

# Inhibition of binding of interferon- $\gamma$ to its receptor by salicylates used in inflammatory bowel disease

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## Abstract

5-Aminosalicylic acid (5ASA), 4ASA, their N-acetylated metabolites N-acetyl-5ASA and N-acetyl-4ASA, olsalazine, and colchicine impair interferon- $\gamma$  (IFN  $\gamma$ ) induced HLA-DR expression on a colonic cell line, HT-29. The mechanism of this effect is now reported. HT-29 cells were cultured with 50 U/ml IFN  $\gamma$  with or without drug, and northern blot analysis was performed using a probe for the  $\beta$  chain of the DR molecule. IFN  $\gamma$  led to a noticeable increase in HLA-DR mRNA which was attenuated by the drugs. Analysis of the specific binding of increasing concentrations of  $^{125}\text{I}$ -IFN  $\gamma$  by non-linear regression showed a  $K_d$  of  $1.35 \times 10^{-10}$  M and  $2.3 \times 10^5$  binding sites per HT-29 cell. Binding of  $^{125}\text{I}$ -IFN  $\gamma$  was reduced by incubation with increasing concentrations of unlabelled IFN  $\gamma$  but not with IFN  $\alpha$ . Incubation with therapeutic concentrations of drugs led to the following reductions in binding: 10 mM 5ASA, 20% ( $p < 0.001$ ); 10 mM N-acetyl-5ASA, 24% ( $p < 0.01$ ); 10 mM 4ASA, 21% ( $p < 0.005$ ); 10 mM N-acetyl-4ASA, 29% ( $p < 0.001$ ); and 1 mM olsalazine, 29% ( $p < 0.001$ ). Colchicine ( $10^{-7}$  M) and  $10^{-5}$  M prednisolone had no effect. Incubation with higher concentrations of the drugs revealed a dose-response effect on binding with complete inhibition by 100 mM 4ASA and 10 mM olsalazine, and lesser degrees of inhibition by 100 mM 5ASA, N-acetyl-5ASA, and N-acetyl-4ASA. At concentrations found in the rectal lumen, the salicylates used in inflammatory bowel disease impair the binding of IFN  $\gamma$  to its receptor on colonic epithelial cells.

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Products of the class II genes of the major histocompatibility complex are cell surface heterodimers consisting of  $\alpha$  and  $\beta$  chains. Three major products are described in man: HLA-DR, DP, and DQ. CD4<sup>+</sup> lymphocytes (the helper/inducer phenotype) recognise foreign antigens carried by class II molecules on antigen presenting cells.<sup>1</sup>

HLA-DR is constitutively expressed on B lymphocytes, some macrophages, dendritic cells, vascular endothelial cells, and some epithelial cells<sup>2</sup> and can be induced in a wide range of tissues in inflammatory diseases.<sup>3</sup> *In vitro*, this is generally mediated by interferon- $\gamma$  (INF  $\gamma$ ), although this effect can be enhanced by other cytokines.<sup>3-5</sup>

In the normal gastrointestinal tract, HLA-DR is constitutively expressed on small intestinal villus cells but not in the crypts or on gastric or colonic epithelial cells.<sup>6</sup> However, epithelial HLA-DR is induced in inflammatory condi-

tions<sup>7-10</sup> including ulcerative colitis and Crohn's disease.<sup>11</sup>

The mode of action of 5-aminosalicylic acid (5ASA), the active moiety of sulphasalazine, and of the other salicylates used in inflammatory bowel disease is unknown.<sup>12</sup> We have recently shown that these drugs and colchicine, but not sulphapyridine, inhibit IFN  $\gamma$  induced HLA-DR expression on colonic epithelial cells<sup>13</sup> and now report our investigations into the mechanism of this effect.

## Materials

### CELL LINE

The HT-29 colonic carcinoma line was obtained from Dr P Brandtzaeg, Oslo, Norway and grown at 37°C in Liebowitz-15 medium (L-15) (Gibco, Paisley, UK) supplemented with 10% fetal calf serum (Flow Laboratories, Irvine, UK),  $5 \times 10^5$  U/ml penicillin, and 50  $\mu\text{g}/\text{ml}$  gentamicin.

### INTERFERONS

Recombinant IFN  $\gamma$  and IFN  $\alpha$  were gifts of Wellcome Biotech (Beckenham, UK). They were diluted in culture medium and aliquots were stored at  $-20^\circ\text{C}$  until the day of use.  $^{125}\text{I}$ -IFN  $\gamma$  (specific activity 675 Ci/mmol) was purchased from Amersham (Aylesbury, UK), diluted in distilled water, and stored at  $-20^\circ\text{C}$ .

### DRUGS

5ASA, 4-aminosalicylic acid (4ASA), prednisolone, and colchicine were purchased from Sigma (Poole, UK). N-acetyl-5ASA, N-acetyl-4ASA, and olsalazine were gifts of Pharmacia AB (Uppsala, Sweden).

Solutions (10 mM) of 5ASA, 4ASA, and their metabolites were used in most experiments, as the effects of IFN  $\gamma$  induced HLA-DR expression were seen at this concentration.<sup>13</sup> Solutions (1 mM) of olsalazine and colchicine ( $10^{-7}$  M) were used for the same reason. The concentrations of 5ASA and N-acetyl-5ASA are similar to those found in the rectal lumen in patients taking therapeutic doses of 5ASA-containing compounds.<sup>14</sup> Prednisolone was studied at  $10^{-5}$  M, which is within the plasma concentration range found in patients taking therapeutic doses of this drug.<sup>15</sup>

Solutions of 5ASA, N-acetyl-5ASA, and N-acetyl-4ASA were made by dissolving the drugs in 0.2 M NaOH, diluting 1 in 10 in L-15, and then adjusting to pH 7.4 with 1 M HCl. Olsalazine and 4ASA were supplied as sodium salts and were dissolved directly in L-15, as was colchicine. Prednisolone was dissolved in

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dimethylsulphoxide and then diluted 1 in 1000 in L-15. All solutions were filtered before use.

## Methods

### NORTHERN BLOT ANALYSIS

Northern blot analysis of HLA-DR and  $\beta$  actin mRNA was performed in the standard manner<sup>16</sup> using diethyl pyrocarbonate treated solutions. Briefly, HT-29 cells were incubated in L-15 medium containing 50 U/ml of IFN  $\gamma$  with or without drug for 72 hours. The cells were detached with 0.2 mg/ml trypsin (Worthington Biochemical Corporation, NJ, USA) and 10 mM EDTA in calcium- and magnesium-free Hank's buffered salt solution and resuspended in L-15.

RNA was extracted with guanidinium thiocyanate (RNazol B, Cinna Biotex, Friendwood, Texas, USA)<sup>17</sup> and electrophoresed on a formamide/agarose gel for 12 hours at 35 V before transfer to a nitrocellulose filter. The filter was probed for HLA-DR mRNA using a probe (provided by Dr J Bell, Oxford) labelled with <sup>32</sup>P (Oligolabelling kit, Pharmacia). After repeated washes in sodium citrate/sodium dodecyl sulphate, an autoradiograph was developed. The filter was then probed for actin (probe provided by Dr M Dallman, Oxford) in the same manner. The autoradiograph was read by eye.

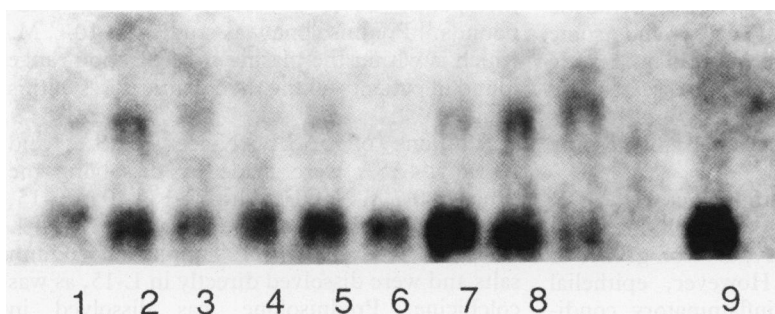
### IFN $\gamma$ BINDING

#### 0–10 mM

HT-29 cells were detached with trypsin/EDTA, washed twice in L-15, and counted. Cells ( $10^6$ ) were added to 5 ml Falcon polystyrene tubes (Becton Dickinson, Lincoln Park, NJ, USA), and centrifuged at 1000 g for 5 minutes. The supernatants were discarded and replaced with solutions of combinations of <sup>125</sup>I-IFN  $\gamma$ , unlabelled IFN  $\gamma$ , IFN  $\alpha$ , and drug dissolved in 1 ml of L-15. In most experiments, 30 nCi (45 fmol) of <sup>125</sup>I-IFN  $\gamma$  were added to each tube. The tubes were then agitated for two to four hours. All binding studies were performed at 4°C to prevent receptor internalisation.

After incubation, the cells were washed three times with 2 ml L-15 and resuspended in 1 ml L-15. Before counting (LKB Wallac 1260 Multi-gamma II counter) they were transferred to a second Falcon tube to exclude <sup>125</sup>I-IFN  $\gamma$  bound to plastic. Four tubes without cells were included in each experiment to measure non-specific binding. Specific binding was calculated as cpm – mean cpm of these four tubes.

**Figure 1:** Northern blot of HLA-DR and  $\beta$  actin mRNA in HT-29 cells incubated for 72 hours with combinations of interferon  $\gamma$  (INF  $\gamma$ ) and drugs. In each lane the upper band is HLA-DR and the lower band is actin. (1) 50 U/ml IFN  $\gamma$  +  $10^{-5}$  M colchicine, (2) 50 U/ml IFN  $\gamma$ , (3) 50 U/ml IFN  $\gamma$  +  $10^{-7}$  M prednisolone, (4) 50 U/ml IFN  $\gamma$  + 1 mM olsalazine, (5) 50 U/ml IFN  $\gamma$  + 10 mM 5ASA, (6) 50 U/ml IFN  $\gamma$  + 10 mM N-5ASA, (7) 50 U/ml IFN  $\gamma$  + 10 mM 4ASA, (8) 50 U/ml IFN  $\gamma$  + 10 mM N-4ASA, (9) no IFN  $\gamma$ . The unlabelled lane is a loading error.



### Dose-response curves

To maintain constant osmolality, experiments requiring drug concentrations greater than 10 mM were performed in Dulbecco's phosphate buffered saline (PBS) with 10% fetal calf serum (FCS). Solutions of 100 mM drug in PBS/10% FCS were made up with the concentration of NaCl reduced to maintain osmolality. Thus, for 100 mM solutions of 5ASA, N-acetyl-5ASA, N-acetyl-4ASA, acetate, propionate, and butyrate, 137 mM NaOH replaced NaCl and the pH was adjusted to 7.4 with HCl. For solutions of the sodium salts 4ASA, olsalazine, and hydrocortisone, the concentration of NaCl was simply reduced by the appropriate amount.

These solutions were mixed with PBS/10% FCS to provide a range of drug concentrations and binding studies were performed as outlined above.

## Results

### NORTHERN BLOT

As shown in Figure 1, IFN  $\gamma$  induced HLA-DR mRNA in HT-29 cells. There was a noticeable reduction in the amount of HLA-DR mRNA induced in cells incubated in 1 mM olsalazine,  $10^{-7}$  M colchicine, and 10 mM 4ASA. Variation in mRNA loading was detected by hybridisation with the  $\beta$  actin probe. Adjustment for RNA loading suggests that IFN  $\gamma$  induction of HLA-DR mRNA was also reduced by 10 mM 5ASA and 10 mM N-acetyl-4ASA but not by 10 mM N-acetyl-5ASA or  $10^{-5}$  M prednisolone. Apart from the lack of effect with N-acetyl-4ASA, the results suggest that these compounds are antagonising the effect of IFN  $\gamma$  at a pretranscriptional level. This led to an investigation of the effect of the drugs on IFN  $\gamma$  binding.

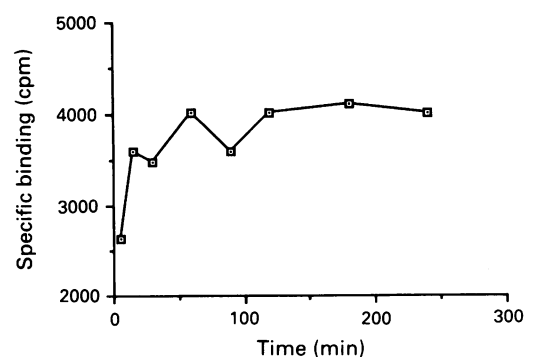
### IFN $\gamma$ BINDING

#### Time course

Maximal binding was attained by 120 minutes (Fig 2). All subsequent experiments were performed with 120–240 minute incubations.

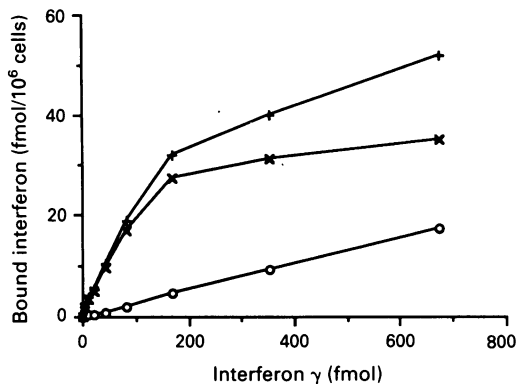
#### Cell viability

Trypan blue exclusion was 90–100% after two hours incubation at 4°C in 10 or 100 mM 5ASA, 4ASA, N-acetyl-5ASA, and N-acetyl-4ASA and



**Figure 2:** Time course of binding of <sup>125</sup>I-interferon  $\gamma$  (INF  $\gamma$ ) to HT-29 cells.

Figure 3: Specific binding of  $^{125}\text{I}$ -interferon  $\gamma$  (INF  $\gamma$ ) to HT-29 cells. Specific binding (+) at each concentration of  $^{125}\text{I}$ -INF  $\gamma$  was calculated by subtracting the cpm in the presence of excess unlabelled INF  $\gamma$  (O) from the cpm without excess unlabelled INF  $\gamma$  (X).



in 1 or 10 mM olsalazine. Viability was lowest with 10 mM olsalazine. In our previous study<sup>13</sup> we performed simultaneous flow cytometric analysis of HLA-DR expression and propidium iodide exclusion to show that the effects of the drugs were not the result of impaired viability.

**Binding isotherm**

Increasing concentrations of  $^{125}\text{I}$ -INF  $\gamma$ , with or without excess unlabelled INF  $\gamma$  (30 000 U), were added to tubes containing  $10^6$  HT-29 cells. Specific binding at each concentration was calculated by subtracting the cpm in the presence of excess INF  $\gamma$  from the cpm without excess INF  $\gamma$ . A representative binding curve is shown in Figure 3. Non-linear regression of the specific binding curve weighted by the inverse of the variance was performed using the Multifit computer programme on an Apple McIntosh computer. This revealed a  $K_d$  of  $1.35 \times 10^{-10}$  M and  $2.3 \times 10^5$  binding sites per cell (assuming one molecule of INF  $\gamma$  binds to one receptor site). Similar results were obtained by analysis of a Scatchard plot.

**Competition with unlabelled INF  $\gamma$  and INF  $\alpha$**

Increasing concentrations of unlabelled INF  $\gamma$  led to complete abolition of  $^{125}\text{I}$ -INF  $\gamma$  binding. INF  $\alpha$  had no effect (Fig 4).

**Effect of therapeutic concentrations of salicylates on INF  $\gamma$  binding**

The effect of each compound on  $^{125}\text{I}$ -INF  $\gamma$  binding was studied by comparing 10 tubes in which cells were incubated in the presence of each of the drugs with 10 control tubes. The coefficient of variation was calculated for each set

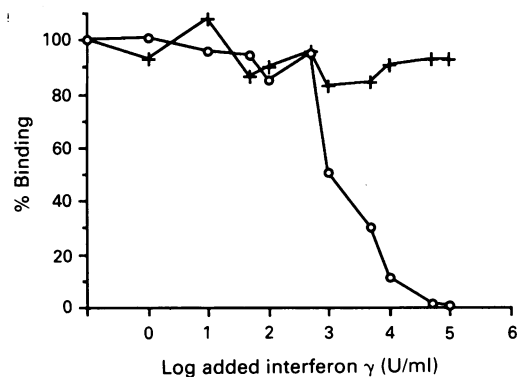


Figure 4: Effect of increasing concentrations of interferon  $\gamma$  (INF  $\gamma$ ) (O) and INF  $\alpha$  (+) on  $^{125}\text{I}$ -interferon  $\gamma$  binding to HT-29 cells.

of 10 tubes. INF  $\gamma$  binding was reduced by 20% by 10 mM 5ASA ( $p < 0.001$ , unpaired Student's  $t$  test), 24% by 10 mM N-acetyl-5ASA ( $p < 0.01$ ), 21% by 10 mM 4ASA ( $p < 0.005$ ), 29% by 10 mM N-acetyl-4ASA ( $p < 0.001$ ), and 29% by 1 mM olsalazine ( $p < 0.001$ ). Prednisolone ( $10^{-5}$  M) and  $10^{-7}$  M colchicine had no effect (Fig 5). The mean coefficient of variation was 12.7%.

**Effect of increasing doses of salicylates**

Increasing concentrations of 5ASA, 4ASA, their N-acetylated metabolites and olsalazine led to increasing inhibition of  $^{125}\text{I}$ -INF  $\gamma$  binding. Olsalazine was the most potent compound, with complete inhibition of INF  $\gamma$  binding to cells incubated in 10 mM olsalazine. Sulphapyridine is insoluble at higher concentrations by acetate, propionate and butyrate, the major anions in the colonic lumen, did not alter binding. The effect is not specific to salicylates, however, as 50 mM hydrocortisone (a much higher concentration than that seen in plasma after conventional doses) also abolished  $^{125}\text{I}$ -INF  $\gamma$  binding (Fig 6).

**Discussion**

We have previously shown that the salicylates used in inflammatory bowel disease, their N-acetylated metabolites, and colchicine impair INF  $\gamma$  induced colonic epithelial cell HLA-DR expression.<sup>13</sup> We now show that this effect is also seen at the transcriptional level, that INF  $\gamma$  binds to colonic epithelial cells, and that this binding is reduced in the presence of salicylates. This inhibition occurs at concentrations of 5ASA and N-acetyl 5ASA found in the rectal lumen of patients taking therapeutic doses of 5ASA containing compounds<sup>14</sup> and is of the same magnitude as the reduction in INF  $\gamma$  induced HLA-DR expression.

INF  $\gamma$  is a pleiotropic cytokine released by T and natural killer (NK) lymphocytes and has similar antiviral, antitumour, and pyrogenic activity to the INF  $\alpha$ s and INF  $\beta$ .<sup>18</sup> INF  $\gamma$  also has a wide range of immunomodulatory properties, including induction of class I and II histocompatibility antigens, Fc receptors, and adhesion molecules; activation of macrophages, T lymphocytes, and NK cells; stimulation of T and B lymphocyte proliferation; and differentiation

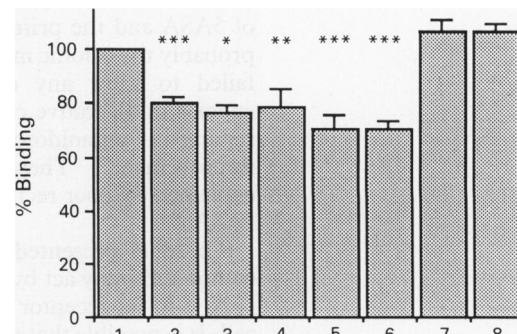


Figure 5: Effect of salicylates on  $^{125}\text{I}$ -interferon  $\gamma$  binding to HT-29 cells. Mean (SEM) expressed as percentage of control ( $n = 10$ ). (1) Control, (2) 10 mM 5ASA, (3) 10 mM N-5ASA, (4) 10 mM 4ASA, (5) 10 mM N-4ASA, (6) 1 mM olsalazine, (7)  $10^{-5}$  M prednisolone, (8)  $10^{-7}$  M colchicine. \*\*\* $p < 0.001$ , \*\* $p < 0.005$ , \* $p < 0.01$ .

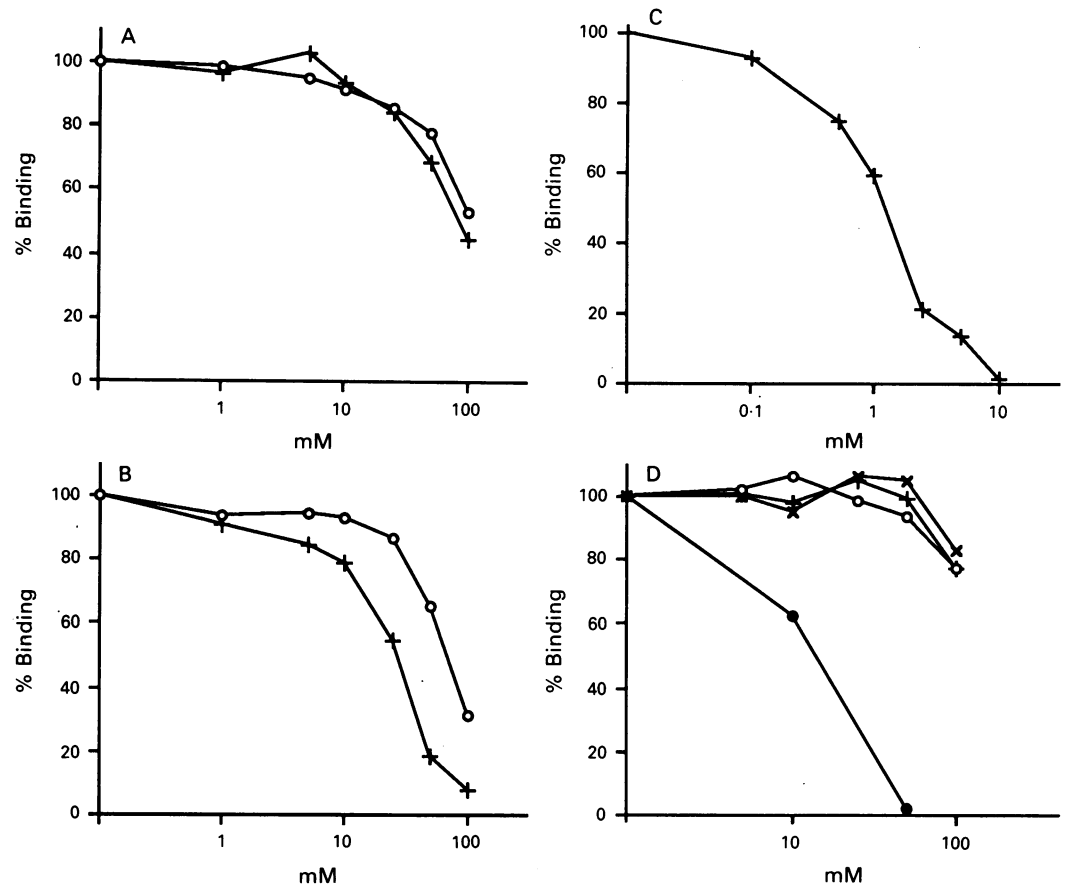


Figure 6: Effect of increasing doses of: (A) 5ASA (+) and N-5ASA (O); (B) 4ASA (+) and N-4ASA (O); (C) olsalazine; and (D) acetate (+), butyrate (x), propionate (O), and hydrocortisone (●) on interferon  $\gamma$  binding to HT-29 cells. All experiments were done in triplicate.

and induction of their cytokines.<sup>19,20</sup> IFN  $\gamma$  has also been shown to increase permeability across colonic epithelial monolayers.<sup>21</sup>

IFN  $\gamma$  binds to a 95 kDa cell surface receptor which is present on most cell lines that have been tested and is distinct from the receptor mediating the actions of the IFN  $\alpha$ s and IFN  $\beta$ . Estimates of its  $K_d$  have varied from  $10^{-11}$  to  $10^{-9}$  M and the number of receptors/cell from 100 to 10 000.<sup>22</sup> The mechanism of signal transduction after IFN  $\gamma$  binding is unknown.

The mode of action of 5ASA and 4ASA in inflammatory bowel disease is unknown. A variety of mechanisms have been proposed, including inhibition of prostaglandins,<sup>23</sup> leukotrienes,<sup>24</sup> or platelet activating factor<sup>25</sup>; free radical scavenging<sup>26</sup>; inhibition of neutrophil, macrophage, or mast cell function<sup>27,28</sup>; alteration of colonic permeability<sup>29</sup>; inhibition of humoral immunity<sup>30</sup>; and impaired cytokine production.<sup>31,32</sup> N-acetyl-5ASA is the major metabolite of 5ASA and the primary site of metabolism is probably the colonic mucosa.<sup>33</sup> Two studies have failed to show any effect of N-acetyl-5ASA enemas in ulcerative colitis,<sup>34,35</sup> although a third reported sigmoidoscopic and histological improvement.<sup>36</sup> These findings are possibly explained by poor rectal absorption of N-acetyl-5ASA.<sup>37</sup>

The data presented here suggest that these compounds may act by impairing the binding of IFN  $\gamma$  to its receptor on the colonic epithelial cell. It is possible that they may also affect IFN  $\gamma$  binding to other cells involved in the colonic mucosal immune response. Although olsalazine was the most potent inhibitor, in vivo measurements have shown that it is almost completely metabolised in the colonic lumen.<sup>14</sup>

In contrast, the reduction of IFN  $\gamma$  induced epithelial HLA-DR expression by colchicine is not caused by interference with receptor binding. It is more likely to be an intracellular effect of tubulin binding.

As IFN  $\gamma$  has such a wide range of effects, an impaired ligand-receptor interaction would modulate the mucosal immune response by a number of mechanisms. Maintenance of low epithelial permeability, inhibition of antigen presentation by epithelial or subepithelial antigen presenting cells, and inhibition of activation or proliferation of immune effector cells could all contribute to the prevention or control of colonic mucosal inflammation.

Some of these data have previously been published in abstract form (*Gastroenterology* 1991; 100: A570).

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