P. Changes in intra-epithelial lymphocyte subpopulations in coeliac disease and enteropathy associated T-cell lymphoma (malignant histiocytosis of the intestine). *Gut* 1989; **30:** 339–46.
5 Brandtzaeg P, Halstensen T, Kett K, Kracci P, Kvale D, Rognum T, *et al.* Immunobiology and immunopathology of human gut mucosa: humoral immunity and intraepithelial lymphocytes. *Gastroenterology* 1989; **97:** 1562–84.
6 Kagnoff MF. Genetic basis of coeliac disease: role of HLA genes. In: Marsh MN, ed. *Coeliac Disease.* Oxford: Blackwell Scientific Publications, 1992: 215–38.
7 Ciclitira P, Sturgess R. Clinicopathologic mechanisms in celiac disease. *Curr Opinion Gastroent* 1992; **8:** 262–7.
8 Sollid L, Gaudernack G, Markussen G, Kvale D, Brandtzaeg P, Thorsby E. Induction of various HLA class II molecules in a human colonic adenocarcinoma cell line. *Scand J Immunol* 1987; **25:** 175–80.
9 Sturgess R, Hopper L, Spencer J, Hung C, Nelufer J, Ciclitira P. Effects of interferon-γ and tumour necrosis factor-α on epithelial HLA class-II expression on jejunal mucosal biopsy specimens cultured in vitro. *Scand J Gastroenterol* 1992; **27:** 907–11.
10 Fais S, Capobianchi M, Pallone F, Di Marco P, Boirivant M, Dianzani F, *et al.* Spontaneous release of interferon γ by intestinal lamina propria lymphocytes in Crohn's disease. Kinetics of in vitro response to inteferon γ inducers. *Gut* 1991; **32:** 403–7.
11 Kusugami K, Yongman K, West G, Fiocchi C. Intestinal immune reactivity to interleukin 2 differs among Crohn's disease, ulcerative colitis, and controls. *Gastroenterology* 1989; **97:** 1–9.
12 Ouyang Q, El-Youssef M, Yen-Lieberman B, Sapatnekar W, Youngman K, Kusugami K, *et al.* Expression of HLA-DR antigens in inflammatory bowel disease mucosa: role of intestinal lamina propria mononuclear cell-derived interferon gamma. *Dig Dis Sci* 1988; **33:** 1528–34.
13 MacDonald T, Hutchings P, Choy M, Murch S, Cooke A. Tumour necrosis factor-alpha and interferon-gamma-

production measured at the single cell level in normal and inflamed human intestine. *Clin Exp Immunol* 1990; **81:** 301–5.
14 Breese E, Braegger C, Corrigan C, Walker-Smith J, MacDonald T. Interleukin-2 and interferon-y-secreting T cells in normal and diseased human intestinal mucosa. *Immunology* 1993; **78:** 127–31.
15 Al-Dawoud A, Nakashabendi I, Foulis A, McMowat A. Immunohistochemical analysis of mucosal gamma-interferon production in coeliac disease. *Gut* 1992; **33:** 1482–6.
16 Dallman M, Montgomery R, Larsen C, Wanders A, Wells A. Cytokine gene expression: analysis using northern blotting, polymerase chain reaction and in situ hybridisation. *Immunol Rev* 1991; **119:** 163–79.
17 Ogilvie A, Wood N, Dickens E, Wojtacha D, Duff G. In situ hybridisation. *Ann Rheum Dis* 1990; **49:** 434–9.
18 Meeuwisse GW. Diagnostic criteria in coeliac disease (European Society of Paediatric Gastroenterology). *Acta Paed Scand* 1970; **59:** 461–3.
19 Sambrook J, Fritsch E, Maniatis T. Molecular Cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory Press, 1989: 7.4.
20 Gray PW, Goeddel DV. Molecular biology of interferon-gamma. In: *Lymphokines.* New York: Academic Press Inc, 1987.
21 Hamid Q, Azzawi M, Ying S, Maqbel R, Wardlow A, Corrigan C, *et al.* Expression of mRNA for interleukin-5 in mucosal bronchial biopsies from asthma. *J Clin Invest* 1991; **87:** 1541–6.
22 MacDonald T. T cell-mediated intestinal injury. In: Marsh M, ed. *Coeliac disease.* Oxford: Blackwell Scientific Publications, 1992: 293–304.
23 Halstensen T, Brandtzaeg P. Activated T lymphocytes in celiac lesion: non-proliferative activation (CD25) of CD4+ α/β cells in the lamina propria but proliferation (Ki-67) of α/β and γ/δ cells in the epithelium. *Eur J Immunol* 1993; **23:** 505–10.
24 Mosmann T, Coffman R. Heterogeneity of cytokine secreting patterns and functions of helper T cells. *Adv Immunol* 1989; **46:** 111–7.
25 Deem R, Shanahan F, Targan S. Triggered human mucosal T cells release tumour necrosis factor-alpha and interferon-gamma which kill human colonic epithelial cells. *Clin Exp Immunol* 1991; **83:** 79–84.
26 Hiribarren A, Heyman M, L'Helgouac'h A, Dejeux J. Effect of cytokines on the epithelial function of the human colon carcinoma cell line HT29 cl 19A. *Gut* 1993; **34:** 616–20.
27 Cambell I, Iscaro A, Harrison L. IFNγ and tumour necrosis factor α cytotoxicity to murine islets of langerhans. *J Immunol* 1988; **141:** 2325–31.
28 Cerf-Bensussan N, Quaroni A, Kurnick J, Bhan A. Intraepithelial lymphocytes modulate Ia expression by intestinal epithelial cells. *J Immunol* 1984; **132:** 2244–52.
29 Madara J, Stafford J. Interferon-γ directly affects barrier function of cultured intestinal epithelial monolayers. *J Clin Invest* 1989; **83:** 724–7.
30 MacDonald T, Spencer J. Evidence that activated T cells play a role in the pathogenesis of enteropathy in human small intestine. *J Exp Med* 1988; **167:** 1341–9.
31 Sturgess R, Kontakou M, Spencer J, Hooper L, Makgoba M, Ciclitira P. Effects of Interferon-γ and Tumour necrosis factor-α on ICAM-1 expression on jejunal mucosal biopsies cultured in vitro. *Gut* 1993; **34** (1): S31.
32 Oppenheim J, Ruscetti F, Faltynek C. Cytokines. In: Stites D, Terr A, eds. *Basic and clinical immunology.* Appleton and Lange, 1988: 78–100.
33 Stamp G, Poulsom R, Chung L, Keshav S, Jeffery R, Longcroft J, *et al.* Lysozyme gene expression in Inflammatory Bowel Disease. *Gastroenterology* 1992; **103:** 532–8.
34 Seckinger P, Lowenthal J, Williamson K, Dayer J, MacDonald H. A urine inhibitor of interleukin 1 activity that blocks ligand binding. *J Immunol* 1987; **139:** 1546–9.