

# Concentrations of interleukin 6 and tumour necrosis factor in serum and stools of children with *Shigella dysenteriae* 1 infection

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## Abstract

Serum interleukin 6 (IL-6) and tumour necrosis factor (TNF) were measured in children with dysentery during an epidemic caused by *Shigella dysenteriae* 1. IL-6 and TNF were also measured in fresh stool filtrates from children with acute gastroenteritis. The median serum IL-6 concentration was raised significantly in the children with complications (haemolytic uraemic syndrome, leukemoid reaction, thrombocytopenia, thrombocytosis, and severe colitis lasting more than one week) during the first week (n=18, 9-7728 pg/ml; median 107) and in the second week (n=13, 5-312 pg/ml; median 77), compared with convalescent sera (n=10, <3-85 pg/ml; median 39; p<0.02 and <0.05 respectively). The median IL-6 concentration during the first week was significantly higher in the group with complicated disease than in those with no complications (n=8, <3-37 pg/ml; median 5; p<0.001). Although serum TNF concentrations were significantly raised in the complicated group during the first and second weeks of the illness and in the uncomplicated group compared with convalescence, there was no significant difference in the TNF concentrations between the complicated and uncomplicated groups. IL-6 was detectable in stool filtrates from eight of 13 children with *S dysenteriae* 1 infection and four of eight children with *S flexneri* infection. It was not detectable in Cryptosporidia, rotavirus, or adenovirus infections, those with pathogen-negative acute diarrhoea or controls. Seven of 13 children with *S dysenteriae* 1 and three of nine children with *S flexneri* infections had TNF detectable in stools. Two of four children with adenovirus infection also had TNF detected in stools. None of the children with Salmonella, Cryptosporidia, rotavirus or children with pathogen-negative diarrhoea and controls had detectable TNF in stool filtrates. It is postulated that the local and generalised vasculitis observed in shigellosis may be related to a direct effect of Shiga toxin on endothelial cells or caused by cytokine production stimulated by endotoxin, or both.

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Most children with dehydration caused by watery diarrhoea can be effectively treated with oral rehydration therapy. By contrast mortality from bacterial dysentery still occurs primarily in patients with *Shigella dysenteriae* 1 infections. The problem of dehydration is eclipsed by the systemic effects of the infection that include

haemolytic uraemic syndrome.<sup>1</sup> In Galle, Sri Lanka, 5-10% of children admitted with bacterial dysentery during epidemics develop haemolytic uraemic syndrome (unpublished observations). Rahaman *et al*<sup>2</sup> first reported the association between *S dysenteriae* 1 infection and haemolytic uraemic syndrome. A potential common factor between haemolytic uraemic syndrome associated with infections caused by *S dysenteriae* 1 and certain strains of *Escherichia coli* (most notably *E coli* serotype O157:H7)<sup>3</sup> is the production of a cytotoxic enterotoxin (Shiga toxin and Verotoxin respectively). Shiga toxin and Verotoxin (VT) share biological effects that may be related to their ability to inhibit protein synthesis.<sup>4,5</sup>

The release of endotoxin during shigellosis could also damage the capillary endothelium, either as part of a generalised Schwartzman reaction<sup>6</sup> or as a result of release of cytokines such as tumour necrosis factor (TNF)<sup>7</sup> or interleukin 6 (IL-6).<sup>8</sup> We therefore measured IL-6 and TNF concentrations in serum and stool from children with bacterial dysentery to determine whether there was a correlation between these measurements and the severity of the disease and appearance of complications, especially haemolytic uraemic syndrome.

## Methods

### PATIENTS

Forty children with clinical dysentery were admitted to the University Paediatric Unit, Teaching Hospital, Karapitiya, Galle, Sri Lanka, during an epidemic of bacillary dysentery caused by *S dysenteriae* 1. Blood samples were taken from the children when they were bled for routine investigations, centrifuged within 30 minutes, and the serum was frozen and stored at -70°. The diagnosis of *S dysenteriae* 1 was established by positive stool cultures or by significantly positive concentrations of IgM antibodies to the endotoxin of *S dysenteriae* 1, measured by ELISA and confirmed by immunoblotting, by methods similar to those used for serological diagnosis of *E coli* O157:H7 infection.<sup>9</sup> Children were divided into a 'complicated' group (aged 7-132 months; median 48) if adverse prognostic features were present including haemolytic uraemic syndrome (n=4) microangiopathic haemolytic anaemia (n=1), leukemoid reactions (neutrophil count >20×10<sup>9</sup>/l; n=5), thrombocytopenia (platelet count <100×10<sup>9</sup>/l; n=1), or thrombocytosis (platelet count >400×10<sup>9</sup>/l; n=2) and severe colitis

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associated with diarrhoea lasting for more than 7 days ( $n=18$ ).<sup>2,10</sup> Haemolytic uraemic syndrome was diagnosed if the blood urea was  $>25$  mmol/l, platelet count  $100 \times 10^9/l$ , and there was evidence of microangiopathic anaemia on blood film. Children with diarrhoea lasting less than 7 days and without any apparent complications, were included in the 'uncomplicated' group ( $n=9$ ; age 24–84 months; median 60). Blood samples were also taken from 12 'convalescent' patients (age 13–132 months; median 54) from the complicated group before discharge from hospital, 1 to 2 days after the diarrhoea had stopped.

Fresh stools from 49 children with acute gastroenteritis were examined by standard methods in the Department of Microbiology University of Colombo, Sri Lanka for *Salmonella*, *Shigella*, *Aeromonas*, *Vibrio cholerae*, enterotoxigenic *E coli* (ETEC; LT and ST), enteropathogenic *E coli* (EPEC), *Campylobacter*, rotavirus (ELISA) adenovirus (ELISA), and *Cryptosporidia*. A measured aliquot of stool was diluted in PBS, filtered through a micropore filter, and frozen at  $-70^\circ\text{C}$ . Control stools from children without diarrhoea were processed identically. The serum and stool filtrate samples were transported to the UK in dry ice.

#### CYTOKINE ASSAYS

Serum and stool IL-6 and stool TNF concentrations were measured using commercial quantitative 'sandwich' enzyme immunoassays (Quantikine, R&D Systems Ltd, MI, USA). TNF concentrations were estimated in serum using the L929 bioassay.<sup>11</sup>

#### STATISTICAL METHODS

Analyses were performed by the Mann-Whitney U test.

Ethical permission was obtained from the Ethical Committees of the Faculty of Medicine, University of Ruhuna, Galle, Sri Lanka and King's College School of Medicine and Dentistry, London, UK.

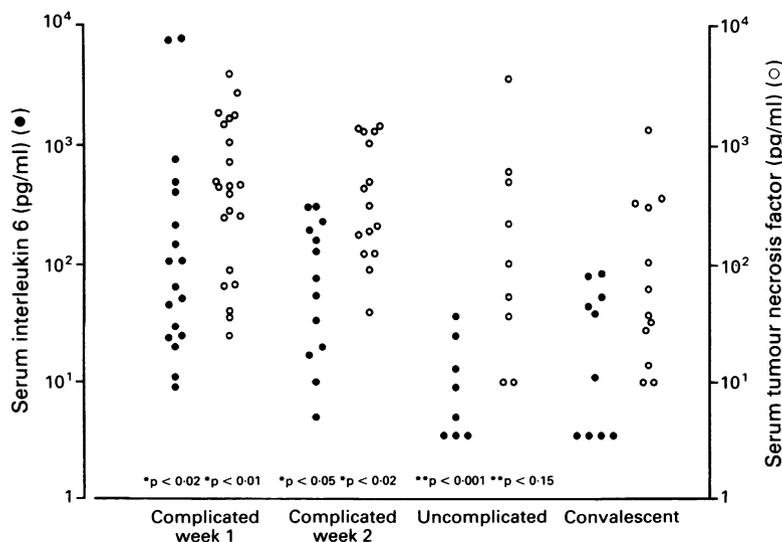


Figure 1: Serum concentrations of interleukin 6 (IL-6) and TNF in children with bacterial dysentery. \* = Complicated v convalescence; \*\* = complicated week 1 v uncomplicated.

## Results

#### SERUM IL-6 AND TNF

Blood samples for IL-6 and TNF measurements were taken at times of venepuncture for clinical indications, and sample volumes were limited. Patients were not studied after discharge from hospital and hence complete data on IL-6 and TNF concentrations were not available throughout the illness. The median serum IL-6 concentration was raised significantly in the children with a complicated course during the first week ( $n=18$ , 11–7728 pg/ml; median 86) and in the second week ( $n=13$ , 5–312 pg/ml; median 77), compared with convalescent sera ( $n=10$ ,  $<13$ –85 pg/ml; median 39;  $p < 0.02$  and  $< 0.05$  respectively; Figs 1–3). The median IL-6 concentration during the first week was significantly higher in the complicated compared with the uncomplicated group ( $n=8$ ,  $<3$ –37 pg/ml; median 5;  $p < 0.001$ ). Serum TNF concentrations were significantly raised in the complicated group during the first week ( $n=22$ , 25–3942 pg/ml; median 451 pg/ml) and in the second week of the illness ( $n=15$ , 40–1462 pg/ml; median 317 pg/ml) when compared with convalescence ( $n=12$ ,  $<10$ –1368 pg/ml; median 63 pg/ml;  $p < 0.01$  and  $< 0.02$  respectively). The median serum TNF concentration was also raised in the uncomplicated group ( $n=9$ ,  $<10$ –3613 pg/ml; median 103 pg/ml), but there was no statistically significant difference in the TNF concentrations between the complicated and uncomplicated groups ( $p < 0.15$ ). One patient whose serum concentration of TNF was very high (1368 pg/ml) during convalescence had had disseminated intravascular coagulation during the acute phase.

#### STOOL IL-6 AND TNF

IL-6 was measured in stool filtrates from 48 children and was detectable in filtrates from eight of 13 children with *S dysenteriae* 1 infection (20–8044 pg/ml), and four of eight children with *S flexneri* (12–68 pg/ml) infection. IL-6 was also detected in the one patient with *S sonnei* infection (24 pg/ml) and was just detectable (6 pg/ml) in one patient with *Salmonella* infection. It was not detectable in *Cryptosporidia* ( $n=5$ ), rotavirus ( $n=4$ ), or adenovirus ( $n=4$ ) infections, those with acute diarrhoea in whom no pathogen was detected ( $n=4$ ), or healthy control children (Fig 4).

Seven of 13 children with *S dysenteriae* 1 and three of nine children with *S flexneri* infections had TNF detectable in stools (12–2545 and 25–358 pg/ml respectively; Fig 5). Two of four children with adenovirus infection also had TNF detected in stools (10 and 109 pg/ml). In contrast none of the children with *S sonnei*, ( $n=1$ ) *Salmonella* ( $n=5$ ), *Cryptosporidia* ( $n=5$ ), rotavirus ( $n=4$ ), or children with pathogen-negative acute diarrhoea ( $n=4$ ) and healthy controls ( $n=4$ ) had detectable TNF in stool filtrates.

## Discussion

Severe shigellosis may be complicated by disseminated intravascular coagulation, haemolytic uraemic syndrome, and occasionally gangrene

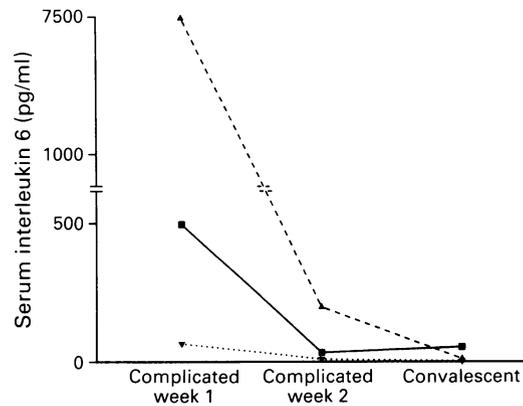


Figure 2: Serum interleukin 6 concentrations in children with complicated shigellosis in whom serum samples were available during the first and second weeks of the illness and convalescence. Each symbol represents a single patient.

and perforation of the large bowel.<sup>10</sup> Endotoxaemia,<sup>12</sup> raised fibrin degradation products,<sup>13</sup> and deposition of fibrin in glomeruli<sup>14</sup> and in rectal microvasculature<sup>15</sup> have been reported in patients with uncomplicated shigellosis. Infusion of TNF or endotoxin in rabbits produced similar pathology including disseminated intravascular coagulation with thrombocytopenia, damage to glomeruli with leukocyte infiltration, segmental ischaemia, haemorrhage, and necrosis in the liver, bowel, adrenals, pancreas, lung, and other tissues.<sup>7</sup> Microscopy showed fibrin deposits, polymorphonuclear infiltration, and arterial thromboses.<sup>7, 16, 18</sup> Similarities between the pathology of shigellosis and endotoxin or TNF infusions prompted us to investigate cytokine concentrations in sera and stool extracts from children with shigellosis.

Serum IL-6 and TNF concentrations were significantly raised during the acute phase of *S dysenteriae* 1 infection in children with a complicated course compared with convalescence, and IL-6 concentrations correlated with the presence of complications such as haemolytic uraemic syndrome, microangiopathic haemolytic anaemia, leukemoid reactions, thrombocytopenia or thrombocytosis, and severe colitis associated with persistent diarrhoea. IL-6 has been shown previously to be a better indicator of

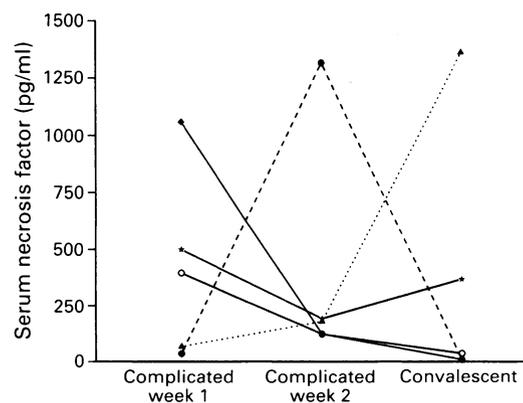


Figure 3: Serum tumour necrosis factor concentrations in children with complicated shigellosis in whom serum samples were available during the first and second weeks of the illness and convalescence. Each symbol represents a single patient.

disease severity in other septic states,<sup>19, 20</sup> although TNF release is essential for the initiation or amplification of IL-6 release.<sup>21</sup> IL-6 and IL-1 are probably important mediators of the pathological effects of TNF<sup>8</sup> including endothelial cell damage caused by increasing the adhesiveness of neutrophils and endothelial cells,<sup>22</sup> endothelial rearrangement,<sup>23</sup> production of a procoagulant factor by endothelial cells, reduced expression of thrombomodulin,<sup>24</sup> increased production of IL-1, which can in turn activate leukocytes to initiate coagulation,<sup>25</sup> and stimulation of endothelial cells, polymorphs, and macrophages to produce platelet activating factor.<sup>26</sup>

Although IL-6 concentrations discriminated between children with severe and mild dysentery, there was considerable variation in concentrations of IL-6 and TNF between members of the same patient groups and in individual patients studied longitudinally. By analogy, injection of endotoxin in rabbits produced a surge of TNF secretion lasting 4–5 hours and TNF secretion was refractory to a second injection of endotoxin.<sup>27</sup> These fluctuations may not be detected unless repeated estimations are made.<sup>28</sup> All children in the convalescent group had had a complicated course and had had blood taken on the first or second day of convalescence; this may explain the persistent noticeable increase in serum TNF in one patient.

The finding of IL-6 and TNF in some stool filtrates shows that the colonic mucosa was exposed to cytokines from both serosal and mucosal surfaces. The highest concentrations were found in stools from children infected with *S dysenteriae* 1, that is the most virulent species of Shigella. The IL-6 and TNF may have originated from blood loss or transudate from the bloodstream, but the absence in the stool of other invasive micro-organisms, and the presence of TNF in stools from children with watery diarrhoea caused by adenovirus raise the possibility of local production. Interestingly, adenovirus infection has been reported in association with haemolytic uraemic syndrome<sup>29, 30</sup> and other viruses can stimulate production of cytokines.<sup>31</sup> The intestinal mucosa is a potential source of local cytokine production, as small intestinal cells have been shown to produce IL-6,<sup>32</sup> and, in inflammatory bowel disease, production of IL-1 and TNF by colonic mucosae has been demonstrated.<sup>33</sup> Extensive infiltration by mononuclear cells including macrophages was seen in mucosal biopsy specimens of the large bowel in shigellosis.<sup>15</sup> These macrophages, stimulated by endotoxin released from invading shigellae represent a further potential source of local cytokine production.<sup>34</sup>

We postulate that the degree of local tissue destruction of the colonic mucosa in shigellosis is related to the amount of Shiga toxin produced,<sup>35</sup> which in turn exposes inflammatory cells, especially macrophages, to endotoxins. The local vasculitis observed in shigellosis,<sup>15, 36</sup> may be related to a direct effect of Shiga toxin on endothelial cells, as shown in *in vitro* studies of O'brig *et al*,<sup>4</sup> or caused by local cytokine production or both. TNF and IL-6 are also known to cause vasculitis.<sup>36</sup> The vasculitis in turn could

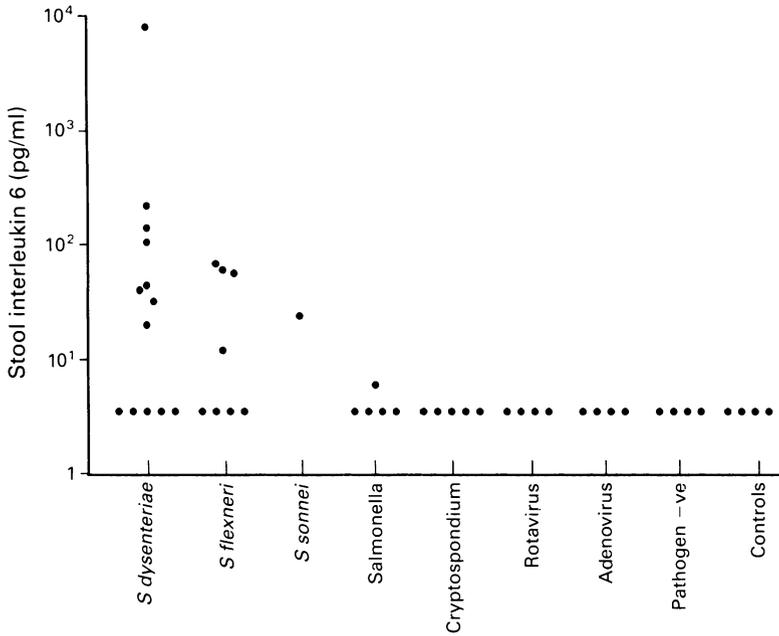


Figure 4: Stool concentrations of interleukin 6 in children with acute diarrhoea of various aetiologies.

produce further tissue necrosis, ulceration, and in severe cases, necrotising enteritis.

Sequestered TNF release is thought to be imported in the pathogenesis of other diseases including adult respiratory distress syndrome,<sup>37</sup> malaria,<sup>38</sup> meningitis,<sup>39</sup> and urinary tract infection.<sup>40</sup> A generalised release of cytokines in shigellosis could lead to disseminated intravascular coagulation and haemolytic uraemic syndrome that is associated with *S. dysenteriae 1* infection. Monoclonal antibodies to TNF are a successful adjunct in the treatment of septic shock.<sup>41</sup> Further studies on the role of Shiga toxin, TNF, and other cytokines in the complications of shigellosis are indicated since the early treatment of patients with poor prognostic signs, with anti-TNF monoclonal antibodies might

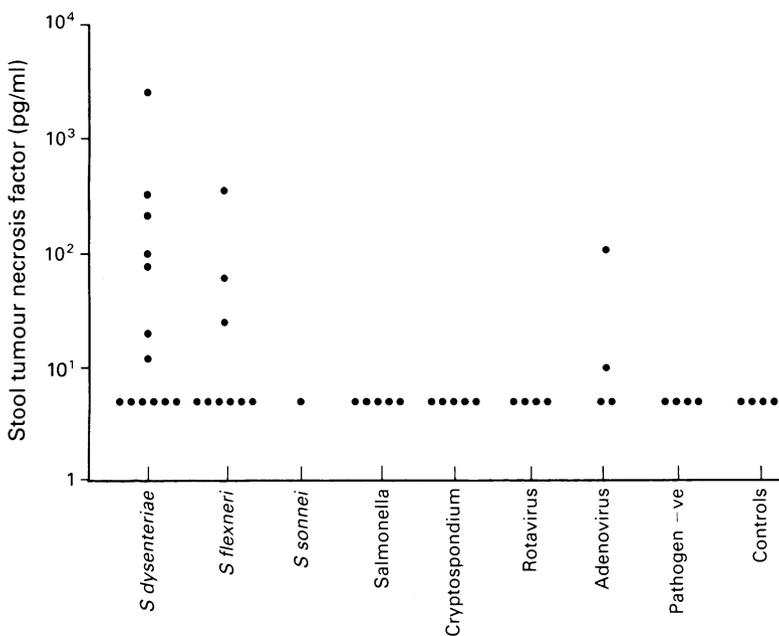


Figure 5: Stool concentrations of tumour necrosis factor in children with acute diarrhoea of various aetiologies.

prevent disseminated intravascular coagulation and haemolytic uraemic syndrome.

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