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). These cytokines may exert local cytopathic effects, or may serve to upregulate endothelial adhesion molecules, thereby promoting the extravasation of neutrophils and monocytes into the gut mucosa.

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, and there is an increased incidence of TNFα secreting cells in the mucosa in inflammatory bowel disease. Significantly high concentrations of TNFα are also present in the stools of children with active inflammatory bowel disease; these fall to normal after treatment. IL-1 values are also noticeably raised in the mucosa in active inflammatory bowel disease. As yet there are fewer data on IL-6. Plasma IL-6 concentrations are raised in active Crohn’s disease, but surprisingly, not in active ulcerative colitis.

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 lambia*, enteropathogenic *Escherichia coli*, *Shigella sonnet*, *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%

Figures 1A, 1B, 1C, and 1D:

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

**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 pg/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).
Cytokines in stools of children with IBD or infective diarrhoea

Survival of cytokines in stool samples

<table>
<thead>
<tr>
<th>Cytokine (pg/ml)</th>
<th>TNFa n = 8</th>
<th>IL-6 n = 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount in stool</td>
<td></td>
<td></td>
</tr>
<tr>
<td>45-2 (13-5-833-5)</td>
<td>23-3 (16-5-37-6)</td>
<td></td>
</tr>
<tr>
<td>After heat inactivation</td>
<td>7-8 (5-0-17-0)</td>
<td>5-3 (3-0-12-5)</td>
</tr>
<tr>
<td>No heat inactivation + spike</td>
<td>1003 (832-1598)</td>
<td>47-8 (15-2-447)</td>
</tr>
<tr>
<td>Heat inactivation + spike</td>
<td>893 (833-932)</td>
<td>2032 (1846-2127)</td>
</tr>
</tbody>
</table>

Samples spiked with either 600 pg TNFa 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 TNFa 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 TNFa (fig 1C). Despite this, however, stool IL-6 was undetectable in most samples (even when high concentrations of TNFa were present). The diarrhoeal control children (fig 1D) fell clearly into two groups. In most stool samples TNFa was detectable but low, and IL-6 was only occasionally present. By contrast, three children with S flexneri infection had extremely high TNFa 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. TNFa values were raised, however, in patients with active inflammatory bowel disease (p < 0.002; Mann-Whitney U test).

We considered that our failure to detect IL-6 in samples containing high TNFa concentrations could have been attributed to degradation of IL-6 in the stools. When TNFa was spiked into eight stool supernatant fluids, we were able to identify immunoreactive TNFa in both heat inactivated and non-heat-inactivated stool supernatant fluids, the latter giving higher concentrations than the former because of endogenous TNFa (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 TNFa 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 TNFa, had undetectable IL-6. IL-6 is produced by many cell types, particularly activated T cells, B cells, monocytes and macrophages, 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. 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, TNFa 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. 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 TNFa and IL-6 were greatly increased in the stools of children with S flexneri diarrhoea. This confirms a recent observation that Shigelllosis 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 shigelllosis. As it has been shown that these contain TNFa, this is the likely source of the high concentrations of this cytokine in these particular patients. Presumably, in shigelllosis, there is such an excess of IL-6 that the inhibitors of IL-6 in stool cannot 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 TNFa in the stools of most of the healthy control children. This TNFa 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 TNFa in the subepithelial lamina propria of colonic biopsy specimens from children with no detectable gut disease. This may reflect local production of TNFa by subepithelial macrophages in response to the products of the normal flora leaking at low concentrations across normal epithelium.


