Inhibition of leucocyte adhesion molecule upregulation by tumour necrosis factor α: a novel mechanism of action of sulphasalazine

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Abstract

The effects of the cytokine tumour necrosis factor α and the calcium ionophore A23187 upon CD11a, CD11b, CD11c and CD18 leucocyte membrane expression was analysed in whole blood using monoclonal antibodies and flow cytometry. Both agents significantly increased the density of CD11b/CD18 *membrane* expression on monocytes and granulocytes, but had no effects on adhesion molecule expression on lymphocytes. The effects of sulphasalazine, 5-aminosalicylic acid (5-ASA) and sulphapyridine upon adhesion molecule upregulation were then examined; 10^{-3} and 10^{-4}M sulphasalazine and 5–ASA significantly reduced tumour necrosis factor α induced CD11b/CD18 upregulation on monocytes and granulocytes but had no effects upon A23187 mediated upregulation. Sulphapyridine was inactive. These results suggest that sulphasalazine and 5-ASA may interfere with mechanisms of leucocyte recruitment in inflammatory bowel disease.

(*Gut* 1993; 34: 252–256)

Tumour necrosis factor α is a cytokine produced primarily by activated macrophages and mononuclear cells and is involved in the regulation of the immune system. It is a major effector molecule in macrophage mediated tumour cell cytotoxicity and can cause severe tissue injury and wasting.[1] In addition, tumour necrosis factor α activates leucocytes, stimulating increased membrane expression of leucocyte adhesion molecules (Leu-CAMs) which results in adhesion dependent phenomena such as leucocyte aggregation and adherence to endothelial cell monolayers.[2–5] Increased numbers of tumour necrosis factor α secreting cells have been described in the colonic mucosa of children with inflammatory bowel disease as well as raised serum and stool concentrations of tumour necrosis factor α in patients with active inflammatory bowel disease.[6–8] Because cellular adhesion to vascular endothelium is required before leucocyte diapedesis and entry into parenchymal tissue, it is possible that tumour necrosis factor α may be responsible for leucocyte recruitment into the bowel wall in inflammatory bowel disease.

5-ASA is the active moiety of sulphasalazine[9] although it is possible that the parent compound, which has a number of pharmacologically distinct properties from 5–ASA may have intrinsic activity when used in inflammatory bowel disease.[10] The mode of action of these drugs remains unclear; however, possibilities include scavenging of toxic reactive oxygen metabolites and inhibition of prostaglandin and leukotriene production from the colonic mucosa.[11–15] These properties may explain the therapeutic effects of sulphasalazine and 5-ASA during an acute attack of inflammatory bowel disease but do not adequately explain their effectiveness in maintaining clinical remission. The latter effect might be because of the ability of these drugs to prevent leucocyte recruitment into the bowel wall thus reducing the inflammatory infiltrate that characterises inflammatory bowel disease.

Sulphasalazine has previously been shown to inhibit neutrophil degranulation[16] and we therefore determined whether this drug and its constituents could prevent the upregulation of intracellular storage pools of CD11b/CD18 and CD11c/CD18, which occurs upon leucocyte activation and is thought to contribute to increased cellular adhesion.[2–5] We investigated the ability of sulphasalazine, 5-ASA and sulphapyridine to prevent Leu-CAM upregulation on leucocyte cell membranes induced by either tumour necrosis factor α or the calcium ionophore A23187. In addition we investigated whether circulating concentrations of tumour necrosis factor α were present in the sera of adults with inflammatory bowel disease at concentrations that cause CD11/CD18 upregulation.

Methods

SUBJECTS

Aliquots of 0·5 ml heparinised blood from 10 healthy volunteers who had received no drugs for 14 days were incubated in the presence or absence of A23187 (Sigma) at a final concentration of 10^{-6}M for 15 minutes or of recombinant human tumour necrosis factor α (British Biotechnology) at 160 pg/ml for 30 minutes, both at 37°C. In addition varying concentrations of freshly prepared sulphasalazine, 5-ASA or sulphapyridine (all from Sigma), dissolved in 0·02 M sodium hydroxide were added to some of these incubations. Control cells contained an equal volume of diluent and were incubated at 37°C for the relevant time period. Incubations were performed in the dark to prevent oxidation of drugs. Previous work established that the above concentrations of A23187 and tumour necrosis factor α caused optimal Leu-CAM upregulation on monocytes and granulocytes cell membranes. We confirmed that none of the drugs altered the pH of the incubation.

After incubation, leucocytes were prepared for labelling using a recently developed technique which prevents artefactual increases in Leu-

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Accepted for publication 21 July 1992

TABLE I *Demographic details of 26 patients with inflammatory bowel disease in whom tumour necrosis factor α was measured*

Ulcerative colitis	
Male:female	8:9
Mean age (SEM)	43·6 (4·0)
Pancolitis:left sided	6:11
Inactive	2
Mild:moderate:severe	6:6:3
Body Mass Index<20 and albumin<36 g/l	3
Oral/IV steroids	5
Sulphasalazine/mesalazine	10
Crohn's disease	
Male:female	2:7
Mean age (SEM)	42·9 (7·1)
Ileal:Ilecocolonic:Colonic	4:1:4
Harvey Bradshaw<5	7
Harvey Bradshaw>5	2
Body Mass Index<20 and albumin<36 g/l	4
Oral/IV steroids	3
Sulphasalazine/mesalazine	2

CAM membrane expression associated with cell separation.[17] Briefly, the blood was fixed immediately in prewarmed 0·13 M formaldehyde/phosphate buffered saline (PBS-Oxoid) pH 7·4. The cells were incubated at 37°C for four minutes and erythrocytes lysed with 20 ml 0·16 M ammonium chloride/0·01 M tris-methylamine buffer, pH 7·4. The cells were centrifuged at 160 g, the supernatant discarded and the leucocytes resuspended in 5 ml phosphate buffered saline. This washing procedure was repeated once before the leucocytes were resuspended and kept on ice until the antibody labelling procedure, which was performed within 30 minutes.

In order to determine the effects of drugs on cell viability, heparinised blood mixed with an equal volume of RPMI-1640 (Gibco Ltd) tissue culture medium was separated over Lymphoprep (Nycomed) density gradient. The mononuclear cell and granulocyte layers were aspirated and cells incubated with the above drugs in RPMI/PBS for 15 or 30 minutes before viability was assessed by exclusion of trypan blue. On each occasion viability was greater than 97% for mononuclear cells and 95% for granulocytes.

ANTIBODIES

The mouse monoclonal antibodies recognising CD11a, CD11b, CD11c, CD18 and the fluorescein conjugated F(ab')$_2$ rabbit antimouse antibody were obtained from DAKO Ltd (High Wycombe, Bucks). The monoclonal antibodies UCHT1, UCHM1 and 29 were used for leucocyte subpopulation identification detecting CD3 on T-lymphocytes, CD14 on monocytes and CD15 on granulocytes respectively. We thank Dr N Hogg (ICRF) for antibody 29 and Professor P Beverley (University and Middlesex Hospital Medical School) for the antibodies UCHT1 and UCHM1.

IMMUNOFLUORESCENCE LABELLING

The methods for antibody labelling of leucocytes and flow cytometry have been described previously.[17] Twenty five microlitres of each monoclonal antibody in RPMI/5% fetal calf serum (FCS, Gibco Ltd) was added to the wells of a microtitre plate kept at 0°C. 25 μl leucocyte suspension was then added to every well and incubated for 30 minutes. Unbound antibody was then removed by two washes with RPMI/FCS. Double label immunofluorescence was established by adding 25 μl F(ab')$_2$ antibody (diluted 1:50) to every well. The cells were incubated in the dark for 30 minutes before the unbound F(ab')$_2$ antibody was washed out with RPMI/FCS. The cells were then resuspended in 25 μl RPMI/FCS and transferred to FACScan tubes (Falcon 2054) containing 300 μl 1% paraformaldehyde/phosphate buffered saline.

Leucocyte labelling was analysed by flow cytometry using a FACScan analyser (Becton-Dickinson, Mountain View, California, USA) equipped with Consort 30 software (Hewlett Packard) 24 hours later.

IMMUNOFLUORESCENCE FLOW CYTOMETRY

On each occasion 10 000 cells were analysed and the lymphocytes, granulocytes, and monocytes separated by dot plot. Each population of cells was gated and its identity confirmed by analysing the fluorescence that arose from the antibodies to CD3, CD15 and CD14. Leucocyte purity was assessed by determining the degree of staining of the gated lymphocyte population by UCHM1 and the granulocyte and monocyte population by UCHT1. Fluorescence data were collected on a four decade logarithmic scale in histogram form and cell purity was greater than 95% for lymphocytes and granulocytes, and 90% for monocytes. When analysing the histogram, markers were set on the negative control antibody which was UCHM1 for lymphocytes and UCHT1 for granulocytes and monocytes. Values of mean fluorescence intensity (MFI), a quantitative measurement of antigen density, were automatically recorded by the FACScan analyser for both the fluorescent and non-fluorescent cells. The percentage of labelled or non-labelled cells was also recorded. The non-specific binding of the F(ab')$_2$ fragment was determined each time by incubating a leucocyte sample with the fluorescent label only and was negligible on each occasion.

TUMOUR NECROSIS FACTOR α ENZYME LINKED IMMUNOADSORBENT ASSAY

Twenty six patients with inflammatory bowel disease were venesected and sera obtained within 30 minutes of blood clotting. Samples were stored at −70°C until assayed. The demographic data of the patients is depicted in Table I. Disease activity was assessed by the criteria of Truelove and Witts for ulcerative colitis and by the Harvey-Bradshaw Index for Crohn's disease.[18 19] Malnourishment was defined as a serum albumin <36 g/l and body mass index <20. Sera were also obtained from 16 healthy laboratory personnel (mean age (SEM) 36·7 (3·1)) who had not received any drugs for 14 days. Tumour necrosis factor α concentrations in sera were measured by enzyme linked immunoadsorbent assay (ELISA) using a commercial assay (T Cell Sciences, Cambridge, MA, USA). A standard curve was created with a lower limit of detection of 10 pg/ml.
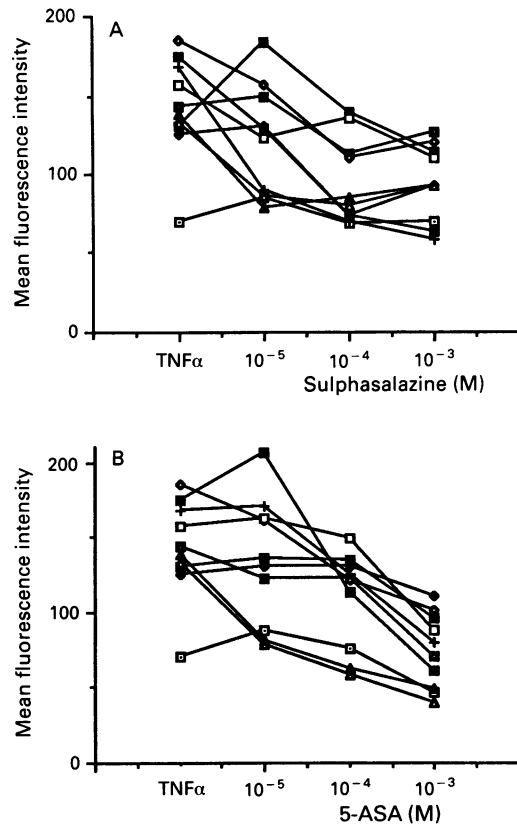
Figure 1: Dose response curves of CD11b membrane expression on granulocytes in ten volunteers. Cells were either incubated with tumour necrosis factor α alone or tumour necrosis factor α with sulphasalazine (a) or 5-ASA (b). Sulphasalazine and 5-ASA reduced tumour necrosis factor α induced CD11b upregulation in eight and nine volunteers respectively.

STATISTICAL ANALYSIS

Results were analysed by Student's paired *t* test. Significance was taken at the 5% level.

Results

EFFECTS OF TUMOUR NECROSIS FACTOR α

The effects of tumour necrosis factor α upon CD11b/CD18 granulocyte membrane expression for each individual are shown in Figs 1 and 2 whilst mean values are shown in Table II. Tumour necrosis factor α increased CD11b expression by a mean (SEM) of 62 (16)% compared with the 30 minute control (p<0·005) and sulphasalazine and 5-ASA reduced this

upregulation in eight and nine volunteers respectively (Figs 1a and 1b). From Table II it can be seen that 10^{-3} and 10^{-4} M sulphasalazine inhibited tumour necrosis factor α induced CD11b upregulation by a mean of 82 (29)% and 92 (23)% respectively (p<0·01, p<0·005, respectively), while 10^{-3} M 5-ASA completely abrogated CD11b upregulation (p<0·005) and 10^{-4} M 5-ASA inhibited it by 64 (12)% (p<0·05). Tumour necrosis factor α increased CD18 granulocyte membrane expression by 51 (18)% (p<0·01) and 10^{-3} and 10^{-4} M sulphasalazine reduced this upregulation in eight volunteers (Fig 2a) with a fall in mean CD18 levels back to control values (p<0·005, both values). The response to 5-ASA was more varied with only five volunteers, however, showing a large reduction in CD18 upregulation (Fig 2b) and only 10^{-3} M 5-ASA caused a significant reduction in mean CD18 upregulation (p<0·01).

The effects of tumour necrosis factor α upon CD11b/CD18 monocyte membrane expression on monocytes are shown in Figs 3 and 4. Tumour necrosis factor α increased CD11b expression by 137 (29)% (p<0·001) compared with controls and sulphasalazine and 5-ASA greatly reduced this upregulation in seven and six volunteers respectively; both 10^{-3} and 10^{-4} M sulphasalazine inhibited CD11b upregulation by approximately 65% (both p<0·01) and 10^{-3} and 10^{-4} M 5-ASA inhibited CD11b upregulation by 55 (16)% and
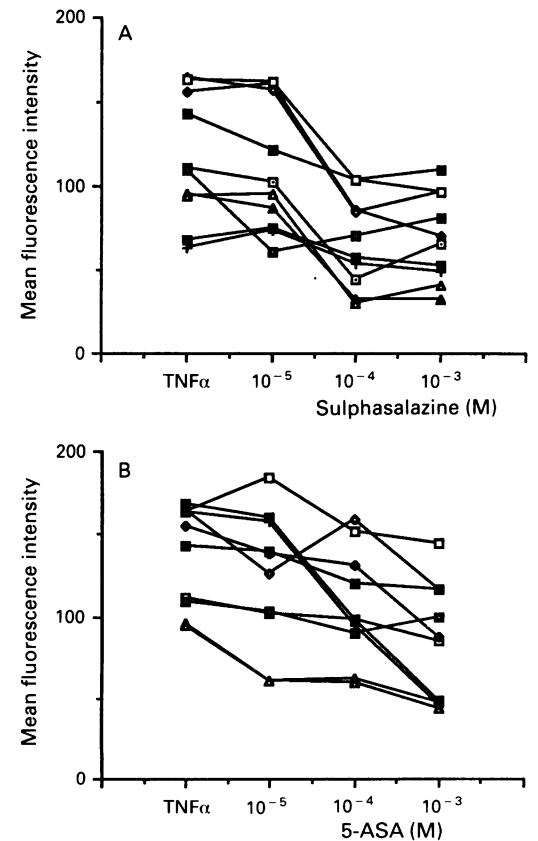

Figure 2: Dose response curves of CD18 membrane expression on granulocytes in 10 volunteers. Cells were either incubated with tumour necrosis factor α alone or tumour necrosis factor α with sulphasalazine (a) or 5-ASA (b). Sulphasalazine and 5-ASA reduced tumour necrosis factor α induced CD18 upregulation in eight and five volunteers respectively.

TABLE II *Mean fluorescence intensity (n=10) of CD11b/CD18 membrane expression on granulocytes and monocytes. Cells were either incubated alone (control) or in the presence of tumour necrosis factor α ± varying concentrations of sulphasalazine or 5-ASA*

| | Mean fluorescence intensity (SEM) | | | |
| | Granulocytes | | Monocytes | |
	CD11b	CD18	CD11b	CD18
Control	88·8 (10·2)‡	77·3 (8·2)†	94·9 (18)§	105·3 (10·9)‡
TNFα alone	142·3 (10·2)	116·8 (6·6)	189·5 (18·8)	213·6 (32·6)
10⁻⁵ M Sulphasalazine +TNF	98·7 (7·0)†	74·8 (6·6)‡	124·9 (16·7)†	112·9 (18·4)‡
10⁻⁴ M Sulphasalazine +TNF	93·0 (9·6)‡	66·3 (8·5)‡	122·9 (23·1)†	105·9 (22·5)‡
10⁻³ M Sulphasalazine +TNF	114·7 (11·3)	109·4 (12·2)	168·8 (21·9)	175·5 (33·1)
10⁻⁵ M 5-ASA +TNF	73·1 (13·9)‡	87·8 (12·9)†	137·1 (20·8)*	133·4 (17·6)†
10⁻⁴ M 5-ASA +TNF	108·1 (9·8)*	106·2 (10·7)	118·2 (21)†	110·2 (8·1)‡
10⁻³ M 5-ASA +TNF	137·0 (14·9)	114·9 (8·9)	170·2 (18)	196·5 (20·5)

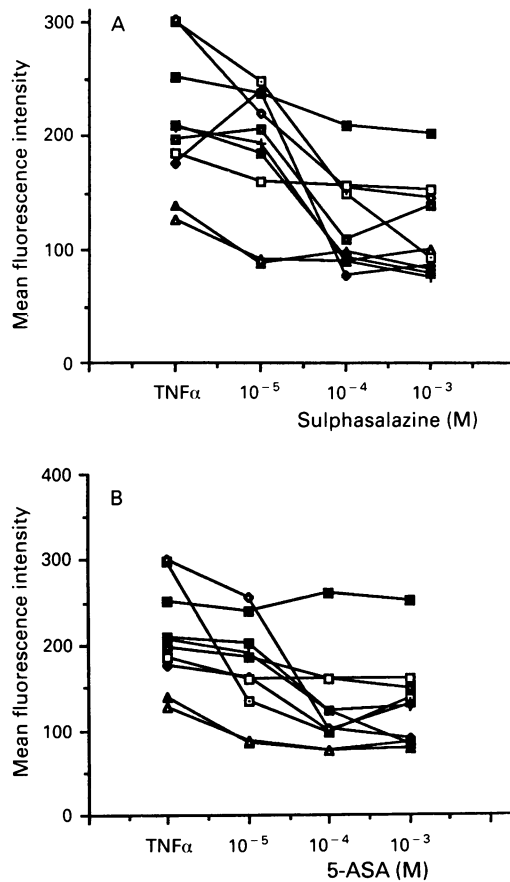*p<0·05, †p<0·01, ‡p<0·005, §p<0·001 versus tumour necrosis factor α.

Figure 3: Dose response curves of CD11b membrane expression on monocytes in 10 volunteers. Cells were either incubated with tumour necrosis factor α alone or tumour necrosis factor α with sulphasalazine (a) or 5-ASA (b). Sulphasalazine and 5-ASA reduced tumour necrosis factor α induced CD11b upregulation in seven and six volunteers respectively.

control. All other results were below the lower limit of detection of the assay of 10 pg/ml.

Discussion

Our study has confirmed that tumour necrosis factor α can increase adhesion molecule expression on the cell membranes of monocytes and granulocytes.[2][3] As raised numbers of tumour necrosis factor α secreting cells are present in the colon of children with inflammatory bowel disease and increased concentrations of tumour necrosis factor α are present in the stools of these patients,[7][8] this cytokine may activate circulating leucocytes within the gut vasculature resulting in leucocyte recruitment into the bowel wall in inflammatory bowel disease. Indeed CD11a and ICAM-1 upregulation has been documented in a histological study of mononuclear phagocytes in colonic mucosa from inflammatory bowel disease patients.[20]

A recent study has shown that 5-ASA can prevent an increase in markers of cellular activation, namely interleukin-2 (IL-2) and transferrin receptors, on peripheral blood mononuclear cells in response to pokeweed mitogen.[21] In this study we have also shown that sulphasalazine and 5-ASA can inhibit cellular activation, reducing tumour necrosis factor α induced upregulation of CD11b/CD18 on both monocytes and granulocytes. This property suggests a

75 (21)% respectively (p<0·05, p<0·01 respectively). Tumour necrosis factor α increased CD18 membrane expression by 103 (26)% (p<0·005) and 10⁻³ and 10⁻⁴ M sulphasalazine greatly reduced this upregulation in eight volunteers with a fall in mean CD18 levels back to control values (both p<0·005). Table II shows that 10^{-3} M 5-ASA caused a 74 (25)% inhibition of CD18 upregulation (p<0·01) but 10^{-4} M 5-ASA completely inhibited CD18 upregulation (p<0·005). Tumour necrosis factor α did not alter the mean fluorescence intensity of any adhesion molecules on lymphocytes and had no effect upon CD11a or CD11c expression on granulocytes and monocytes. Sulphapyridine had no effect upon tumour necrosis factor α induced adhesion molecule upregulation.

EFFECT OF A23187
A23187 caused a 79 (23)% and 46 (14)% increase of CD11b and CD18 respectively on granulocytes (p<0·01, p<0·05, respectively) compared with the 30 minute control. A23187 caused a 52 (14)% and 32 (13)% increase of CD11b and CD18 respectively in monocytes (both p<0·05) compared with the 30 minute control. Neither sulphasalazine nor 5-ASA, however, altered adhesion molecule upregulation caused by A23187.

TUMOUR NECROSIS FACTOR α ASSAY
Tumour necrosis factor α concentrations were recorded at 15 pg/ml in one patient with severe left sided ulcerative colitis and one healthy
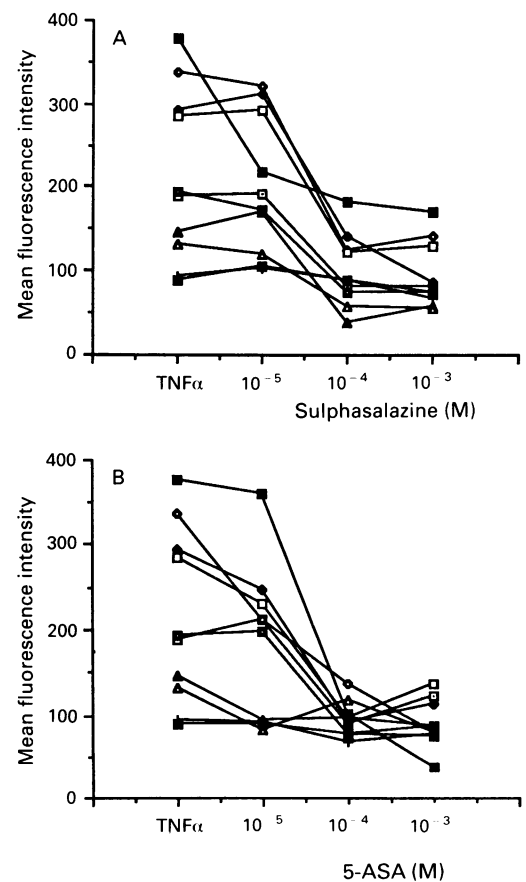

Figure 4: Dose response curves of CD18 membrane expression on monocytes in ten volunteers. Cells were either incubated with tumour necrosis factor α alone or tumour necrosis factor α with sulphasalazine (a) or 5-ASA (b). Both Sulphasalazine and 5-ASA reduced tumour necrosis factor α induced CD18 upregulation in eight volunteers.

mechanism of action by which these drugs may prevent leucocyte recruitment into parenchymal tissue in inflammatory bowel disease and may explain why these drugs prevent disease relapse.

Neither sulphasalazine nor 5-ASA affected adhesion molecule upregulation induced by A23187 implying that these drugs may interrupt the cell signal delivered by tumour necrosis factor α to cause degranulation of adhesion molecule stores. The mechanism by which sulphasalazine and 5-ASA exerted their effect in this study remains unclear; previous binding studies suggested that sulphasalazine but not 5-ASA can inhibit the binding of tumour necrosis factor α its receptor, although not at the lower concentrations that we found to be effective in our study.[22] Further evidence that sulphasalazine is able to interfere with cellular receptors is provided by a study that showed that sulphasalazine and to a lesser extent 5-ASA is able to inhibit binding of the proinflammatory peptide N-formyl-methionyl-leucyl-phenylalanine (FMLP) to its receptor on neutrophils.[23]

Sulphasalazine and 5-ASA inhibited tumour necrosis factor α induced adhesion molecule upregulation at concentrations of 10^{-3} and 10^{-4} M and these concentrations are reached in the colonic luminal contents of patients taking sulphasalazine.[24] Sulphapyridine which is the inactive moiety of sulphasalazine was without effect, however. It is interesting that sulphasalazine which has previously been shown to be a more effective inhibitor of thromboxane B_2 synthesis and neutrophil degranulation than 5-ASA as well as being able to block the binding of tumour necrosis factor α to its receptor[13 16 22 25] was effective in this study. As it seems unlikely that sulphasalazine was split into its constituent parts in the incubations, our work further supports the suggestion that sulphasalazine may be more than just a delivery system for 5-ASA.[10]

Despite 11 patients having moderate or severe inflammatory bowel disease of whom seven were malnourished, we did not find raised tumour necrosis factor α concentrations in their sera. This is in contrast with the raised concentrations found in children[6] and suggests that either tumour necrosis factor α is not secreted into the adult circulation or that a tumour necrosis factor α inhibitor, which has been found in the sera of adults with Crohn's disease[26] may neutralise circulating tumour necrosis factor α and block its detection by the ELISA used here.

5-ASA has been shown to have a number of possibly important immunological effects, inhibiting IL-1 production in colonic biopsy specimens from inflammatory bowel disease patients, reducing antibody and IL-2 receptor secretion by mononuclear cells and also decreasing interferon-γ induced HLA-DR expression on colonic epithelial cells.[21 27-29] In our study sulphasalazine and 5-ASA have been shown to inhibit cytokine mediated adhesion molecule upregulation by which means they may prevent leucocyte recruitment into the bowel wall in inflammatory bowel disease.

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