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Immunohistochemical analysis of mucosal gamma-interferon production in coeliac disease

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Abstract

The role of γ-interferon in the pathogenesis of enteropathies with an immunological basis such as coeliac disease, is unclear. γ-interferon immunoreactive lymphocytes were quantified in jejunal biopsies from patients with coeliac disease and from normal controls. In coeliac disease, there was an apparent decrease in the percentage of both intra-epithelial (3·5% *v* 13·5%) and lamina propria (10·3% *v* 47·2%) lymphocytes expressing γ-interferon compared with controls. In patients successfully treated with a gluten free diet, the percentage of γ-interferon immunoreactive intra-epithelial lymphocytes was 10·3%. Intraepithelial lymphocytes were immunonegative for class II major histocompatibility complex, while epithelial cells showed increased expression of this product in coeliac disease. The results show that a relatively large proportion of lymphocytes in normal small bowel express γ-interferon. They also indicate that in coeliac disease the major increase in the numbers of mucosal lymphocytes is the result of infiltration by lymphocytes not expressing γ-interferon.

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Gamma-interferon plays a critical role in protective immune responses because of its ability to stimulate the effector functions of several non-specific cells such as macrophages and natural killer cells, and to enhance the expression of class II major histocompatibility complex antigens on antigen presenting cells and other tissue cells.[1] γ-interferon has also been shown to modulate the proliferation and function of tissue cells from a variety of organs, including thyroid, pancreas, brain bone, and kidney. Therefore, this T lymphocyte product has been implicated as an important mediator of pathology in a variety of autoimmune and inflammatory conditions.

Gamma-interferon may play a similar role in enteropathies with an immunological basis such as coeliac disease, and this has been supported by several recent findings. Enhanced expression of class II major histocompatibility complex antigens is a characteristic feature of coeliac disease[2 3] and γ-interferon is a potent inducer of major histocompatibility complex expression by human enterocytes in vitro.[4] Furthermore, the similar form of enteropathy which occurs during a graft-*versus*-host reaction in experimental animals can be prevented by in vivo administration of anti γ-interferon monoclonal antibodies.[5] There is no direct evidence, however, that γ-interferon plays a pathogenetic role in clinical enteropathy. In addition, it is not known whether γ-interferon producing cells are present within the intestinal mucosa or if the production of γ-interferon correlates with pathological changes in the epithelium. In this report, we have attempted to define the role of γ-interferon in intestinal immunopathology by enumerating γ-interferon producing lymphocytes in paraffin embedded sections of normal and coeliac disease intestine using an immunohistochemical technique. In parallel, we have correlated the number of γ-interferon secreting cells with the epithelial expression of class II major histocompatibility complex antigens and have examined the effects of treatment with a gluten free diet.

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Methods

JEJUNAL BIOPSIES

Formol saline fixed, paraffin embedded Crosby capsule jejunal biopsy specimens were collected by reviewing the archives of the Royal Infirmary Pathology Department over a five year period.

Thirty six biopsy specimens from 34 adult patients with coeliac disease were retrieved and these were classified into four groups as described below and in the Table.

Group 1 comprised biopsies from patients with untreated coeliac disease (n=19).

Group 2 comprised biopsies from patients previously diagnosed as having coeliac disease. The biopsies studied in this group showed no histological improvement despite the patients claiming to adhere strictly to the gluten free diet (eight).

Group 3 comprised biopsies from patients previously diagnosed as having coeliac disease who admitted not adhering to a gluten free diet. These biopsies (two) showed no histological improvement.

Group 4 comprised follow up of histologically normal biopsy specimens from five patients, previously diagnosed as having coeliac disease, who had strictly adhered to a gluten free diet.

The interval between the initial diagnosis of coeliac disease and subsequent jejunal biopsies (Groups 2, 3, and 4) varied between three and 12 months (average seven months).

An additional 32 biopsies from adults investigated for diarrhoea but with a normal jejunal mucosa on histology were selected as controls. The age and sex distribution of all patients are detailed in the Table.

STAINING TECHNIQUES

Five micron serial sections were cut from the tissue blocks and placed on 3-aminopropyltriethoxysilane coated slides. The first serial section from each block was stained by haematoxylin and eosin. Subsequent sections were stained by

Clinical details, total number of lymphocytes in the epithelium and lamina propria, and proportion of interferon expressing lymphocytes, in the groups of coeliac patients and controls detailed in Methods

						IEL (n)			*IPL (n)*		
						Total	*γ-IFN+*		*Total*	*γ-IFN+*	
Group	*Patients (n)*	*Biopsies (n)*	*M*	*F*	*Age (yr)*	*Median (range) p value*	*Median (range) p value*	*% γ IFN p value*	*Median (range) p value*	*Median (range) p value*	*% γ IFN+ p value*
Group 1	19	19	4	15	16–74	20·3 (11·8–61·1) 0·06	0·7 (0·1–2·7) 0·2	3·5% 0·06	7·8 (4·3–15·7) 0·13	0·8 (0·3–5·9) 0·2	10·3% 0·09
Group 2	8	9	3	5	25–31	23·0 (13·4–32·8) 0·06	1·0 (0·1–3·0) 0·4	4·3% 0·1	9·1 (6·0–10·7) 0·1	0·9 (0·4–3·8) 0·1	9·9% 0·05
Group 3	2	2	0	2	45–46	17·9 (15·8–20·0) 0·08	0·2 (0·1–0·3) 0·04	1·1% 0·05	5·9 (5·1–6·7) 0·4	1·1 (1·0–1·1) 0·04	18·6% 0·04
Group 4	5	6	2	3	18–30	15·6 (7·8–18·0) 0·2	1·6 (0·1–2·5) 0·5	10·3% 0·3	7·6 (4·8–9·9) 0·2	3·2 (2·2–4·3) 0·3	42·1% 0·2
Control	32	32	14	18	13–72	11·1 (2·3–20·8)	1·5 (0·3–3·8)	13·5%	5·3 (3·1–10·6)	2·5 (1·1–4·8)	47·2%

Absolute counts of total lymphocytes and of γIFN-expressing cells are expressed as IEL/0·19 mm epithelium and LPL/0·0025 mm² lamina propria, or as the percentage of CD45+ cells which express γIFN. p Values refer to differences between each group and the control group.
IEL=intra epithelial lymphocytes; IPL=lamina propria lymphocytes; γ-IFN=gamma-interferon.

indirect immunoperoxidase techniques using overnight incubation at 4°C of the following primary antisera: (1) mouse monoclonal anti-leucocyte common antigen (CD45 Dako, High Wycombe, UK), was used to identify total lymphocyte numbers; (2) rabbit antihuman class II major histocompatibility complex[6] (a gift from Dr J J Neefjes, Amsterdam, the Netherlands, and (3) sheep antihuman interferon-gamma (a gift from Dr K Cantell, Helsinki, Finland). The rabbit antibody to class II major histocompatibility complex was used at a dilution of 1:100, while the sheep anti interferon-gamma antiserum was absorbed twice with a 1:1 mixture of porcine and guinea pig liver powders (Sigma, Dorset, UK) before being used on sections at a dilution of 1:100. Normal tonsil and inflamed nasal polyps were used as positive controls for class II major histocompatibility complex and interferon-gamma staining respectively. Details of these techniques have been given previously.[7 8]

The antibody to γ-interferon was raised to highly purified natural human γ-interferon and had a neutralising titre against γ-interferon of 1:10 000 but had no detectable activity against human alpha or beta interferons.[9] As negative controls, additional sections were stained with appropriately diluted normal rabbit or sheep sera.

The following secondary antisera were used in the indirect techniques: peroxidase conjugated swine antirabbit immunoglobulin (dilution 1:50, Dako, High Wycombe, UK), peroxidase conjugated swine antisheep immunoglobulins (dilution 1:100, Serotec, Oxford, UK) and peroxidase conjugated rabbit anti mouse immunoglobulins (dilution 1:50, Dako). Diaminobenzidine was used as substrate in the techniques.

ENUMERATION OF POSITIVELY STAINED CELLS

Quantification of cells expressing CD45 or γ-interferon was achieved by using an eye piece graticule in a Laborlux microscope (Leitz) under ×400 magnification. The number of positive intra-epithelial lymphocytes was counted per unit length of epithelium (0·19 mm) and the number of positive lymphocytes within the lamina propria was counted per unit area (0·0025 mm²). In each case, the numbers of γ-interferon positive cells were expressed both per unit length (or area) and as a percentage of the total number of CD45+ cells.

The extent and intensity of class II major histocompatibility complex staining of epithelial cells was assessed in both surface epithelium and crypt epithelium.

STATISTICAL ANALYSIS

The Mann-Whitney U-test was used to compare results for different groups.

Results

EXPRESSION OF CLASS II MAJOR HISTOCOMPATIBILITY COMPLEX ANTIGENS ON ENTEROCYTES

Class II major histocompatibility complex antigen could be detected on the epithelial cells of all control cases. This was usually at low concentrations and was found predominantly in the upper two-thirds of the villi, with crypt epithelial cells being entirely negative. In coeliac disease, patients with abnormal histology have increased intensity of epithelial staining which in some cases now extended into the crypts. In patients whose mucosa had returned to normal after a gluten free diet, the intensity and extent of class II major histocompatibility complex expression were similar to that seen in control biopsies.

GAMMA-INTERFERON EXPRESSING LYMPHOCYTES IN NORMAL MUCOSA

Gamma-interferon expressing cells which all had the morphology of small lymphocytes, were readily detected both in the lamina propria and epithelium of the normal controls (Fig 1). Indeed, the median number of γ-interferon expressing cells in the lamina propria was almost

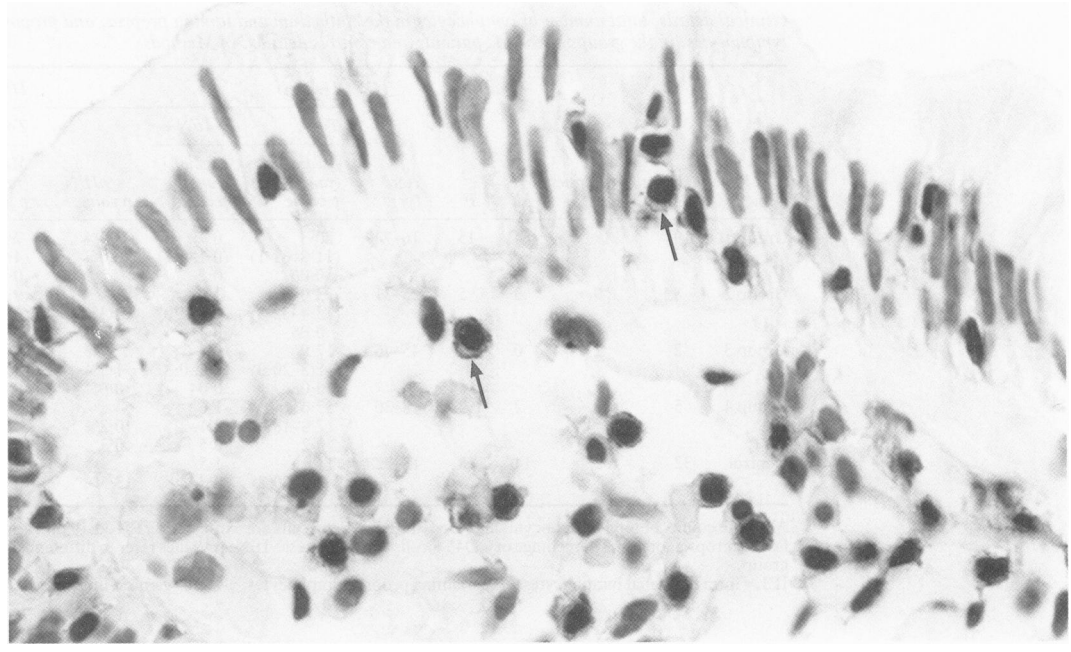

Figure 1: Distribution of γ-interferon expressing lymphocytes in normal jejunal mucosa as detected by indirect immunoperoxidase technique. Positive lymphocytes are abundant in the lamina propria and are also easily identified in the epithelium (arrow).

half the total of leucocytes identified by the anti-CD45 antibody (range: 20–88%), while 13·5% of normal intraepithelial lymphocytes were positive for γ-interferon (range: 2–35%). The staining was specific for γ-interferon as it could be blocked by preincubating the antibody with recombinant human γ-interferon.[8]

GAMMA-INTERFERON EXPRESSION BY INFILTRATING LYMPHOCYTES IN COELIAC DISEASE

As anticipated, patients with untreated coeliac disease had an increased density of CD45+ intra epithelial lymphocytes compared with disease controls (although this did not reach statistical significance), as did patients on a gluten free diet with abnormal histology (Table). The density of lamina propria lymphocytes was somewhat increased in most groups of coeliac patients, but this showed considerable variability and did not exhibit the same relationship to dietary status (Table).

Despite the increased total intra epithelial lymphocyte count, there was no corresponding increase in the absolute number of γ-interferon expressing intra epithelial lymphocytes in coeliac patients with abnormal histology (Table, Fig 2). As a result, the proportion of intra epithelial lymphocytes which expressed γ-interferon was considerably reduced in these patients, compared with disease controls (Fig 3). Although this did not attain statistical significance in individual groups, the proportion of γ-interferon expressing intra epithelial lymphocytes in untreated coeliacs was only 25% of the value found in normal mucosa and a similar reduction was found in other groups with mucosal pathology (Fig 3). Furthermore, in those patients with resolution of pathology on a gluten free diet, the proportion of γ-interferon expressing intra epithelial lymphocytes returned towards normal concentrations, indicating a true disease related effect (Fig 2). The proportion of γ-interferon expressing lymphocytes in the lamina propria of coeliac patients showed an identical pattern, with reduced levels (33%–43% of control values) in all groups of patients with abnormal histology and a return towards normal during a successful gluten free diet (Table, Fig 4).

Discussion

Our study is the first to enumerate γ-interferon producing cells in sections of human intestine and the results show that a remarkably large proportion of lymphocytes in the lamina propria and epithelium of normal small bowel mucosa contain γ-interferon. As γ-interferon is produced primarily by activated T cells, these findings confirm other impressions that intestinal T lymphocytes are in a constant state of immune

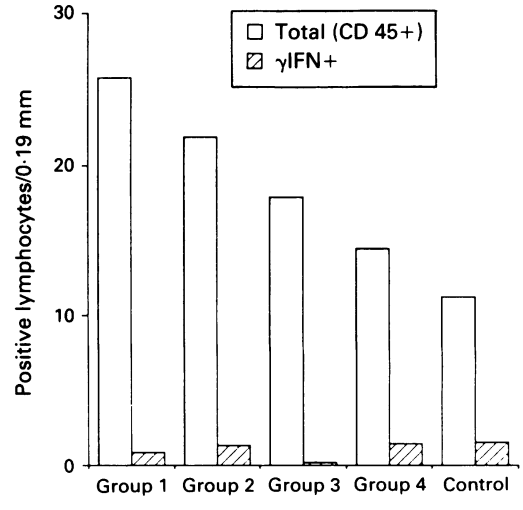

Figure 2: Numbers of total (CD45+) and γ-interferon expressing intraepithelial lymphocytes in jejunal biopsy specimens from different groups of patients with coeliac disease. Results shown are mean number of positive intra epithelial lymphocytes/0·19 mm epithelium.

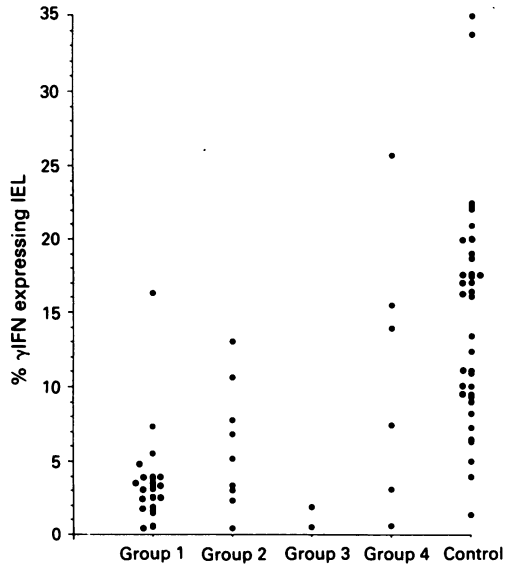

Figure 3: Proportion of intraepithelial lymphocytes (IEL) expressing γ-interferon in normal and coeliac jejunal biopsies. Results shown are the proportion of γ-interferon positive intra epithelial lymphocytes expressed as a percentage of the total number of CD45+ intra epithelial lymphocytes in the same biopsy. p values comparing groups of patients with coeliac disease with the control group are as follows: Gp.1=0·06, Gp.2=0·1, Gp.3=0·05, Gp.4=0·3.

activation, with a large proportion of human mucosal T cells expressing memory cell markers[10 11] associated with the capacity to produce large amounts of a range of different cytokines.[12]

Our results contrast with recent reports which found few γ-interferon secreting cells in normal human intestinal mucosa,[13 14] but this absence could reflect the effects of isolating mucosal lymphocytes. In addition others have shown in mice that a high proportion of mucosal lymphocytes produce cytokines such as γ-interferon and interleukin 5 in vitro.[15] These findings support our own observations and it would be of interest to use immunohistochemistry to examine for the presence of other cytokines in the normal human mucosa.

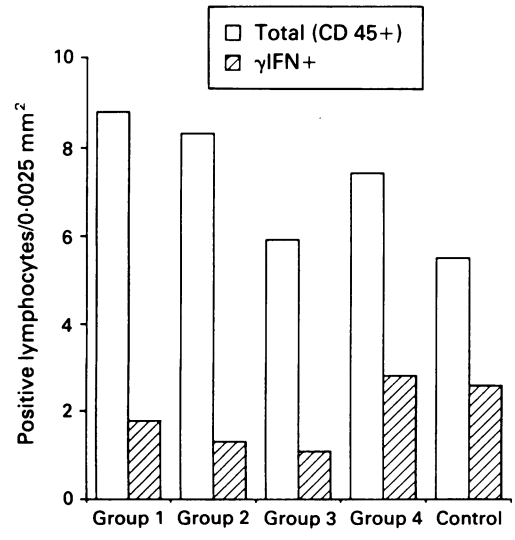

Figure 4: Number of total (CD45+) and γ-interferon expressing lymphocytes in the lamina propria of jejunal biopsies from different groups of patients with coeliac disease. Results shown are mean of positive lymphocytes/0·0025 mm² lamina propria.

We were unable to analyse the phenotype of the lymphocytes producing γ-interferon because the specimens had been fixed in formalin. CD4+ T cells are normally held to be the principal source of this mediator[1] and the higher proportion of γ-interferon producing cells we found among lamina propria lymphocytes compared with intra epithelial lymphocytes is consistent with the relative numbers of CD4+ T cells in these sites.[5] Nevertheless, CD8+ T cells from mouse intestine can produce significant amounts of γ-interferon in vitro,[15] emphasising the need to determine the phenotype of γ-interferon producing lymphocytes directly. Furthermore, direct confirmation is needed that the cytoplasmic γ-interferon we detected was being synthesised actively by these cells, using appropriate techniques to assess the presence of cytoplasmic messenger ribonucleic acid.

An unexpected finding from our study was that, in untreated coeliac disease, the proportion of γ-interferon positive cells in the lamina propria and epithelium was reduced compared with normal mucosa. This was despite the fact that our patients had the expected increases in intra epithelial lymphocyte count, as well as increased expression of epithelial class II major histocompatibility complex antigens. The proportional reduction in γ-interferon production appeared to be truly disease related as the numbers of γ-interferon positive intra epithelial lymphocytes and lamina propria lymphocytes returned towards normal on a gluten free diet, in parallel with the improvement in mucosal pathology and in other indices of immunopathology. In addition, all parameters remained abnormal in patients not adhering to their gluten free diet, or in whom villous atrophy had not resolved.

The reduced concentrations of γ-interferon were surprising in view of its involvement in other forms of immunopathology[16–20] and of the recent finding that there is increased spontaneous production of γ-interferon by lamina propria cells in Crohn's disease.[14] There is also experimental evidence implicating γ-interferon in the epithelial manifestations of immunologically mediated enteropathy,[5] while coeliac disease is associated with the presence of increased numbers of activated T lymphocytes.[11 21] One possible reason for these findings is that increased synthesis of γ-interferon does occur in coeliac disease, but that this is accompanied by more rapid secretion from the cell. As a result there could be a decrease in stainable immunoreactive product in the cell. A similar phenomenon seems to occur in Crohn's disease,[22] but direct confirmation of this idea would need examination of messenger ribonucleic acid concentrations.

We consider it more likely that increased numbers of non-γ-interferon producing lymphocytes infiltrate the mucosa in coeliac disease. This is suggested by our observation that the absolute number of γ-interferon positive cells remains close to normal in coeliac disease but that the total number of intra epithelial lymphocytes and lamina propria lymphocytes is increased. Therefore, it may be these additional lymphocytes which are responsible for the enteropathy of coeliac disease and γ-interferon

may play no direct pathogenic role in this condition. The nature of the lymphocytes which dilute out the interferon positive cells must remain speculative, but could reflect increased numbers of cells producing other cytokines which have a primary role in the mucosal damage. Tumour necrosis factor α and α/β interferon are enteropathic in mice,[23] while mucosal tumour necrosis factor α production is increased in Crohn's disease.[13] It would be of interest to use an immunohistochemical approach to examine the production of these and other mediators in coeliac disease.

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