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

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Abstract

In situ hybridisation has been used to study interferon gamma (IFN γ) 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 IFN γ 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 IFN γ may play a part in the immunopathogenesis of coeliac disease.

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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 (IFN γ) is produced during immune reactions, mainly by antigen, mitogen, or lectin stimulated T lymphocytes and also by large granular leukocytes. IFN γ 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 IFN γ in other enteropathies such as Crohn's disease suggest that there is an increase in the number of IFN γ secreting cells compared with controls.¹⁰ Conflicting results have been reported, however, on interleukin 2 and IFN γ 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 IFN γ 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 IFN γ . The proportion of lymphocytes that produced IFN γ 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 IFN γ . It was suggested that although IFN γ 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 IFN γ 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

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

IFN γ .1	5'AAA GAG TTC CAT TAT CCG CTA CAT TCG AAT ³
IFN γ .2	5'GTA TTG CTT TGC GTT GGA CAT TCA AGT CAG ³
IFN γ .3	5'ACC CAA AAC GAT GCA GAG CTG AAA AGC CAA ³

sections were cut at 5 μ m 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 treatment¹⁹ 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. IFN γ 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 IFN γ 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 IFN γ , isolated from blood mononuclear cells that had been cultured in the presence of 1 μ g/ml phytohaemmagglutinin or 10 μ g/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/ μ g.

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 μ g/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 $\times$ saline-sodium citrate buffer, 50%

(v/v) formamide and 1 $\times$ 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 $\times$ 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). Post-hybridisation stringency washes were carried out at 39°C , for 30 minutes each, in 2 $\times$ sodium citrate buffer, 1 $\times$ sodium citrate buffer+50% formamide and 0.1 $\times$ 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 IFN γ .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 mm²) under $\times 400$ 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 mm²). 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 $\times 200$ 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 IFN γ . Positive cells are abundant in the lamina propria but not the epithelium (original magnification $\times 200$).

made and the mean and standard deviation calculated.

Results

IFN γ mRNA EXPRESSION

In situ hybridisation, using ^{35}S -labelled antisense oligonucleotide probe for IFN γ , showed that numerous cells infiltrating the jejunal mucosa contained IFN γ 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 IFN γ expressing cells in the lamina propria of the untreated coeliac disease patients was increased significantly

Figure 2: Distribution of IFN γ 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 $\times 200$).

($p < 0.001$) compared with treated patients and controls. The difference in the numbers of IFN γ 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).

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 IFN γ 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 IFN γ is expressed by lamina propria cells, increasingly so in active coeliac disease, by assessing the presence of cytoplasmic messenger RNA. IFN γ 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 IFN γ 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 IFN γ 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, IFN γ 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 IFN γ , in response to local presentation of antigen.²⁴

IFN γ , 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 IFN γ probe, used as a negative control (original magnification $\times 200$).

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 IFN γ also inhibits the proliferation of rodent intestinal epithelial cell lines²⁸ 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 IFN γ 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 IFN γ production. This hypothesis should be confirmed by studying the effect of IFN γ 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 IFN γ are produced which could lead to increased expression of HLA class II antigens on surface enterocytes⁹ 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.

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 IFN γ 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 IFN γ is probably associated with the damage to the villi that is observed in this condition.

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TABLE II Number of interferon gamma mRNA (IFN γ) 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	IFN γ 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)

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