Cyril Fernando Oration

Cytokines in the pathogenesis of shigellosis

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Madam President, Prof Priyani Soysa, distinguished guests, members of the council of the Ceylon College of Physicians, members of the family of late Dr. Cyril Fernando, ladies and gentlemen. May I first of all thank you, Madam President for the kind words of introduction, and to you and the council, for the honour of giving me the opportunity to deliver this oration in memory of Dr. Cyril Fernando.

Dr. Cyril Fernando was born in Colombo in April 1900. He was educated at St. Benedict's College and University College Hospital, London. Here he was inspired by, Sir Thomas Lewis, Sir John Rose Bradford and Dr. Betty Shaw. He graduated in 1926, and the same year he passed the MRCP examination. He did extremely well in the MRCP examination that he was exempted from the final viva. He returned to Sri Lanka then Ceylon, and was appointed Medical Registrar to the General Hospital, Colombo. In 1929 he returned to London and took the M.D., and was awarded the Gold Medal as the best candidate of the year. He was then appointed as additional assistant pathologist. He later became the Physician to Out-patients, and in 1934 was appointed Physician to the General Hospital, Colombo, where he served till his death in 1955.

Dr. Cyril Fernando was an active member of the Ceylon Medical Association and before that of the Ceylon Branch of the British Medical Association, being successively Honorary Secretary and Treasurer, Vice-President and in 1948, President. In 1949 he was appointed an Officer of the Most Excellent Order of the British Empire and in 1955 he was made a Companion of the Most Distinguished Order of St. Michael and St. George.

Dr. Cyril Fernando was a physician of great repute. His ability as a clinician and diagnostician was highlighted by Professor Bruce Perry in his inaugural memorial oration in 1971. We are gathered here today to remember this outstanding physician and teacher.

Madam President, ladies and gentlemen, I have chosen to speak to you this evening on "Cytokines in the pathogenesis of shigellosis".

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Endemic and epidemic bacterial dysentery due primarily to *Shigella dysenteriae* 1 is an important health problem in less developed countries[1]. Sri Lanka experienced a major epidemic in 1976 and since then it is a major cause of morbidity and mortality[2]. Reports from the epidemiological[3] department show high morbidity rates during the initial epidemic, with a gradual fall in morbidity from 1979-1981. However, since 1985 there is a gradual increase in the morbidity rates. The mortality rate was extremely high during the initial epidemic. It is alarming to note a rising mortality from 1986. During a study carried out in the University Paediatric Unit in Galle, in 1989/90, systemic manifestations of Shigellosis was one of the major causes of mortality in the over 1 year population[4].

Effective treatment exists for the majority of children with watery diarrhoea who can be treated as outpatients with oral rehydration therapy. Mortality from bacterial dysentery continues, because the problem of dehydration is eclipsed by the systemic effects of the infection. The prevention and treatment of these manifestations is mainly symptomatic, since the aetiology is unknown. The major systemic complication being haemolytic uraemic syndrome (HUS)[1]; a combination of thrombocytopaenia, microangiopathic haemolytic anaemia, and acute renal failure[5]. The other systemic manifestations include, seizures, cholestasis, leukaemoid reactions, hypertension, shock and possibly adult respiratory distress syndrome and multi-organ failure. It is important to realize that there is a spectrum of manifestations, and mortality is not always due to HUS, but due to other systemic manifestations as well.

The ability of shigellae to invade colonic mucosal cells is critical to their pathogenesis, and the production of cytotoxic enterotoxin (shiga toxin) plays a synergistic role in determining the severity of disease[6].

The presently available literature point to two possible aetiological agents in the pathogenesis of these systemic manifestations. These being shigatoxin and endotoxin.

Shigatoxin is a complex molecule consisting one "A" subunit and 5 "B" subunits. Once it enters a cell it inhibits ribosomal protein synthesis leading to cell death[7].

Endotoxin or lipopolysaccharide (LPS) is a compo-

nent of the outer membrane of gram negative bacteria[8]. The outermost part consists of a series of oligosaccharides that are structurally and antigenically distinct in different organisms, and are responsible for the 0 serotype of gram negative bacteria. Internal to this are the core oligosaccharides, which are structurally similar in different organisms. Next to the core oligosaccharides is the Lipid-A which is responsible for most of the toxicity of endotoxins.

What evidence is there to link shigatoxin to the pathogenesis of Shigellosis?. Rahaman et al (1974)[1] reported the association between *S. dysenteriae* infection and HUS, while Karmali et al (1983)[9] reported the association between 'epidemic' diarrhoea associated HUS seen in developed countries, and *E.coli* 0157: H7 infection. *E.coli*0157 produce verotoxin which was subsequently shown to be similar in structure to shigatoxin by O'Brien et al[10], suggesting it to be an important link in the pathogenesis of HUS. In-vitro toxicity of shiga toxin to human umbilical vein endothelial cells[11], and the characteristic vascular changes of capillary microangiopathy reproduced in rabbits given intravenous injections of verotoxin[12], point to the noxious effect of Shiga toxin on the endothelium. However, circulating shiga toxin has not been demonstrated in patients or in experimental animal models.

The possibility of endotoxins as a causative agent was first highlighted by Koster et al (1978)[13], who demonstrated endotoxaemia preceding the onset of HUS. They also demonstrated circulating immune complexes to endotoxins after the onset of haemolysis. Butler et al (1985) injected intravenous endotoxins of Shigellae to rabbits, and reproduced features of HUS[14]. These observations were attributed to a generalized Shwartzman reaction. It is now known that the administration of LPS to animals and man, intravenously, stimulates secretion of cytokines including tumour necrosis factor (TNF), to reproduce features of disseminated intravascular coagulation (DIC), and impaired renal function[15,16,17]. Infusions of TNF alone produce similar changes,[18,19,20] indicating it to be an important mediator responsible for these changes.

Cytokines such as TNF and interleukins are, soluble mediators which communicate between cells especially of the immune system. They are extremely potent, being effective at picomolar concentrations. Cytokines are produced by a variety of cells especially mononuclear and endothelial cells, and are essential for the normal host reaction to infection. They are released as a cascade and act as network. These cytokines have synergistic, additive or antagonistic effects. However, when cytokines are produced in excessive amounts, it has deleterious effects on cells and organs.

How do endotoxins cause septic shock and D.I.C.?

Endotoxaemia in patients stimulate macrophages and also other cells like endothelial cells to produce cytokines. Endotoxins could also activate other pathways like complement. These factors either acting directly or through polymorphs and platelets cause endothelial cell damage leading to a consumptive coagulopathy.

Recent work suggests that the deleterious effects associated with TNF in septic shock is not caused by TNF alone but by TNF in association with other factors such as interleukin-1 (IL-1) and IL-6[21,22]. The clinical features and pathological changes seen after administration of endotoxins or TNF are similar to some of the systemic manifestations observed in Shigellosis. Since endotoxaemia is known to influence the prognosis in shigellosis, it is likely that TNF and IL-6 released during endotoxaemia, play a role in the pathogenesis of the systemic complications in Shigellosis. Elevated levels of cytokines have not been demonstrated previously in shigellosis.

Serum cytokines

Blood was taken from children admitted to the University Paediatric Unit, Teaching Hospital, Galle, with dysentery, during an epidemic of bacterial dysentery in 1989. The blood was centrifuged within 1/2 hour, and plasma frozen and stored at — 70°C. The samples were transported to the U.K. in dry ice. Ethical permission was obtained from the Ethical Committees of the Faculty of Medicine, University of Ruhuna, and King's College Hospital, London.

TNF levels were estimated using the L929 mouse fibroblast bioassay[23]. Briefly, The L 929 cells were seeded in 96 well tissue culture plates, for 24 hours. The following day, the samples and standards were added. The plates were then incubated for a further 24 hours in the presence of Actinomycin D. The following day the cells were fixed in 5% formaldehyde, and stained with 2% crystal violet. The stain was then eluted using 33% acetic acid. The colour change was then measured using an ELISA reader. The principle of the test is based on the specific sensitivity of the L 929 cells to the cytotoxic effects of TNF. Samples which contain more TNF will cause more cytotoxicity. Therefore the number of cells remaining, as measured by the optical density in the well, will inversely correlate to the amount of TNF present. A standard curve is plotted using the optical densities measured in the wells with standards of TNF in serial dilution. The TNF levels in the test wells is measured by extrapolating the optical density measured. This assay was initially standardized. The use of a higher concentration of cells in wells gave a better standard curve, but was less sensitive to measure

lower concentrations of TNF. The recommended time to measure the optical density was immediately after the addition of acetic acid. However, it was found that by allowing 1 hour for the dye to elute gave the best standard curve. 10% foetal calf serum (FCS) in the incubating medium gave a better standard curve compared to 5% FCS.

Interleukin-6 levels were estimated using a commercial immunoassay. The plates already come coated with monoclonal antibody to IL-6. The samples and standards were added in duplicate and incubated at 37°AC for 2 hours. The polyclonal antibody conjugate was then added and incubated for a further 2 hours. The substrate was subsequently added and the colour reaction stopped after 20 minutes with 2N sulphuric acid. Along with the samples to be tested, a series of wells were prepared using known concentrations of the IL6 standards. A curve, plotting the optical density versus the concentrations of IL6 in these standard wells, was prepared. By comparing the optical density of the test samples to this standard curve, the concentrations of IL6 in the unknown samples were determined.

The diagnosis of *S. dysenteriae 1* was established by positive stool cultures and/or by significantly positive IgM antibodies to the LPS (endotoxin) of *S. dysenteriae 1*. It was measured by an ELISA method and confirmed by immunoblotting[24] by previously described methods similar to those used for serological diagnosis of E.coli 0157:H7 infection[25].

Blood was taken from patients admitted at various stages of the disease, and therefore serial samples were not taken from all patients. Patients were allocated into 3 groups depending on the clinical features. Uraemia, microangiopathy, thrombocytopenia or thrombocytosis, leukaemoid reactions, and severe colitis associated with prolonged diarrhoea are features associated with a poor prognosis[1,2], and were included in the 'complicated' group, while children with diarrhoea lasting less than seven days without any apparent systemic complications, were included in the uncomplicated group. In both groups 1 & 2, the blood was taken during the acute phase of the illness. Blood samples taken before discharge from hospital, 1-2 days after the diarrhoea had subsided, were included in the convalescent group.

Statistical analysis was done by the Mann-Whitney test using the 'Minitab' statistical micro computer program.

Results

Serum cytokines

Serum IL-6 levels were raised in patients with a complicated course during the first week (range 11-7700 pg/ml). IL-6 was also elevated in the second week (range 5-300 pg/ml). The levels of IL-6 during the acute phase of the illness was significantly raised compared to convalescent sera (range, 3-85 pg/ml). [significant at 0.0165 and 0.0377 respectively]. The IL-6 levels in the uncomplicated group ranged, 3-37 pg/ml. The levels of IL-6 during the acute stage between the complicated, and uncomplicated groups were also significantly different [significant at .001.]

Serum TNF levels were significantly elevated during the acute phase, in the complicated group during the first week (range, 25-3900 pg/ml). The TNF levels of convalescent sera ranged, 10-1300 pg/ml, and this was significantly lower than the acute levels. [significant at 0.0065]. The serum TNF levels in the acute phase was also raised in the uncomplicated group (range, 10-3600 pg/ml). There was no statistically significant difference in the TNF levels between the complicated and uncomplicated groups.

Another interesting finding was the LPS antibody levels correlated to the severity of disease. The antibody levels in the complicated group was significantly higher than in the uncomplicated group, indicating that the immune system was more exposed to the endotoxins in the complicated cases.

The results of this study show that IL-6 and TNF levels were significantly higher during the acute phase of *S. dysenteriae* 1 infection compared to convalescence. The IL-6 levels correlated to the severity of disease.

It is interesting to note that some of the samples taken from patients with a complicated course had normal TNF levels. Animals given intravenous LPS show a surge of TNF secretion, with a rapid fall within 2-3 hours due to it's short half life[17], compared IL-6 which has a relatively longer half life. Since there is a latent period between the pathological changes and peak TNF secretion, at the time of systemic clinical manifestations the TNF may be normal[26].

In-vitro studies have shown TNF to cause endothelial cell damage by increasing the adhesion of neutrophils to endothelial cells, and by altering the adhesiveness of the endothelial cells[30]. The other effects on the endothelial cells include, endothelial rearrangement,[31] production of a procoagulant factor by endothelial cells, reduced expression of thrombomodulin[32], and increased production of interleukin 1, which can in turn activate leucocytes to initiate coagulation[33]. It also stimulates endothelial cells, polymorphs, and macrophages to produce platelet activating factor[34].

IL-6 has been shown to be a better indicator of the severity of disease in other septic states[21,22]. Although TNF release is essential for the initiation or amplification

of interleukin-6 release[35], IL-6 is likely to be an important mediator of the pathological effects of TNF[36].

Damage to glomeruli by infusing TNF to rabbits has been demonstrated[37]. Microscopically it is associated with fibrin deposits, polymorphonuclear infiltration, thrombosis of the arteries and necrosis. Macroscopically, areas of segmental ischaemia, haemorrhage, and necrosis in the liver, bowel, adrenals, pancreas, lung and other tissues have been demonstrated[15,18,20,37]. Thrombocytopenia following TNF administration is probably secondary to disseminated intravascular coagulation. Similar pathological changes are also seen in shigellosis.

Barret et al[38], recently showed that bacterial endotoxins enhanced the toxic effects of shiga like toxin. It is likely that the toxic effects observed by them were partly due to cytokines, and the possibility of synergism between shiga toxin, cytokines and endotoxins should be considered.

The successful treatment of septic shock with monoclonal antibodies to TNF is encouraging[39]. Prediction of complications, before it's onset is possible in many patients with Shigellosis[2,40]. Further studies on the role of LPS, TNF and IL-6 in the complications of Shigellosis are indicated, since the early treatment of patients with poor prognostic signs, with anti TNF monoclonal antibodies or antibodies to core polysaccharide of LPS may prevent systemic manifestations.

Stools cytokines

Shigellosis, especially S. dysenteriae infection is characterized by lower abdominal colics associated with tenesmus and straining, and the passage of blood and mucous in stools. The rectal mucosa may be oedematous, ulcerated, and necrotic[2,41]. In the more severe infections pseudo-polyps and pus with sloughed out mucosal shreds may be passed in the stools. Occasionally it may be associated with gangrene and perforation of the large bowel[2,41]. These clinical features suggest the local tissue damage to be due to a vasculitis.

To look at the possibility of local cytokine production, we looked at TNF and IL-6 levels in stools of patients with infective diarrhoea. Fresh stools from patients with acute gastroenteritis sent for routine microbiological examination to the Department of Microbiology University of Colombo were used. The stools were examined for evidence of *Salmonella, Shigella, Aeromonas, Vibrio cholera, enterotoxigenic Escherichia coli* (for both LT and ST producing strains), enteropathogenic *Escherichia coli, Campylobacter,* rotavirus, adenovirus, and *Cryptosporidium*. A measured volume of stool was diluted in phosphate buffered saline (PBS), filtered through a micropore filter, and frozen at — 70°C. The specimens were transported to the UK in dry ice. TNF and IL-6 was measured using a commercial quantitative ELISA method.

Results

Stools cytokines

7/13 patients with *S. dysenteriae* infection had detectable TNF in stools (range-12-2500 pg/ml). 3/9 patients with *S. flexneri infection had TNF in stools (range-25-350 pg/ml). In contrast, none of the patients with S. sonnei,* Salmonella, Cryptosporidium, rotavirus, non specific diarrhoea, or healthy controls had detectable TNF in stools. However, 2 out of 4 patients with adenovirus infection also had small quantities of TNF in stools.

IL-6 was detected in 8/13 patients with *S. dysenteriae*-1 infection (range-20-8000 pg/ml), and in 4/8 patients with *S. flexneri* (range-12-68 pg/ml). IL-6 was also detected in the only patient with *S. sonnei* infection (24 pg/ml) and was weakly positive (6pg/ml) in one patient with Salmonella infection. It was not detectable in Cryptosporidium (n=5), rotavirus (n=4), adenovirus (n=4), non diarrhoeal controls (n=4), and in diarrhoeal patients in whom no known pathogens were isolated.

Mathan and Mathan (1986)[29], in electron microscopic studies, demonstrated endothelial cell damage, fibrin deposits, and thrombosis within vessels in the lamina propria of the large bowel of patients with uncomplicated shigellosis. Their study strongly supports vasculitis as a cause of tissue damage and necrosis shigellosis.

Fontaine et al (1988)[42], demonstrated that infection of monkeys by species of *Shigella dysenteriae*-1, capable of both producing shiga toxin and invading cells, caused blood in stools, a sharp drop in polymorphs followed by increased myelocytes on day 3, destruction of capillary vessels within the connective tissue of the colonic mucosa, and death on day 4. Organisms not capable of producing shiga toxin, but capable of invasion, also caused death on day 4, indicating that shiga toxin is not essential in causing lethality. However, these animals did not have any blood in stools, and there was no purulent necrosis of the mucosa suggestive of a vasculitis. These workers postulated a local release of shiga toxin by invading organisms within the tissues, enhancing the severity of the mucosal lesions, due to alteration of blood flow to the mucosa.

The results of our study suggests a local release of TNF & IL-6 in shigellosis, and more so in *S. dysenteriae* infection. TNF and IL-6 are known to mediate effects of vasculitis[43], and the observations of a sharp drop in polymorphs followed by increased myelocytes by Fontaine et al is similar to effects produced by TNF[18,44]. It is likely that the local vasculitis is mediated by locally produced TNF and other cytokines.

Evidence of local cytokine production in the colon in shigellosis, leads to the postulate that, invasion of the colonic mucosal cells by the shigellae stimulate an inflammatory cell response including macrophages and other mononuclear cells. These cells when exposed to LPS (endotoxins) of shigellae would be stimulated to produce cytokines. The cytokines thus produced probably acts in synergism with shiga toxin leading to local endothelial cell damage and vasculitis. The vasculitis in turn could produce further tissue necrosis, ulceration, and even gangrene in the more severe cases. It is likely that the extent of tissue destruction would depend on the degree of cytokine production and also the amount of shigatoxin produced by the invading shigellae. Extensive infiltration by mononuclear cells including macrophages seen in mucosal biopsies of the large bowel[29] in shigellosis also supports this hypothesis, since macrophages are a major source of cytokines[45].

It is interesting not to observe an increase in cytokines in the stools of patients with Salmonellosis which is also an invasive organism. This may be due to the fact that shigellae multiply rapidly in the epithelium, destroy the cells, and spread both intracellularly and extracellularly, leading to a marked local inflammatory cell response and mucosal abscess formation, while Salmonellae use the epithelium only as a route to gain access to the mesenteric lymph nodes[46].

Sequestered (or local) Cytokine release is thought to be important in the pathogenesis of other diseases including adult respiratory distress syndrome[47], malaria[48] and meningitis[49]. A recent study had demonstrated local production of IL-6 in the bladder mucosa in patients with urinary tract infection, which was thought to be the main effect in the pathogenesis[50]. Small intestinal cells have shown to be capable of producing IL-6[51]. Recent work has demonstrated colonic mucosal TNF production in inflammatory bowel disease[52].

The only relevance of the finding of TNF in stools of patients with adenovirus infection was, the reported cases of HUS in association with adenovirus infection by Ray et al[53], and Van Wieringen et al[54].

Ongoing preliminary studies by us have shown elevated levels of TNF and IL-6 in rectal biopsies of adults with bacillary dysentery.

In-Vitro Studies

Mononuclear cells produce cytokines in response to endotoxins and other agents. Therefore *in-vitro* studies were done to compare the ability of *shigella* and *E.coli* endotoxins to release cytokines *in-vitro,* and whether shigatoxin is able to stimulate mononuclear cells to release cytokines.

The potency of shigatoxin available was not known and it was necessary to quantitate the shigatoxin in the preparation used. The available quantitative assays for shiga toxin include an ELISA method[56] and also an assay using Radio-isotope uptake by vero cells[57]. The ELISA method has a disadvantage, that it measures only the protein content but not bioactivity. The other method has the disadvantage that it uses radio-isotopes. Therefore it was necessary to develop a new quantitative bioassay for shigatoxin.

MTT a toluidine dye is converted in active mitochondria in live cells to a purple dye[58]. The amount of dye produced would correlate to the number of live cells present in cell cultures[58]. This principle was used to develop the new bio-assay for shigatoxin.

Vero cells were grown in 96 well tissue culture plates, for 48-72 hours, in 10% FCS. Tenfold serial dilutions of shigatoxin was added to the wells in triplicate. Following a further period of incubation, the plates were washed, stained with MTT and the toluidine dye was then eluted using alcohol. The amount of toluidine dye converted was measured by an ELISA reader by measuring the optical density (OD). When the OD was plotted against the concentration of shigatoxin a negative dose response curve was obtained, typical of a cytotoxic bioassay, as shown previously in the TNF bioassay.

Peripheral blood mononuclear cells (PBMC) were isolated using a ficoll-hypaque gradient[59]. Blood diluted 1:1 in normal saline, was carefully layered over lymphoprep^{R} (Sodium metrizoate / ficoll). It was Centrifuged at 1800 RPM for 10 minutes. After centrifugation the mononuclear cells form a distinct band between the plasma and the separation medium and the cells were removed from the interphase using a Pasteur pipette. The cells were diluted in 2-4 ml of RPMI 1640, and centrifuged at 1500 RPM for 7 minutes. The washing procedure was repeated to remove the platelets. The supernatant was decanted, and a measured volume (2-3 ml) of RPMI was added and mixed thoroughly. The cell concentration was adjusted to 1×10^{6} cells per ml. This suspension of cells were incubated in the presence of different concentrations of endotoxin and shigatoxin with and without polymyxin B for 4 hours in Falcon tubes. At the end of incubation, the tubes were centrifuged and the supernatant aspirated. TNF and IL-6 was estimated using a commercial ELISA kit.

The endotoxin activity of the two endotoxin preparations used were quantitated by the limulus amoebocyte lysate assay (LAL test), and, the concentrations adjusted according to endotoxic activity. Contamination of LPS in the shiga toxin preparation was also measured by the limulus amoebocyte lysate (LAL) test and was found to

be 880 pg/ml in undiluted toxin.

Results

A dose response curve for TNF and IL-6 was obtained when mononuclear cells were incubated in the presence of endotoxin of *Shigella dysenteriae*-1. A maximum TNF and IL-6 response was seen with 250 pg/ml of endotoxin. With increasing concentrations of endotoxin, a slight reduction of TNF release was observed, but not with IL-6. This may be because IL-6 is a negative modulator of TNF[60]. TNF and IL-6 release by control mononuclear cells were minimal.

Incubation of mononuclear cells with endotoxins of *S. dysenteriae* and *E. coli* showed that maximal TNF release was seen at similar concentrations of the two endotoxins, indicating that endotoxin of *S. dysenteriae* is of similar potency to that of *E. coli* endotoxin in stimulating mononuclear cells to release TNF.

A dose response curve of TNF secretion by mononuclear cells was seen with increasing concentrations of shigatoxin. Since the shigatoxin used contained a small amount of contaminating endotoxin, this TNF response could be due to the contaminating endotoxin. To exclude this possibility, a parallel incubation of mononuclear cells with shigatoxin in the presence of polymyxin B was done. Polymyxin B is known to inhibit endotoxin activity but not completely[61,62]. TNF release by mononuclear cells in the presence of polymyxin B was reduced, suggestive of partial inhibition of the contaminating endotoxins. Polymyxin B, however is also known to inhibit TNF release by mononuclear cells, and this reduced TNF response may have been for the same reason[63]. To exclude this possibility, shigatoxin was inactivated by boiling, which does not cause inactivation of endotoxins[64]. When the inactivated shigatoxin was used, the TNF response was similar to the response seen in the preparation with active shigatoxin. Similarly, inactivated shigatoxin was used with polymyxin B, the TNF response was similar to TNF release with active shigatoxin with polymyxin B. This confirms that the TNF release was due to contaminating endotoxins.

The conclusions of the *in-vitro* studies are:

Mononuclear cells were extremely sensitive to endotoxins of *shigella dysenteriae*-1, with cytokine release by as little as 2.5 pg/ml, and a maximal response at 250 pg/ml. Endotoxins of *S. dysenteriae* 1 equipotent in stimulating TNF release by mononuclear cells as compared to *E. coli* endotoxin. TNF release with shigatoxin was due to contaminating endotoxins and not due to shigatoxin.

Madam President, ladies and gentlemen, In conclusion these studies lead to the postulate that, invasion of the colon by shigellae and release of shigatoxin predispose to a local inflammatory response leading to local cytokine production in the colon. Cytokines thus produced and shigatoxin are likely to cause endothelial cell damage and a vasculitis, leading to further tissue destruction and necrosis. In the event of extensive tissue destruction and or endotoxaemia, a more generalized release of cytokines would produce the systemic manifestations of shigellosis, including DIC and HUS.

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