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Salicylates used in inflammatory bowel disease and colchicine impair interferon-γ induced HLA-DR expression

B Crotty, P Hoang, H R Dalton, D P Jewell

Abstract

Colonic epithelial cells express HLA-DR in inflammatory bowel disease. The effect of drugs used in the treatment of inflammatory bowel disease and colchicine on interferon-γ (IFN-γ) induced DR expression has been investigated. HT-29 cells were cultured in 25 cm² flasks. At 48 hours interferon-γ (0, 50, or 100 U/ml)±drug were added. At 120 hours the cells were stained for HLA-DR and analysed by flow cytometry. 10^{-2} M 5ASA reduced DR expression induced by 50 U/ml interferon-γ from 62 (12)% of cells (mean SD) to 29 (20)% ($p<0.005$). Corresponding figures for 10^{-2} M N-acetyl 5ASA were 68 (16)% to 39 (17)% ($p<0.05$); for 10^{-2} M 4ASA, 61 (4)% to 57 (4)% ($p=0.6$); for 10^{-2} M N-acetyl 4ASA, 60 (12)% to 35 (13)% ($p<0.05$); for 10^{-2} M olsalazine, 72 (9)% to 3 (1)% ($p<0.001$); for 10^{-3} M olsalazine, 72 (9)% to 16 (10)% ($p<0.001$); for 10^{-6} M colchicine, 62 (13)% to 5 (3)% ($p<0.001$); and for 10^{-7} M colchicine, 62 (13)% to 10 (3)%. Similar results were obtained when DR was induced by 100 U/ml of interferon-γ except with 10^{-2} M 4ASA which reduced expression from 77 (4)% to 68 (3)% ($p<0.05$). Sulphapyridine, prednisolone, indomethacin and cyclosporin A had no effect. Concurrent staining with propidium iodide showed that these results were unchanged when viable cells alone were analysed. Prior incubation of cells with drug, followed by washing, had no effect on interferon-γ induced DR expression. 5ASA, N-acetyl 5ASA, 4ASA, N-acetyl 4ASA, olsalazine and colchicine reduce interferon-γ induced HLA-DR expression. In inflammatory bowel disease these compounds may impair antigen presentation by the colonic epithelium.

Products of the class II genes of the major histocompatibility complex are cell surface heterodimers consisting of α and β chains. Three major products are described in man: HLA-DR, DP, and DQ. Lymphocytes bearing CD4 (the helper/inducer phenotype) recognise foreign antigens associated with class II molecules on the surface of antigen presenting cells.[1]

HLA-DR is constitutively expressed on B lymphocytes, some macrophages, dendritic cells, vascular endothelial cells, and some epithelial cells.[2] Expression can be induced, however, in a wide range of tissues in inflammatory diseases.[3] In vitro this induction is generally mediated by interferon-γ, although the effect of interferon-γ can be enhanced in the presence of tumour necrosis factor-α[3-5] and reduced by interleukin 1-α.[6] Granulocyte-macrophage colony stimulating factor can also induce class II expression.[7]

In the normal gastrointestinal tract HLA-DR is expressed on small intestinal epithelial cells but not on gastric or colonic epithelial cells. Only fully differentiated villus cells and the epithelium overlying lymphoid follicles (except M cells) constitutively express HLA-DR; there is no expression on crypt cells and goblet cells.[8]

This pattern is markedly altered in inflammatory diseases of the gastrointestinal tract. Expression of HLA-DR has been shown in gastritis.[9,10] Villus cells show an increased level of expression and there is expression by crypt cells in coeliac disease,[11] dermatitis herpetiformis,[11] seronegative spondylarthropathy,[12] and graft versus host disease.[13] Moreover, the villus cells express DP and DQ antigens in addition to DR in untreated coeliac patients.[14] Epithelial DR expression is also found in inflamed colonic mucosa in ulcerative colitis, Crohn's disease, radiation colitis, and infectious colitis.[15,16]

Over the last few years several groups have presented data suggesting that class II antigen bearing gastrointestinal epithelial cells are capable of antigen presentation to lymphocytes.[17-20] If this occurs in vivo the HLA-DR expression seen in inflammatory bowel disease and other inflammatory diseases of the gut may augment the inflammatory response by increasing the amount of antigen presented to the mucosal immune system.

The mode of action of 5-amino salicylic acid, the active moiety of sulphasalazine, and of the other salicylates used in inflammatory bowel disease is unknown.[21] In this paper we report the results of studies examining the effect of these compounds on interferon-γ induced HLA-DR

TABLE I *Effect of drugs on percentage of HT-29 cells induced to express HLA-DR by interferon-γ*

	HLA-DR expression (cells %)			
Interferonγ	*50*	*50+drug*	*100*	*100+drug*
10^{-2} M 5ASA	62 (12)	29 (20)†	76 (8)	44 (24)‡
10^{-2} M N-ac 5ASA	68 (16)	39 (17)‡	82 (11)	63 (14)§
10^{-2} M 4ASA	61 (4)	57 (4)‖	77 (4)	68 (3)‡
10^{-2} M N-ac 4ASA	60 (12)	35 (13)‡	75 (6)	62 (11)‡
10^{-2} M colchicine	72 (9)	3 (1)*	85 (3)	4 (3)*
10^{-3} M olsalazine	72 (9)	16 (10)*	85 (3)	33 (14)*
10^{-6} M olsalazine	62 (13)	5 (3)*	82 (6)	6 (5)*
10^{-7} M colchicine	62 (13)	10 (3)*	82 (6)	7 (5)*

Data are expressed as the mean (SD) of five experiments. At both concentrations of interferon-γ the effect of colchicine was analysed at 0, 10^{-8}, 10^{-7}, and 10^{-6} M and all other compounds at 0, 10^{-4}, 10^{-3}, and 10^{-2} M. There was no effect at concentrations not listed in this Table. Data were analysed by one way analysis of variance of the data for each drug at 50 and 100 U/ml of interferon-γ. *$p<0.001$, †$p<0.005$, ‡$p<0.05$, §$p=0.05$, ‖$p=0.6$.

Gastroenterology Unit, Radcliffe Infirmary, Oxford
B Crotty
P Hoang
H R Dalton
D P Jewell

Correspondence to:
Dr D P Jewell, Gastroenterology Unit, Radcliffe Infirmary, Oxford OX2 6HE.

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expression on colonic epithelial cells and propose this effect as a possible mode of action for these drugs.

Methods

MATERIALS

Cell line

The HT-29 colonic carcinoma line was obtained from Dr P Brandtzaeg, Oslo, Norway and grown at 37°C in 25 cm^2 tissue culture flasks (Sterilin, Feltham, UK) in Liebowitz-15 (L-15) medium (Gibco, Paisley, UK) supplemented with 10% fetal calf serum (Flow Laboratories, Irvine, UK), 5×10^5 U/ml penicillin and 50 μg/ml gentamicin.

Interferon-γ

Recombinant interferon-γ was a gift of Wellcome Biotech (Beckenham, UK). On receipt the solution was diluted to 5×10^5 U/ml in Roswell Park Memorial Institute medium (RPMI) culture medium (Gibco) and aliquots were stored at −20°C until the day of use. After thawing the solution was diluted to a final concentration of 1000 U/ml in L-15.

Drugs

5-amino salicylic acid (5ASA), 4-amino salicylic acid (4ASA), and colchicine were purchased from Sigma (Poole, UK). N-acetyl 5ASA, N-acetyl 4ASA, and olsalazine were gifts of Pharmacia AB (Uppsala, Sweden). 5ASA, N-acetyl 5ASA, and N-acetyl 4ASA were dissolved in 0·2 M NaOH, diluted 1:10 in L-15 and then adjusted to pH=7·4 with 1 M HCl indomethacin was dissolved in 0·03 M NaOH, diluted 1:100 in L-15 and then adjusted to pH=7·4 with 0·1 M HCl olsalazine, 4ASA and colchicine were dissolved in L-15. Prednisolone, sulphapyridine, and cyclosporin A (Sandoz, Feltham, UK) were dissolved in dimethyl sulphoxide and then diluted 1:100 (sulphapyridine) or 1:1000 (prednisolone and cyclosporin A) in L-15. All solutions were filtered before use.

All other chemicals were purchased from Sigma unless stated.

INCUBATION

HT-29 cells were counted in 0·05% trypan blue in a Neubauer chamber and 10^6 cells in L-15 were added to 25 cm^2 tissue culture flasks and incubated at 37°C for 48 hours. The medium was then removed and replaced with a solution of interferon-γ and/or drug in L-15 and the cells were incubated for a further 72 hours.

The effect of 5ASA, N-acetyl 5ASA, and olsalazine on HLA-DR expression induced by the supernatant of incubated lamina propria lymphocytes was also examined. These experiments were carried out as described above except that lamina propria lymphocyte supernatant, diluted 1:4 in L-15 was used instead of interferon-γ. Aliquots of HT-29 cells were incubated with 100 U/ml of interferon-γ in each batch.

A number of experiments were done where the cells were preincubated with drug for 48 hours before the addition of interferon-γ. After removal of the drugs by washing three times in L-15, interferon-γ was added and the cells were incubated for a further 72 hours.

TABLE II *Effect of drugs on percentage of HT-29 cells induced to express HLA-DR by lamina propria lymphocyte supernatant (sn)*

	HLA-DR expression (cells %)	
	Patient 1	*Patient 2*
LPL sn	96	93
LPL sn + 10^{-2} M 5ASA	82	71
LPL sn + 10^{-2} M N-acetyl 5ASA	84	72
LPL sn + 10^{-3} M olsalazine	88	70

LPL=lamina propria lymphocyte.

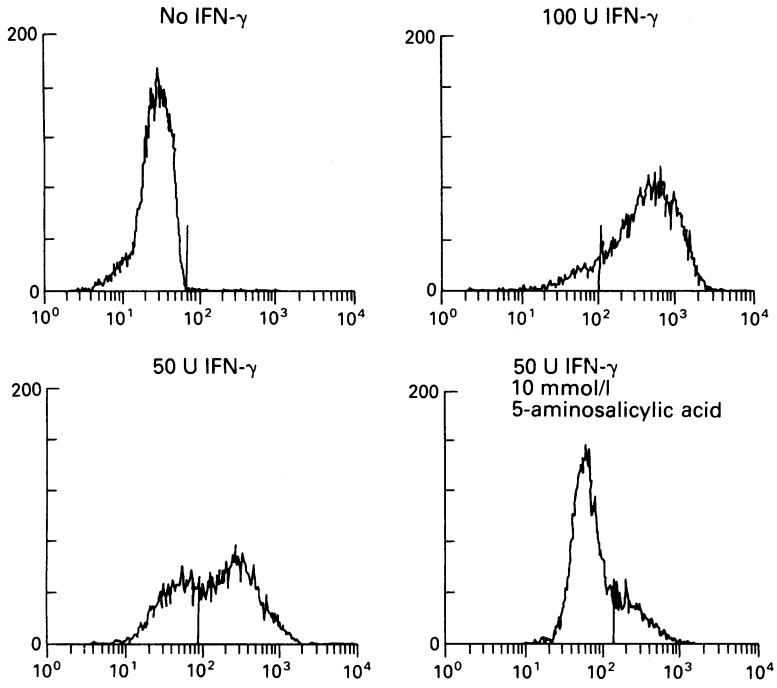
LAMINA PROPRIA LYMPHOCYTE SUPERNATANT

The effect of supernatants of lamina propria lymphocytes isolated from two colonic resection specimens was also examined. Patient 1, a 63 year old man underwent a hemicolectomy for carcinoma of the colon and patient 2, a 60 year old man, underwent a proctocolectomy for active ulcerative colitis. The lamina propria lymphocytes were isolated as previously described[22] and 1 ml aliquots of a suspension of 10^6 cells/ml in RPMI supplemented with 10% fetal calf serum, penicillin and gentamicin were added to 24 well tissue culture plates (Flow Laboratories). Phytohaemagglutinin (Wellcome Diagnostic, Dartford, UK) was added to half the wells at a final concentration of 10 μg/ml and the plates were incubated for 72 hours in 5% CO_2 at 37°C. The cell suspension was then aspirated into Eppendorf tubes, centrifuged for 10 minutes, filtered and the supernatant stored at −20°C until use.

STAINING

The cells were detached from the flasks with 0·2 mg/ml trypsin (Worthington Biochemical Corporation, NJ, USA) 10 mM ethylenediamenetetra acetic acid (EDTA) in calcium and magnesium free Hank's buffered salt solution (CMF-HBBS) (Flow Laboratories), washed once in L-15, resuspended in L-15 and counted in 0·05% trypan blue. Two aliquots of 10^6 cells from each flask were transferred into (5 ml) Falcon polystryrene test tubes (Becton Dickinson, Lincoln Park, NJ, USA) and centrifuged at 100 *g* for five minutes. The supernatant was discarded and the cells were resuspended in 50 μl phosphate buffered saline (PBS) (Gibco) with 0·1% sodium azide (PBS/azide).

Ten microlitres of fluorescein isothiocyanate conjugated anti-HLA-DR (Becton-Dickinson, Mountain View, CA, USA), a murine IgG_{2a} monoclonal antibody to human HLA-DR was then added to one of the two Falcon tubes, the other serving as a negative control. The tubes were briefly vortexed then incubated for 30 minutes at 4°C in the dark. Unbound antibody was removed by washing the cells in a further 2 ml PBS/azide, centrifuging at 750 *g* for five



Effect of increasing concentrations of interferon-γ on HLA-DR expression on HT-29 cells. Four flow cytometry traces showing the number of cells (y axis) expressing HLA-DR (measured by fluorescence along the x axis) after incubation with 0, 50, and 100 U/ml interferon-γ and 50 U/ml interferon-γ and 10^{-2} M 5ASA.

minutes and discarding the supernatant. The cells were fixed by resuspension in 0·5 ml of 1% paraformaldehyde in PBS/azide and kept at 4°C in the dark until analysis which was carried out within 48 hours in all cases.

A separate control experiment was done using murine fluorescein isothiocyanate conjugated IgG_{2a} monoclonal antibodies to keyhole limpet haemocyanin and human transferrin receptor to confirm that changes in fluorescence induced by interferon-γ were caused by specific binding by anti-HLA-DR. HT-29 cells were incubated for 72 hours with 50 U/ml interferon-γ (10^{-2}) M 5ASA, 10^{-2} M N-acetyl 5ASA, or 10^{-3} M olsalazine.

A number of analyses were carried out using simultaneous staining with fluorescein isothiocyanate conjugated anti-HLA-DR and propidium iodide. In these experiments the staining procedure was similar to that described above except that the centrifuged cells were suspended in 10 μg/ml propidium iodide in PBS/azide before addition of fluorescein isothiocyanate conjugated anti-HLA-DR. After incubation the cells were centrifuged but were then suspended in 0·5 ml of PBS/azide instead of paraformaldehyde. The tubes were kept on ice and analysed within one hour.

FLOW CYTOMETRY

Flow cytometry was performed on a FACScan (Becton Dickinson). The initial samples of each run were analysed according to size (forward light scatter) and granularity (side light scatter) and a homogeneous population of cells selected (gated) by restricting the analysis to cells falling within a defined forward light scatter and side light scatter range which was then used for all samples in that run.

Five thousand cells within this gate were then analysed for green fluorescence. The control sample of unstained cells was used to set the lower limit of positive fluorescence and the percentage of cells with fluorescence above this limit was measured in the stained sample.

Where cells had been stained with fluorescein isothiocyanate conjugated anti-HLA-DR and propidium iodide the gated population was first analysed for red fluorescence. This revealed a bimodal pattern of fluorescence as viable cells (low fluorescence) exclude propidium iodide whereas non-viable cells (high fluorescence) take up propidium iodide. A second gate was applied to analyse the viable cells for green fluorescence.

EXPERIMENTAL PROTOCOL

A gradient of HLA-DR expression with increasing concentrations of interferon-γ was established. The effect on HLA-DR expression induced by 50 and 100 U/ml interferon-γ was then examined at different concentrations of each drug. 5ASA, N-acetyl 5ASA, 4ASA, N-acetyl 4ASA and sulphapyridine were studied at 0, 10^{-4}, 10^{-3}, and 10^{-2} M. These were chosen on the basis of reported concentrations of 5ASA and N-acetyl 5ASA in rectal dialysates in patients taking therapeutic doses of sulphasalazine or olsalazine, the highest concentration being similar to that found in the dialysate.[23] Prednisolone was studied at 0, 10^{-7}, 10^{6}, and 10^{-5} M; colchicine and cyclosporin A at 0, 10^{-8}, 10^{-7}n ad 10^{-6} M and indomethacin at 0, 10^{-6}, 10^{-5}, and 10^{-4} M. In each case the middle concentration approximates to that found in plasma after therapeutic doses.[24-26] Each experiment was carried out five times unless indicated.

Results were analysed by one way analysis of variance of the data for each drug at 50 and 100 U/ml interferon-γ.

Results

Cell viability assessed by trypan blue exclusion after 72 hours incubation with interferon-γ and/or drug was greater than 90% in all cases except for cells exposed to 10^{-2} M olsalazine and 10^{-6} M colchicine when it was between 60 and 95%. The number of cells after incubation with these two compounds at these concentrations was up to 90% lower than after other incubations, presumably because of inhibition of cell growth. Exposure to 10^{-7} M colchicine resulted in a lesser degree of inhibition of proliferation.

Staining with fluorescein isothiocyanate conjugated anti-keyhole limpet haemocyanin was

TABLE III *Effect of preincubation of HT-29 cells with drug on percentage of cells induced to express HLA-DR by interferon-γ*

	HLA-DR expression (cells %)	
	50 U interferon-γ	*100 U interferon-γ*
No drugs	43·4	69·2
10 mM 5ASA	42·3	67·0
10 mM N-ac 5ASA	40·4	70·7
1 mM olsalazine	61·5	84·1
10 mM olsalazine	70·8	64·7

TABLE IV *Simultaneous staining with anti-HLA-DR and PI. The reduction in the percentage of HT-29 cells induced to express HLA-DR is almost identical when the entire cell population (stained with anti-HLA-DR alone) or the viable population (simultaneous staining with anti-HLA-DR and PI) are analysed*

	50 U interferon-γ		*100 U interferon-γ*	
	Anti-DR	*Anti-DR +PI*	*Anti-DR*	*Anti-DR +PI*
No drug	69·7	74·7	89·6	90·0
10 mM 4ASA	65·3	65·7	70·8	73·2
10 mM 5ASA	40·6	42·8	58·5	59·1
10 mM N-ac 5ASA	27·3	26·9	59·3	57·3
1 mM olsalazine	8·5	9·1	26·4	24·4
10 mM olsalazine	7·6	1·5	7·0	1·3

negative in all cases. Staining with fluorescein isothiocyanate-conjugated anti-human transferrin receptor was positive but unaffected by interferon-γ±drug.

HLA-DR was not detected on HT-29 cells in the absence of interferon-γ but incubation with increasing concentrations of interferon-γ led to increasing expression (Figure). None of the drugs induced HLA-DR expression in the absence of interferon-γ.

HLA-DR EXPRESSION INDUCED BY 50 U/ML INTERFERON-γ

HLA-DR expression induced by incubation with 50 U/ml interferon-γ was significantly reduced by 10^{-2} M 5ASA (Figure), 10^{-2} M N-acetyl 5ASA, and 10^{-2} M N-acetyl 4ASA. In contrast 10^{-2} M 4ASA had no effect. The effect of incubation with olsalazine was more pronounced with a greater inhibition of DR expression at 10^{-2} M and 10^{-3} M. This was the only salicylate to have an effect at 10^{-3} M and none had any effect at 10^{-4} M. Both 10^{-6} M and 10^{-7} M colchicine markedly reduced DR expression (Table I).

HLA-DR EXPRESSION INDUCED BY 100 U/ML INTERFERON-γ

Similar results were obtained when the same concentrations of these drugs were added to cell suspensions induced to express HLA-DR by 100 U/ml interferon-γ. At this concentration of interferon-γ, however, 10^{-2} M 4ASA had a statistically significant effect whereas the effect of 10^{-2} M N-acetyl 5ASA just failed to reach statistical significance (Table I). These effects were not altered by the addition of 1:100 dimethyl sulphoxide.

Sulphapyridine (n=five), prednisolone (n=two), cyclosporin A (n=two), and indomethacin (n=two) had no effect on DR expression induced by 50 or 100 U/ml interferon-γ at any of the concentrations studied.

HLA-DR EXPRESSION INDUCED BY LAMINA PROPRIA LYMPHOCYTES SUPERNATANT

Incubation of HT-29 cells with a 1:4 dilution of the supernatant of lamina propria lymphocytes incubated with phytohaemagglutinin resulted in induction of HLA-DR expression similar to that seen with 100 U/ml interferon-γ. There was a similar degree of induction by both patients' lamina propria lymphocytes supernatants. The supernatant of cells which had not been incubated with phytohaemagglutinin had no effect. Addition of 10^{-2} M 5ASA or N-acetyl 5ASA or 10^{-3} M olsalazine resulted in a similar reduction in DR expression to that seen with interferon-γ (Table II).

PREINCUBATION

Preincubation of HT-29 cells with 10^{-2} M 5ASA, 4ASA, N-acetyl 5ASA or olsalazine, or 10^{-3} M olsalazine before incubation with interferon-γ did not reduce HLA-DR expression. In fact preincubation with olsalazine slightly augmented the effect of interferon-γ (Table III).

SIMULTANEOUS STAINING WITH ANTI-HLA-DR AND PROPIDIUM IODIDE

When simultaneous staining with fluorescein isothiocyanate conjugated anti-HLA-DR and propidium iodide was used to examine HLA-DR expression by the viable cell population, the effect of 10^{-2} M 5ASA, 4ASA, N-acetyl 5ASA and olsalazine, and 10^{-3} M olsalazine was almost identical to that seen when the total cell population was analysed (Table IV).

Discussion

These results show that the salicylates used in inflammatory bowel disease (5ASA, 4ASA, and olsalazine), their major metabolites (N-acetyl 5ASA and N-acetyl 4ASA) and colchicine impair colonic epithelial cell HLA-DR expression induced by interferon-γ. This occurs at concentrations of 5ASA and N-acetyl 5ASA previously shown in the rectal lumen[23] and of colchicine found in plasma[25] in patients taking therapeutic doses of these drugs. Trypan blue exclusion and propidium iodide staining confirm that this effect is not the result of cell toxicity.

This effect was also seen when HLA-DR expression was induced by the supernatant of phytohaemagglutinin stimulated lamina propria lymphocytes. The ability of stimulated lamina propria lymphocytes supernatant to induce DR expression on monocytes[27] and HT-29[28] has previously been reported and is probably caused by interferon-γ as induction on monocytes is neutralised by antiserum to interferon-γ.[27]

The mode of action of 5ASA and 4ASA is unknown. A large number of possible mechanisms, however, have been proposed. These include interference with prostaglandins,[29] leukotrienes,[30] or platelet activating factor[31]; free radical scavenging[32]; inhibition of neutrophil, macrophage or mast cell function[33 34]; alteration of colonic permeability[35]; inhibition of cellular[36] or humoral[37] immunity and impaired cytokine production.[38 39] There is no evidence or a priori reason that treatment of active disease and prevention of relapse occur by the same mechanism.

N-acetyl 5ASA is the major metabolite of 5ASA[40] and the primary site of this metabolism is probably the colonic mucosa.[41] Three studies

have evaluated N-acetyl 5ASA in the treatment of mild ulcerative colitis[42–44] with only one reporting efficacy.[42] N-acetyl 5ASA is poorly absorbed from the rectum in vivo, however,[45] and by epithelial cells in vitro.[46] There are no studies of the therapeutic effect of N-acetyl 5ASA in maintaining remission. N-acetyl 4ASA is the major metabolite of 4ASA[47] but has not been used in ulcerative colitis or Crohn's disease.

Colchicine binds to tubulin and its effects include the arrest of mitosis and impaired neutrophil chemotaxis. It has been shown to influence a wide range of cellular processes through its binding to microtubules. These include intracellular protein translocation, movement and fusion of endosomes and lysosomes and DNA synthesis.[48] Colchicine has not been used in ulcerative colitis or Crohn's disease. Mekori and colleagues[49] used immunofluorescence to show that incubation or preincubation of HT-29 cells with colchicine reduced interferon-γ induced HLA-DR expression. They obtained the same effect with vinblastine, another tubulin binding drug, but not with the antimetabolite methotrexate. The results of our experiments with colchicine are identical with theirs.

In recent years several groups have studied the capacity of intestinal epithelial cells to act as antigen presenting cells. Bland and Warren[17 18] showed that isolated rat enterocytes could present ovalbumin to primed autologous peripheral lymph node T lymphocytes. Subsequently Mayer and Schlein[19] carried out a similar experiment using human colonic epithelial cells to present tetanus toxoid to autologous peripheral blood lymphocytes. In both cases antigen presentation was inhibited by anticlass II antibodies and phenotypic and functional studies suggested that the responding cells were suppressor T cells, although the suppression was antigen specific in the former case and antigen non-specific in the latter. Mayer has recently presented data suggesting that epithelial cells isolated from patients with inflammatory bowel disease present antigen to allogeneic $CD4^+$ lymphocytes with antigen non-specific helper activity[50] and to allogeneic lamina propria lymphocytes.[51] Kaiserlian and colleagues have reported class II restricted antigen presentation by murine enterocytes to $CD4^+$ T cell hybridoma cells.[20] Thus it seems clear from these studies that colonic epithelial cells expressing class II antigens can present antigen to T lymphocytes. The phenotype of these lymphocytes, their anatomical location and the function of this process in vivo, however, are still unresolved.

This study shows that 5ASA and related compounds impair class II antigen expression by colonic epithelial cells and thus suggests that one mode of action of these drugs may be to prevent an immune response by reducing antigen presentation by these cells.

The mechanism of this effect is not clear from these data but it seems likely that the salicylates and colchicine impair DR expression in different ways. Interferon-γ binds to a 95 kDa cell surface receptor which is present on most cell lines and is distinct from the receptor which mediates the actions of interferon-α and β.[52] Binding of interferon-γ to the receptor is followed by transcription of HLA-DR genes and assembly of HLA-DR molecules by the endoplasmic reticulum and Golgi complex. They transiently associate with an additional subunit known as the invariant chain and are transported to a peripheral endocytic compartment where they dissociate from the invariant chain and can bind processed, endocytosed antigen before export to the cell surface.[53] It seems logical to attribute the effect of colchicine on class II expression to impaired HLA-DR synthesis or transport. The salicylates could conceivably interfere with any part of this process and further studies are planned to investigate their site of action.

Some of these data have previously been published in abstract form (*Gut* 1990; **31**: A1187).

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