Clin. exp. Immunol. (1990) **81**, 301–305

Tumour necrosis factor-alpha and interferon-gamma production measured at the single cell level in normal and inflamed human intestine

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(Accepted for publication 27 February 1990)

SUMMARY

The spot-ELISA technique has been used to enumerate the frequency of cells secreting tumor necrosis factor-alpha (TNF-α) and interferon-γ (IFN-γ), isolated from biopsies of normal intestine and from biopsies of children with inflammatory bowel disease. TNF-α production was undetectable in six out of 12 biopsies from normal intestine and in the other six biopsies it ranged from 60 to 580 TNF-α-secreting cells/10^6 isolated intestinal cells. In contrast, cells isolated from biopsies of children with Crohn's disease ($n=9$) all showed elevated frequencies of TNF-α-secreting cells (500–12 000 secreting cells/10^6 cells). In ulcerative colitis, four out of eight children had increased production of TNF-α and in children with indeterminate colitis two out of three had elevated levels. There was no correlation between plasma TNF-α levels and the number of intestinal cells secreting TNF-α. In controls and all groups of patients IFN-γ-secreting cells were uncommon. These results suggest that TNF-α is an important mediator of inflammation in the human gut, and, furthermore, may play a role in the growth failure frequently seen in children with inflammatory bowel disease.

Keywords tumour necrosis factor interferon-gamma Crohn's disease

INTRODUCTION

The aetiology of chronic inflammatory bowel disease (IBD) remains unknown. Whatever the initial stimulus, there is a general consensus that the immune system contributes towards the local intestinal inflammation. The normal intestinal mucosa is infiltrated with T cells, B cells, plasma cells, macrophages, dendritic cells, eosinophils and mast cells (Parrott, 1987). In IBD the density of the infiltrate of all of these cell types is increased (reviewed by Brandtzaeg, 1987). There have been numerous immunohistochemical studies on the changes of plasma cell isotype, T cell populations, macrophages and dendritic cells in IBD (Selby *et al.*, 1983a, 1983b, 1984; MacDermott, *et al.*, 1986; Brandtzaeg, 1987; Allison *et al.*, 1988; Seldenrijk *et al.*, 1989; Mahida *et al.*, 1989b). There have also been several studies on local T cell immunoregulation and immunoglobulin production in IBD but these have been uninformative (Fiocchi, Youngman & Farmer, 1983; Elson, Machelski & Weiserbs, 1985; James *et al.*, 1985). In contrast, there have been very few studies on local production of T cell-derived and macrophage-derived mediators of inflammation in IBD, although it is well established that mediators such as leukotrienes and prostaglandins are produced at increased levels (Zifroni *et al.*, 1983; Lauritsen *et al.*, 1988).

The spot-ELISA technique allows quantification at the single cell level of the frequency of cytokine secreting cells in any cell population (Hutchings *et al.*, 1989). We therefore used this technique to quantify the frequency of cells secreting tumour-necrosis factor-alpha (TNF-α) (cachectin) and interferon-gamma (IFN-γ) in normal and inflamed human intestine. The choice of these two mediators was not random since children with IBD frequently show growth failure (Silverman, 1966; O'Donogue & Dawson, 1977) which may be related to the presence of increased levels of circulating TNF-α as well as other factors, such as impaired nutrition and protein-losing enteropathy. In addition, the intestinal lesion in IBD shows some of the phenomena associated with local production of IFN-γ, such as increased HLA-DR expression and granuloma formation (Rappaport, Burgoyne & Smetana, 1951; Lockhart-Mummery & Morson, 1960; Selby *et al.*, 1983a; MacDonald, Weinel & Spencer, 1988).

MATERIALS AND METHODS

Subjects

These studies were carried out with the approval of the Hackney and District Health Authority Ethical Committee. Tissue was

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Table 1. Clinical details of the patients with inflammatory bowel disease

Patient no.	Sex	Age (years)	Disease	Treatment*
Crohn's disease				
1	F	13·3	Ileum, *pan*-colitis	Pred (25 mg in d.) Azath (25 mg in d.) Asacol (400 mg in d.)
2	M	13·1	Ileum, severe colonic disease, rectal sparing	Asacol (400 mg in d.)
3	M	11·0	Ileum, colon, rectal sparing	None
4	M	11·6	Ileum, colon	Asacol (800 mg b.i.d.)
5	F	15·0	Ileum only	SZP (2000 mg t.i.d.)
6	M	7·9	Ileum, *pan*-colitis	SZP (1500 mg t.i.d.)
7	M	9·9	Ileum, right colon	None
8	F	16·1	Ileum, right colon	Flexical
9	M	11·6	Ileum, right colon	Cimetidine (400 mg b.i.d.)
Ulcerative colitis				
1	M	15·5	pancolitis	Asacol (1200 mg b.i.d.)
2	M	9·4	proctitis	None
3	M	3·1	pancolitis	SZP (600 mg t.i.d.)
4	F	11·0	disease from sigmoid colon to caecum	SZP (1500 mg t.i.d.)
5	M	16·0	descending colon	Asacol (1200 mg t.i.d.) Pred (2 mg b.i.d.)
6	F	11·2	pancolitis	Pred (30 mg b.i.d.)
7	F	9·8	pancolitis	Pred (10 mg b.i.d.)
8	M	17·7	Left colon, rectum	Asacol (800 mg b.i.d.) Pred (15 mg in d.)
Indeterminate colitis				
1	F	9·8	pancolitis	Asacol (800 mg b.i.d.) Pred (20 mg b.i.d.)
2	F	12·7	pancolitis normal rectum	SZP (750 mg t.i.d.) Pred (10 mg t.i.d.)
3	M	7·1	caecum	Pred (5 mg b.i.d.) Asacol (800 mg b.i.d.)

* Treatment during the 4 weeks prior to colonoscopy.
Pred, prednisolone; Azath, azathioprine; SZP, salazopyrine.

obtained from 20 children with IBD: nine with active Crohn's disease; eight with active ulcerative colitis; and three with active indeterminate colitis (intestinal inflammation with none of the characteristics of Crohn's disease or ulcerative colitis). Details of these patients are given in Table 1. None had prior intestinal surgery. As controls, 12 patients were studied in whom IBD was suspected (seven girls and five boys, age range 1–14·5 years). They were admitted for colonoscopy because of recurrent abdominal pain, diarrhoea, and in some cases unexplained weight loss. However, on clinical and histologic analysis, a diagnosis of IBD was excluded. None of these children has subsequently developed IBD and all are healthy. Clinical diagnosis was based on the criteria of Chong *et al.* (1985).

Biopsies

During investigative colonoscopy to assess the extent of the inflammation in patients in relapse, or in patients under initial investigation, two biopsies were taken from regions of the mucosa showing macroscopic inflammation. Biopsies were taken for histologic analysis from adjacent sites and confirmed that the tissues were inflamed. In patients with Crohn's disease, samples were taken from the ileum (patients 2, 5, 7) and from the ascending or transverse colon in the rest. In patients with ulcerative colitis, if the patient had pancolitis the biopsies were taken from the right colon or transverse colon, and in two patients with distal disease one was biopsied in the rectum and the other in the left colon. All biopsies from the control patients were made in the right colon or transverse colon. The biopsies were immediately placed in tissue culture medium, transported to the Paediatric Gastroenterology Laboratory, and the cells isolated by collagenase digestion as previously described (MacDonald *et al.*, 1987). Since these cells contain numerous IgA plasma cells they are predominately from the lamina propria although some intra-epithelial lymphocytes may also be present. Cell yields varied from 4×10^5 to $1{\cdot}5 \times 10^6$ from the two biopsies and there was a tendency for higher cell yields from patients with IBD. Plasma was also collected from some of the children at the time of colonoscopy and stored at $-70°C$.

Spot-ELISA assays

These were carried out exactly as described elsewhere (Hutchings *et al.*, 1989). Aliquots of the cells were plated in triplicate into microtitre wells coated with monoclonal anti-TNF-α or IFN-γ antibody. After overnight incubation the cells were

washed off and bound TNF-α or IFN-γ was detected using rabbit anti-TNF-α or anti-IFN-γ followed by alkaline phosphatase-conjugated sheep anti-rabbit IgG. Antibody binding was visualized using 5-bromo-4-chloro-3-indolyl phosphate as enzyme substrate and fast blue. Blue spots per well were counted and the frequency of spot-forming cells/10^6 input cells (SFC/10^6) was then derived based on the number of cells placed into the wells. Results given are the mean of the triplicate cultures. Comparison between groups was made using the Kolmogorov–Smirnov two-group test or by χ^2 analysis with continuity correction factor.

Plasma TNF-α levels

These were carried out using a commercial ELISA kit with a lower limit of sensitivity of 10 pg TNF-α/ml of plasma (T Cell Sciences, Cambridge, MA).

Separation of E^+ and E^- mucosal lymphocytes

T cell rosettes were made using AET-treated sheep erythrocytes. The rosetted cells were separated by centrifugation through Ficoll/Paque (Pharmacia). Due to the low numbers of cells available from biopsies it proved impossible to recover the E^+ cells from the erythrocyte pellet by water lysis. Thus the comparison was made between unseparated mucosal lymphocytes and the E^- cells recovered from the buffy coat (between 10 and 50% of the original cell number).

RESULTS

TNF-α production in normal and diseased mucosa

In six of 12 controls, TNF-α was undetectable and in the other six patients, levels range was 60–580 SFC/10^6 mucosal cells (median 200 SFC, Fig. 1). In contrast, patients with Crohn's disease had between 510 and 12 000 SFC/10^6 cells (median 1280 SFC). Four of the eight patients with ulcerative colitis had no TNF-α-secreting cells, and in the other four, levels ranged from 620 to 78 000 SFC/10^6 mucosal cells. In three patients with indeterminate colitis, TNF-α-secreting cells were 480, 1700 and 1730 SFC/10^6 intestinal cells.

No correlation was apparent between local production in the gut of TNF-α and plasma levels (Table 2). Of nine patients tested with raised levels in the gut, five had no detectable plasma TNF-α and in the others the values ranged from 18 to 150 μg/ml. Comparing the frequency of TNF-α-secreting cells in unseparated cells and the E^- cells, it was found that in four patients, most of the activity appeared to be associated with the E^+ cells (Table 3). In another patient, the E^- cells had around 50% of the activity of the unseparated cells. It is unlikely that E rosetting non-specifically removes TNF-α-secreting cells, since in a single patient with ulcerative colitis the E^- cells were enriched for TNF-α-secreting cells compared with unseparated cells.

IFN-γ production in normal and inflamed mucosa

In contrast to the results with TNF-α, IFN-γ-secreting cells were uncommon in all of the patient groups studied. Four out of nine Crohn's patients showed some activity (Table 4); however, two out of 11 controls tested also had low levels of IFN-γ-secreting cells.

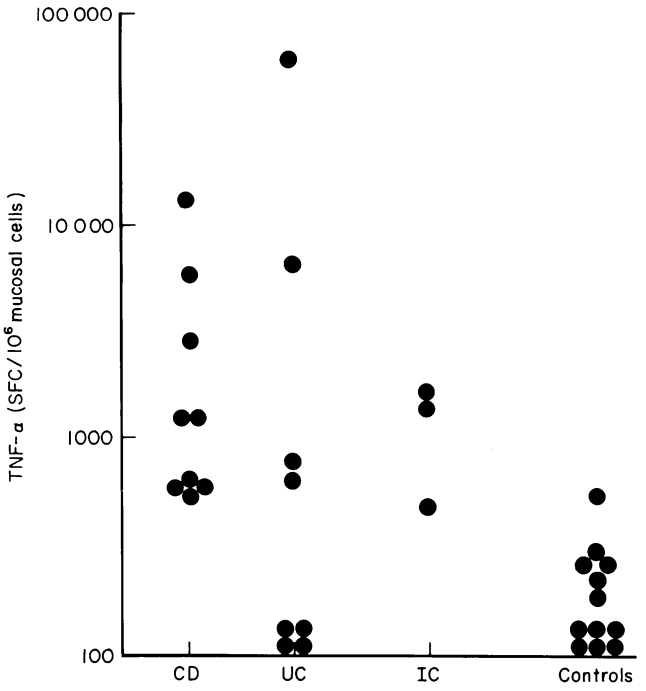

Fig. 1. TNF-α production by mononuclear cells isolated from intestinal biopsies of children with Crohn's disease (CD), ulcerative colitis (UC), indeterminate colitis (IC) or disease controls. Means are not given since only for the Crohn's disease patients did the values correspond to samples from a normally distributed population. Note the log scale. There were more TNF-α-secreting cells in Crohn's disease tissue than in controls ($P<0.0005$, Kolmogorov–Smirnov two-group test). Using 600 SFC as a cut-off point for the upper limit of normal, there was significantly greater TNF-α production in ulcerative colitics ($P=0.03$, χ^2) and patients with indeterminate colitis ($P=0.038$, χ^2) than controls.

Table 2. Lack of association between TNF-α production in inflamed bowel and plasma levels

Patient no.	TNF (SFC/10^6 cells) in the mucosa	Plasma TNF-α (pg/ml)
Crohn's disease		
1	1280	< 10
2	510	< 10
3	1283	30
4	2940	18
5	535	< 10
Ulcerative colitis		
1	5400	< 10
2	763	< 10
3	78 660	27
4	1730	150

Table 3. TNF-α secreting cells in unseparated and E^- mucosal cells

	TNF-α (SFC/10^6 cells)	
	Unseparated	E^-
Crohn's disease	5920	60
Crohn's disease	12 066	5700
Indeterminate colitis	1730	60
Indeterminate colitis	480	0
Ulcerative colitis	78 660	2820
Ulcerative colitis	1710	10 510

Table 4. IFN-γ production is not a prominent feature of inflammatory bowel disease

Patients	No. positive/total	Positives (SFC/10^6 cells)
Controls	2/11	93, 113
Crohn's disease	4/9	22, 53, 370, 1500
Ulcerative colitis	2/7	20, 60
Indeterminate colitis	2/3	90, 430

DISCUSSION

To our knowledge this is the first time in which cells secreting inflammatory mediators have been quantified in normal and diseased human intestine. The major observation is that the frequency of cells secreting TNF-α is increased in the mucosa in IBD. All of the Crohn's patients, half of the ulcerative colitis patients and two out of three patients with indeterminate colitis showed raised levels. We cannot yet explain the heterogeneity in the ulcerative colitis patients, since all the biopsies were taken from apparently inflamed mucosa, and the results did not correlate with steroid treatment.

There has been interest in TNF-α as a mediator of inflammation in recent years (reviewed by Beutler & Cerami, 1987; Cerami & Beutler, 1988), and it clearly has many diverse effects. However, we would like to comment briefly on features of its biological activity of relevance to gastrointestinal disease and its systemic sequelae.

TNF-α infused systemically into rats causes intestinal haemorrhage and necrosis (Sun & Hsueh, 1988). This is due to break-down of the endothelium in the intestinal vascular network caused by TNF-induced pro-coagulant activity of the endothelial cells and increased neutrophil adherence and mediator release (Cerami & Beutler, 1988). TNF-α is also angiogenic and may help in the healing of diseased mucosa (Frater-Schroder *et al.*, 1987).

TNF-α has also been shown to be important in the generation of the granulomatous response to *Mycobacteria* in mice (Kindler *et al.*, 1989). TNF-α acts in an autocrine fashion, being secreted by activated macrophages, and inducing other macrophages to differentiate into epithelioid cells. A macrophage influx is a feature of Crohn's disease and to a lesser extent ulcerative colitis (Selby *et al.*, 1983b; Allison *et al.*, 1988; Seldenrijk *et al.*, 1989; Mahida *et al.*, 1989b); however, it is still unknown why granulomas are seen only in Crohn's disease.

TNF-α also has cachectic activity (Oliff *et al.*, 1987; Tracey Manogue *et al.*, 1988; and may contribute to weight loss frequently seen in children with IBD (as do other factors, such as poor nutrition).

Raised plasma C-reactive protein (CRP) levels are also associated with IBD (Walker-Smith, 1988). Infusion of graded doses of TNF-α into patients leads to a dose-dependent increase in plasma CRP levels (Michie *et al.*, 1988).

In preliminary experiments we attempted to identify the mucosal cell type secreting the TNF-α. Our results were equivocal in that in some patients all of the activity appeared to be in the T cell fraction of mucosal cells, in another patient activity was associated with the non-T cell fraction, and in another patient, both fractions had activity. There is however no *a priori* reason to assume that only macrophages secrete TNF-α in inflamed bowel since it is now well established that T cells can also secrete this mediator (Cuturi *et al.*, 1987; Pawlec *et al.*, 1989). It is likely that there are numerous mediators being produced in inflamed bowel. For example, it has recently been shown that interleukin-1 secretion by lamina propria macrophages is increased in patients with IBD (Mahida, Wu & Jewell, 1989a). It is also unlikely that TNF-α production is specific for IBD and that as more enteropathies are studied TNF-α production may be found to be a common feature.

Class II MHC expression on epithelial cells is increased in IBD (Selby *et al.*, 1983a), a feature usually attributed to local production of IFN-γ by activated T cells (MacDonald *et al.*, 1988). However, a striking feature of our study was the relative infrequency of cells secreting IFN-γ in diseased mucosa. This was surprising in view of the large numbers of activated T cells in the mucosa in Crohn's disease (Mahida *et al.*, 1988; Choy *et al.*, 1990). However, a single previous report also showed that mitogen induced IFN-γ secretion was lower in mucosal lymphocytes from patients with Crohn's disease compared with controls (Ouyang *et al.*, 1988).

ACKNOWLEDGMENTS

A. Cooke and T. T. MacDonald are Wellcome Senior Lecturers. M. Choy is supported by a grant from the National Foundation for Ileitis and Colitis. This work is also supported by the MRC, and Crohn's Disease in Childhood Research Association.

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