Characterisation and quantification of mucosal cytokine that induces epithelial histocompatibility locus antigen-DR expression in inflammatory bowel disease

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Abstract
Epithelial histocompatibility locus antigen (HLA) class II expression was studied to evaluate its induction by mucosal mononuclear cells in inflammatory bowel disease and to characterise the responsible cytokine. Unstimulated cells of the HT-29 epithelial cell line did not produce class II molecules. After being stimulated with the mitogenic lectin phytohaemagglutinin mucosal mononuclear cells released a cytokine that induced epithelial HLA-DR expression. The cytokine had the physicochemical and immunological characteristics of interferon- γ , and no additional cytokines were detected.

The expression of major histocompatibility complex antigen-class II molecules by non-lymphoid cells is well recognised.^{1,4} The function of these molecules remains the subject of debate.⁵ It has been claimed that epithelial cells expressing class II histocompatibility molecules can act as antigen presenting cells to T cells.^{6,7} It has been suggested that normally, epithelial cells present antigen to CD8+ antigen specific suppressor cells,⁸ or antigen non-specific suppressor cells.⁶ Normal murine enterocytes however, have been shown to present soluble antigen in a class II restricted fashion to an antigen specific T cell hybridoma producing interleukin-2.⁹

Mayer has further shown that enterocytes from idiopathically inflamed intestine may support the proliferation of CD4+ cells unrelated to the level of HLA-DR expression, and suggest that in idiopathic inflammatory bowel disease there is an inability to present antigen to antigen non-specific T suppressor cells.¹⁰ In inflammatory bowel diseases such as ulcerative colitis and Crohn's disease there is evidence of activation of immunological effector mechanisms in the mucosa.¹¹ It is not clear if the activated immunological processes are an appropriate response to an as yet unidentified pathogen or a pathologically increased response to a relatively otherwise innocent challenge. An increase in colonic epithelial cell class II histocompatibility locus antigen expression has been noted in both ulcerative colitis and Crohn's disease when in relapse.^{2,12-14} If these cells act as antigen presenting cells they may amplify the mucosal immune response by increasing antigen presentation to the mucosal and intraepithelial T cell population. It is likely that epithelial cell class II histocompatibility antigen expression

is induced by cytokines acting in a paracrine fashion in the lamina propria.³ From animal experimentation, interferon- γ is a possible candidate.¹⁵ We have examined the mucosal mononuclear cell population looking for evidence of cytokines that induce epithelial cell class II expression and the hypothesis that there is increased production in ulcerative colitis and Crohn's disease.

Methods

PATIENTS
Fresh surgical specimens were obtained from patients undergoing intestinal resection for active ulcerative colitis (n=13), Crohn's disease (n=5) (ileocaecal resections), colonic neoplasia or chronic constipation (n=11). Patients with inflammatory bowel disease had failed medical treatment for acute relapses of disease. Mucosa from macroscopically inflamed and non-inflamed sites was compared in specimens from patients with distal ulcerative colitis. In patients with colon cancer, mucosa was taken >5 cm from tumour. Histological sections from adjacent mucosa confirmed macroscopic and clinical diagnoses. Patient details are listed in the Table.

ISOLATION OF MUCOSAL MONONUCLEAR CELLS
Mononuclear cells were isolated by a modification of the method of Bookman and Bull.¹⁶ Briefly, the resection specimen was immediately placed in RPMI1640 medium at 4°C and transported to the laboratory. Mucus was scraped from the surface of the specimen during three washes in Hank's balanced salt solution without calcium and magnesium (HBSS-CMF). Strips of mucosa were then placed in 1 mM dithiothreitol for 15 minutes at room temperature to further remove mucus. Epithelial cells were removed by three incubations at 37°C in 5 mM EDTA in a shaking water bath. The mucosal strips were minced and

TABLE 1

Disease	n	M:F	Median (range) Age	Therapy
UC	13	9:4	42 (17-65)	IV Steroids
	4 distal			
Crohn's disease	5	3:2	40 (15-60)	IV steroids (except 1)
Control	11	8:3	64 (50-77)	Nil

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Accepted for publication
2 July 1991

incubated for three hours in collagenase (3 mg/ml) (Boehringer, Mannheim GmbH, Germany) at 37°C. The digest was filtered through a 200 µm

Figure 1: ELISA quantification of HLA-DR/cellular protein (SD) by interferon-γ (Each point represents the mean of five observations)

Fig 2A

Fig 2B

Figure 2: Immunohistochemical quantitation of HLA-DR on HT-29 cells (a) 5 U/ml interferon-γ. Many of the cells are HLA-DR positive. (b) 50 U/ml interferon-γ. The majority of the cells are HLA-DR positive. A significant cytopathic effect is observed.

mesh and viability of the cell suspension calculated using trypan blue exclusion. After 'Ficolpaque' (Pharmacia, UK) density gradient centrifugation, the resulting cell suspension was washed and resuspended at 10⁶ viable cells/ml in RPMI 1640, 10% foetal calf serum. The cells were then cultured for 72 h (10) µg phytohaemagglutinin at 37°C in 24 well plates in a humidified incubator using 10 mM Hepes, or 5% CO₂ as buffer. Supernatants were harvested and filtered through a 22 µm filter (Acrodisc, Gelman Scientific). Supernatants were stored at -20°C for future testing.

EPITHELIAL CELL LINE

The human colonic cancer cell line HT-29 was used (Courtesy of Dr P Brandtzaeg, Oslo, Norway). This was grown in Leibowitz-15 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and gentamicin and amphotericin (Complete Medium).

INTERFERON-γ

Recombinant interferon-γ (courtesy of Dr G Scott, Wellcome Biotechnology, Beckenham Kent) was used as a standard in concentrations up to 10 U/ml after pilot experiments had shown that up to this concentration induced maximal HLA-DR induction on HT-29 and that higher concentrations had a marked cytopathic effect without further increasing HLA-DR induction.

EPITHELIAL HLA-DR INDUCTION

HT-29 cells were seeded at 2×10⁵/200 µl/well into 96 well plates (Linbro, Flow Laboratories). After 24 h the cells are adherent and entering the logarithmic phase of growth, the medium was discarded and replaced with 150 µl complete medium and 50 µl interferon solution (in RPMI 1640, 10% fetal calf serum) or test supernatant. The cells were cultured for a further 48 h at 37°C before assay. In all experiments employing mononuclear cell supernatants, interferon standards were employed in order that interassay variation could be eliminated. HLA-DR induction could then be expressed as interferon-γ equivalent units.

ANTIBODY TO HLA-DR

A monoclonal antibody to a non-polymorphic component of the β chain of the human HLA-DR molecule was used (Dako, High Wycombe).

IMMUNOPEROXIDASE STUDIES

Cells were cultured on plastic cover slips (Thermanox UK) for 72 hours with 0–500 U/ml interferon-γ. Washed three times with Hank's balanced salt solution, air dried, and fixed in absolute ethanol. Endogenous peroxidase activity was blocked by the addition of 3% hydrogen peroxide in phosphate buffered saline. The slides were washed and incubated for 30 minutes in swine serum diluted 1:5 with phosphate buffered saline. The slides were incubated for 30 minutes with 30 µl HLA-DR monoclonal

Figure 3: Interferon- γ equivalents (SD) produced by culture supernatants of resting and phytohaemagglutinin stimulated mucosal mononuclear cells.

antibody diluted 1:5 in phosphate buffered saline and washed. Peroxidase conjugated rabbit antimouse immunoglobulin was added diluted 1:100 in phosphate buffered saline, incubated for 30 minutes. Diaminobenzidine tetrahydrochloride (BDH, Poole, Dorset) was used as the peroxidase substrate.

HLA-DR ENZYME LINKED IMMUNOADSORBENT ASSAY (ELISA)

At the end of the culture period the cells were washed three times in phosphate buffered saline 0.13 M pH 7.3 at room temperature and the cells air dried at 37°C for three to six hours fixed using 200 μ l methanol (AnalaR, BHD, Poole, UK.) and endogenous peroxidase activity was blocked with 1% hydrogen peroxidase. Non-specific binding sites in the wells were blocked by incubation with 200 μ l 1% gelatin (porcine skin, Sigma, Poole, UK) containing 80 μ g heat aggregated rabbit immunoglobulin (Dako, High Wycombe) for two hours at room temperature.

Figure 4: Effects of various physicochemical treatments of culture supernatants on HLA-DR induction.

The monoclonal antibody to HLA-DR was used as a 1:50 dilution of a culture supernatant. Each well was incubated with 100 μ l antibody at 4°C for 16 hours in a humidified chamber. The plates were washed four times with 0.1% gelatin in phosphate buffered saline (Wash solution, pH 7.3, 0.13M) and incubated for one hour with peroxidase conjugated rabbit antimouse antiserum (Dako, High Wycombe, Bucks). The plates were washed four times with wash solution and the peroxidase content per well assayed using 100 μ l orthophenylene diamine 1 mg/ml (Dako, High Wycombe, Bucks) as substrate. The reaction product was terminated after five minutes incubation in the dark at room temperature by the addition of 100 μ l 1M sulphuric acid. The coloured reaction product was read at 492 nm in a multiscan plate reader within 30 minutes. The plate was washed four times with absolute methanol and air dried at 37°C for three hours the determination of cellular protein/well. This was performed before a modification of the Bradford technique.¹⁷ Briefly 30 μ l Bradford reagent (Biorad laboratories, Watford, Herts), was added to each well and plate shaken for 30 minutes. 120 μ l distilled water was added to each well and the plate further shaken for 30 minutes. The reaction product was assayed in a multiscan spectrophotometer at 595 nm. HLA-DR induction/cell was thus expressed as OD_{492 nm}/OD_{595 nm}. Each result was performed in quintuplicate and the results expressed as mean (standard deviation).

CHARACTERISATION OF CYTOKINE

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY
Active supernatants were pooled, desalted by elution from a Sephadex-G25 column and eluted with 0.1 M ammonium carbonate, and lyophilised. The lyophilate was reconstituted with a minimum volume of phosphate buffered saline (0.13 M pH 7.4) and spun in a 'micro-centaur' bench top centrifuge. The supernatant was then applied in a volume of 500 μ l aliquots onto a TSK G2000SW (Pharmacia-LKB, 'Blue column' Milton Keynes.) gel chromatography column and eluted with phosphate buffered saline buffer using an LKB HPLC pump 2150. Forty fractions of 0.45 ml were collected.

PHYSICOCHEMICAL CHARACTERISTICS

Pooled active supernatant was passed onto a 5 ml Sephadex G25 column by Pasteur pipette that had been equilibrated with 0.1 M glycine buffer pH 2 and eluted with phosphate buffered saline after five minutes, one hour, six hours and 24 hours by the manual addition of buffer to the column.

Active supernatant was heated to 100°C for five minutes and aliquots tested before and after treatment.

Active supernatant was incubated at room temperature for 30 minutes with a sheep antiserum to human interferon- γ (H53, - a kind gift of Dr A Meager, National Institute for Biological Standards and Controls, Potter's Bar, Herts).

Figure 5: HLA-DR induction (SD) by high performance liquid chromatography gel fractions of pooled culture supernatants.

Results

INDUCTION BY INTERFERON- γ
HT-29 cells could be induced to express HLA-DR molecules in a dose dependent fashion (Fig 1). Maximal expression occurred at a dose of 10 U/ml interferon- γ . Higher doses produced a decrease in the amount of cellular protein/well in the ELISA and immunohistochemical studies confirmed that higher doses of interferon- γ did not cause an increase in expression but a significant cytopathic effect was exerted (Fig 2a, b).

INDUCTION BY MUCOSAL MONONUCLEAR CELL CULTURE SUPERNATANTS
Supernatants obtained from the culture of unstimulated mucosal cells did not produce any detectable induction of HLA-DR molecules despite the assay being able to detect 2 U/ml interferon- γ . The supernatants obtained from the culture of phytohaemagglutinin stimulated cells all induced detectable quantities of HLA-DR molecules, but there was no evidence of either increased or decreased production of cytokine differences between patients with ulcerative colitis and Crohn's disease and controls (Fig 3). Phytohaemagglutinin alone did not induce class II antigen expression.

Figure 6: Abrogation of HLA-DR induction by anti-interferon- γ antiserum.

PHYSICOCHEMICAL CHARACTERISTICS
Exposure of the supernatants to pH 2 or boiling for 30 minutes destroyed all class II inducing ability (Fig 4). The testing of individual fractions generated by the gel column suggested that the molecular weight of the active fraction was in the region of 20–25 kD (Fig 5).
Incubation of the pooled supernatant with anti-serum to interferon- γ resulted in abrogation of class II induction. At a dilution of the antiserum of 1:100 class II induction was not detectable. The abrogation paralleled that seen with interferon- γ , suggesting that there was not an agent other than interferon- γ present in the supernatant that induced class II expression (Fig 6).

Discussion

This work confirms the work of others that human colonic epithelial cell line can be induced to express HLA class II proteins by recombinant interferon- γ .¹⁸ This occurs in a dose dependent manner up to a concentration of 10 U/ml. Higher concentrations of interferon- γ produce a significant cytopathic effect. These concentrations are much lower than have previously observed to be effective.¹⁸ However previous studies employed a plasmid derived recombinant interferon- γ whereas the product employed here was a culture supernatant of a Chinese hamster ovary cell line transfected with the human interferon- γ gene, had thus undergone eukaryotic glycosylation and hence potentially different biological activities.

Supernatants from unstimulated mucosal mononuclear cell culture did not produce any significant induction of epithelial class II molecules whereas after phytohaemagglutinin stimulation a significant amount was induced. Spontaneous and stimulated interferon- γ production by lamina propria lymphocytes in Crohn's disease has recently been studied.¹⁹ Using an antiviral bioassay for interferon activity and antisera to interferons α and β , spontaneous production of interferon- γ was observed in Crohn's disease. It is possible that the epithelial cell HLA-DR induction assay is less sensitive for detecting interferon activity than the antiviral-bioassay which may explain the differences between these results. No significant differences in HLA-DR induction were obtained using supernatants derived from patients with ulcerative colitis, Crohn's disease, or controls.

The lack of class II induction by cells isolated from inflamed tissue may be considered surprising as the epithelium in cases of inflammatory bowel disease is strikingly HLA-DR positive,¹² but is in accord with observations on interferon- γ production by isolated mucosal mononuclear cells.²⁰ Isolating the viable cells from inflamed tissue, however, may selectively deplete those cells that have been activated and are actively synthesising cytokines or the population of cells responsible for in vivo class II induction are rendered effete by the in vitro isolation procedures.^{21, 22} Furthermore the mucosal mononuclear cell population in inflammatory bowel disease differs from non-inflamed mucosa, and comparisons of cytokine production are therefore meaningless.²²

There is a debate about spontaneous mucosal mononuclear cells production of interferon- γ .^{19,20} Stimulation of mucosal mononuclear cells by PHA or recombinant interleukin-2-, however, has been shown to induce production of interferon- γ .²⁰ In these studies decreased amounts of cytokine inducing monocyte HLA-DR and interferon- γ were produced by cells isolated from patients with inflammatory bowel disease. At first sight the results presented here may seem to be discordant with those of Ouyang *et al*, but the methodology used is quite different. The experiments that are described here quantify the induction of epithelial class II HLA molecules, not the amount of interferon- γ .

There may be significant amounts of interferon- α or interferon- β activity in the supernatant which is indistinguishable from interferon- γ in a bioassay.

Ouyang *et al* used monocytes as model cells for class II induction, and there may be differences between monocyte and epithelial cell lines in induction of class II HLA molecules. There is evidence that many cytokines including interleukin-4, granulocytes macrophage colony stimulating factor and tumour necrosis factor- α may act to increase class II molecule expression,²³ and synergism between interferon- γ and tumour necrosis factor- α has been shown for HT-29 cells.^{24,25}

The immunohistological evidence does not suggest that there is a defect in epithelial class II induction in the colon. All the studies reported thus far show extensive epithelial class II induction in ulcerative colitis and Crohn's disease and no qualitative difference from induction seen in inflammatory controls.^{12,22,25}

The biological and physicochemical properties of the factor in the supernatants are consistent with it being interferon- γ . Human interferon- γ is usually shown to have a molecular weight of about 55 kD, and this has been found to be dimeric with components of 20 and 25 kD.²⁶ The activity in the supernatants is acid labile, has a molecular weight of about 22 kD, and is completely neutralised by antiserum to human interferon- γ . Thus it is possible that the monomeric forms are being detected in these fractions.

Unlike studies using circulating lymphocytes we could not detect any evidence of any class II including factor other than interferon- γ .²⁷ Several explanations for this difference are possible. The assay of class II molecules may be too insensitive, although it was possible to detect the effect of 2 U/ml interferon- γ above background. Epithelial cells may not be susceptible to the same range of cytokines that induce class II HLA molecules on circulating T cells. It is probable that mucosal mononuclear cells do not produce the same range of cytokines as produced by the circulating mononuclear cell population.

The responses measured here may be a selective in vitro response to phytohaemagglutinin and may not represent the in vivo situation.

In conclusion, mucosal mononuclear cells of both 'normal' and inflamed colon have the potential to produce cytokines that induce

epithelial cell class II molecules. These are, however, only produced after mitogenic stimulation of the cells. The cytokine responsible has the characteristics of interferon- γ .

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