Cytokines in stools of children with inflammatory bowel disease or infective diarrhoea

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Abstract

***Aims*—To determine the concentrations of interleukin-6 (IL-6) and tumour necrosis factor α (TNFα) in stools from children.**

***Methods*—Stool samples from 14 healthy children, 32 children with inflammatory bowel disease, and 23 children with acute diarrhoea were emulsified in an equal volume of phosphate buffered saline and then centrifuged to produce a clear supernatant fluid. IL-6 and TNFα were measured by enzyme linked immunosorbent assay (ELISA).**

***Results*—TNFα was detected in the stools of all 14 healthy children (12–130 pg/g stool), but IL-6 was detected only in three. Similar results were seen in children with inactive inflammatory bowel disease. Stool TNFα concentrations were raised in samples from children with active inflammatory bowel disease, but in most (11/18) of these samples IL-6 was undetectable. Stool samples contained a heat-labile factor which rapidly destroyed IL-6 immunoreactivity. Most children with diarrhoea had TNFα concentrations similar to those of healthy controls and most were also negative for IL-6. Three children with *Shigella flexneri* infection had extraordinarily high concentrations of both TNFα and IL-6 in their stools.**

***Conclusions*—There is constant low grade production of TNFα in the intestine of healthy people. Raised values are indicative of mucosal inflammation, but are not specific. Stool IL-6 is of little use in assessing mucosal inflammation because immunoreactivity is rapidly lost in stool samples.**

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Crohn's disease and ulcerative colitis are characterised by chronic intestinal inflammation. Their cause is unknown, but tissue damage is thought to be partly mediated by macrophage derived pro-inflammatory cytokines such as the interleukins as IL-1, IL-6, and tumor necrosis factor (TNFα).[1] These cytokines may exert local cytopathic effects,[2] or may serve to upregulate endothelial adhesion molecules, thereby promoting the extravasation of neutrophils and monocytes into the gut mucosa.[3]

Systemic cytokine concentrations have been measured in inflammatory bowel disease by several investigators. Serum TNFα concentrations are raised in both active ulcerative colitis and Crohn's disease,[4] and there is an increased incidence of TNFα secreting cells in the mucosa in inflammatory bowel disease.[5] Significantly high concentrations of TNFα are also present in the stools of children with active inflammatory bowel disease; these fall to normal after treatment.[6] IL-1 values are also noticeably raised in the mucosa in active inflammatory bowel disease.[7] As yet there are fewer data on IL-6. Plasma IL-6 concentrations are raised in active Crohn's disease, but suprisingly, not in active ulcerative colitis.[8 9]

Because we have already shown that stool TNFα concentrations are an indicator of intestinal inflammation in children, and the samples are easily obtained and studied, we have now investigated stool IL-6 concentrations in children with inflammatory bowel disease. We also examined stools from a large group of children with infectious gastroenteritis.

Methods

Fourteen children with active Crohn's disease (age range 9–17 years) (disease location: colon n = 8, ileum n = 4, ileocolonic n = 2), four children with active ulcerative colitis (age range 3–16 years), nine children with inactive Crohn's disease (age range 7–16 years) and five children with inactive ulcerative colitis (age range 3–15 years) were studied. Fourteen children without gastrointestinal disease (2–16 years) and 23 disease controls (6 months–13 years) with acute diarrhoea were also studied. In this last group neither bacterial nor viral pathogens were isolated from the stools of 11, three had *Shigella flexneri*, three *Campylobacter* spp, and one each had *Giardia lamblia*, enteropathogenic *Escherichia coli*, *Shigella sonnei*, *Clostridium difficile*, rotavirus and adenovirus.

Stool samples from children with chronic inflammatory bowel disease (CIBD) and controls were collected from inpatients at St Bartholomew's Hospital, London, and those with acute diarrhoea from children attending Queen Elizabeth Hospital, Hackney, London.

Stools (10–100 g) were collected in sterile containers and weighed. They were then emulsified in an equal volume of phosphate-buffered saline and centrifuged at 20 000 × *g*. The supernatant fluid was collected and stored at −70°C. Samples were coded and measured blind by one of us (SS).

IL-6 was measured by an enzyme linked immunosorbent assay (ELISA). Stool supernatant fluids were added to the wells of a microtitre plate coated with monoclonal anti-IL-6 (2 μg/ml 5E1). The anti-IL-6 monoclonal antibody 5E1 reacted specifically with IL-6 and did not bind IL-1, TNFα, or β. The plates were washed with phosphate buffered saline and rabbit polyclonal anti-IL-6 (1 in 2000 in phosphate buffered saline/0·1% Tween 20) (polyclonal antibody and 5E1 cell line supplied by Wim Buurman, Department of Surgery, University of Limburg, Maastricht, the Netherlands) was added to the wells. After further incubation and washing, bound rabbit antibody was detected with horseradish-peroxidase-conjugated donkey anti-rabbit serum and tetramethylbenzidine substrate. Optical densities at 630 nm were read on an automated ELISA plate reader. Concentrations of IL-6 were measured by comparison with a recombinant IL-6 standard curve (British Biotechnology, Abingdon, Oxford).

TNFα was also measured by an ELISA method: the microtitre plate wells were coated with monclonal anti-TNFα (CB0006, 8 μg/ml). CB0006 reacts specifically with TNFα by ELISA and not with IL-1, IL-6, or TNF β. After incubation plates were washed and rabbit anti-TNFα (2 μg/ml, from Wim Buurman, Department of Surgery, University of Limburg) was added to the wells. Bound rabbit antibody was detected (as above) with horseradish-peroxidase conjugated donkey anti-rabbit serum. Optical densities were read at 450 nm on an automated ELISA plate reader, and TNFα concentrations in supernatant fluids were measured by comparison with a recombinant TNFα standard curve. Sets of samples for both assays were tested in duplicate.

In each batch of samples assayed several negative controls were run (no sample added to the well). To be considered positive, a test well had to have an optical density greater than 2·5 SD above the mean of these negative controls. The lowest amount of recombinant TNFα or IL-6 used to determine the standard curve was 16 pg/ml, and thus any values below that were determined by extrapolation. Assuming the standard curve was linear at low concentrations of cytokine, the limit of sensitivity of the assay based on 2·5 SD above the means of the negative wells varied from 2–15 pg/ml between different experiments.

The significance of differences among groups of patients was measured using the Mann-Whitney U test, and confidence intervals were calculated using the Wilcoxon method. Two aliquots (1 ml) of individual stool supernatant fluids were taken. One sample was heat inactivated at 80°C for 15 minutes and then either the TNFα or IL-6 concentration were measured in both the samples. The heat inactivated or non-heat inactivated samples were then spiked with either 2000 pg IL-6 or 600 pg TNFα. After, 2 hours of incubation at 37°C, the TNFα or IL-6 concentrations were again measured.

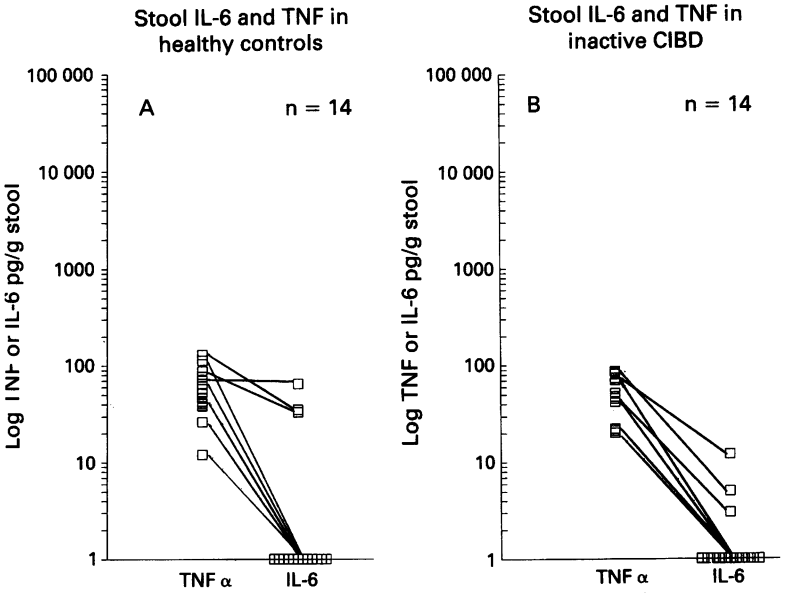

Figure 1A Low TNFα values were detected in all 14 control children; only three of 14 children had detectable IL-6 in their stools.

Figure 1B Similarly, low TNFα values were detected in stools of all 14 children with inactive disease; only three of 14 had IL-6 detectable in their stools.

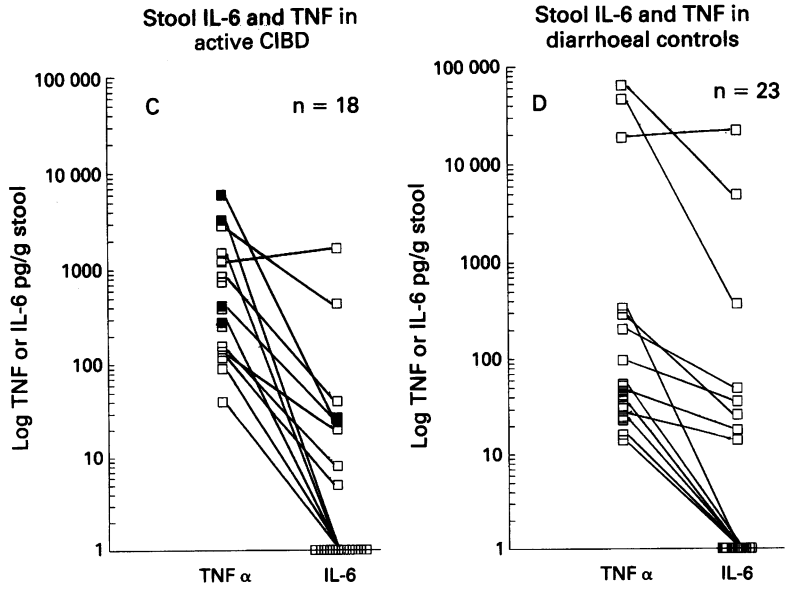

Figure 1C Children with active CIBD had noticeably raised stool TNFα, but stool IL-6 was undetectable in most samples.

Figure 1D There were two distinct groups in the diarrhoeal control children: in most stools TNFα values were detectable, but low, and IL-6 was only occasionally present. In contrast, three children with S flexneri infection had very high concentrations of both TNFα (18 640, 45 600 and 63 600 μg/g stool) and also extremely high IL-6 concentrations (366, 4850 and 21 920 pg/g stool).
A line joining two squares indicates that the results are from the same sample. Open boxes = Crohn's disease, closed boxes = ulcerative colitis.

Results

Low TNFα concentrations were detected in the stools of all 14 control children. Only two had values close to the lower limit of sensitivity of the assay: in the other 12 values ranged from 30–130 pg/g stool. In contrast, only three of the 14 control children had detectable stool IL-6 (fig 1A).

Survival of cytokines in stool samples

	Cytokine (pg/ml)	
	TNFα n = 8	*IL-6 n = 12*
Amount in stool	45·2 (13·5–833·5)	23·3 (16·5–37·6)
After heat inactivation	7·8 (5·0–17·0)	5·5 (3·0–12·5)
No heat inactivation + spike	1003 (832–1598)	47·8 (15·2–447)
Heat inactivation + spike	893 (833–932)	2032 (1846–2127)

Samples spiked with either 600 pg TNFα or 2000 pg IL-6. Data shown as median and 95% confidence limits.

Similar results were seen in children with inactive IBD, with only two of 14 giving values of TNFα close to the lower limit of sensitivity; most of the samples were negative for IL-6 (fig 1B). Children with active IBD had strongly raised values for stool TNFα (fig 1C). Despite this, however, stool IL-6 was undetectable in most samples (even when high concentrations of TNFα were present). The diarrhoeal control children (fig 1D) fell clearly into two groups. In most stool samples TNFα was detectable but low, and IL-6 was only occasionally present. By contrast, three children with *S flexneri* infection had extremely high TNFα concentrations (18 640—63 600 pg/g stool) and also extremely high concentrations of IL-6 (21 920, 366 and 4 850 pg/g stool).

There was no significant difference in IL-6 values among any of the patient groups. TNFα values were raised, however, in patients with active inflammatory bowel disease ($p < 0·0002$; Mann-Whitney U test).

We considered that our failure to detect IL-6 in samples containing high TNFα concentrations could have been attributed to degradation of IL-6 in the stools. When TNFα was spiked into eight stool supernatant fluids, we were able to identify immunoreactive TNFα in both heat inactivated and non-heat-inactivated stool supernatant fluids, the latter giving higher concentrations than the former because of endogenous TNFα (table). In contrast, when IL-6 was added to 12 different samples of non-heat-inactivated stool supernatant fluids, virtually none was recoverable after two hours. If the sample was first heat-inactivated, however, all the immunoreactive IL-6 could be recovered.

Discussion

These results confirm and extend our previous studies on stool TNFα and now show that stool IL-6 is not a reliable marker of intestinal inflammation in childhood inflammatory bowel disease. Many individual stool samples, which contained high concentrations of TNFα, had undetectable IL-6. IL-6 is produced by many cell types, particularly activated T cells, B cells, monocytes and macrophages,[10 11] and it was expected that high concentrations of IL-6 would be present in stools in inflammatory bowel disease because there is a large inflammatory infiltrate into diseased mucosa. Epithelial cells of normal colonic, small intestinal, and gastric mucosa also contain immunoreactive IL-6 and IL-6 mRNA.[12] When we spiked samples of stool supernatant fluids with IL-6, however, virtually none was recoverable two hours later. The ability of stool supernatant fluids to eliminate IL-6 immunoreactivity was heat-labile, indicating that it may be due to enzymatic degradation, but this awaits further studies and confirmation. Interestingly, TNFα seems to be resistant to this effect.

Because IL-6 concentrations were not consistently raised in active inflammatory bowel disease, these studies shed no light on the puzzling observation that high IL-6 concentrations have been found in the serum samples of patients with Crohn's disease but not ulcerative colitis. Serum concentrations, however, may bear no relation to local production because Stevens *et al* recently used the polymerase chain reaction to show that raised IL-6 mRNA values are present in mucosal biopsy specimens from patients with active inflammatory bowel disease but not in specimens from normal or other patients with non-inflammatory bowel disease.[1] A more likely explanation for the raised IL-6 values in Crohn's disease is that because of the transmural nature of the disease, there is more extensive inflammation than in ulcerative colitis, where the disease is confined to the mucosa.

One of the most interesting observations in this study was that both TNFα and IL-6 were greatly increased in the stools of children with *S flexneri* diarrhoea. This confirms a recent observation.[13] Shigellosis is a relatively rare disorder in our patient population, but it is of some interest that one patient with *S sonnei* did not have raised stool cytokine concentrations. Further study is needed to confirm and extend this observation. Large numbers of stool neutrophils are present in shigellosis. As it has been shown that these contain TNFα,[14] this is the likely source of the high concentrations of this cytokine in these particular patients. Presumably, in shigellosis, there is such an excess of IL-6 that the inhibitors of IL-6 in stool can not degrade it all. The source of stool IL-6 in patients with shigellosis is unclear because neutrophils do not seem to contain this cytokine.

A final interesting point from this study is the detection of immunoreactive TNFα in the stools of most of the healthy control children. This TNFα immunoreactivity was far in excess of the lower limit of detection of the assay and was heat labile (some of the samples used in the table 1 were from healthy children). We recently documented, by immunohistology, cells containing TNFα in the subepithelial lamina propria of colonic biopsy specimens from children with no detectable gut disease.[15] This may reflect local production of TNFα by subepithelial macrophages in response to the products of the normal flora leaking at low concentrations across normal epithelium.

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