Peripheral Blood Neutrophil Responses in Children with Shigellosis

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Alterations in peripheral blood neutrophil function are known to occur in patients with colitis and may have a role in precipitating nonspecific tissue injury. It is not known whether neutrophil function is altered in patients with Shigella dysenteriae type 1 infection, during which there is extensive colitis and which may be associated with life-threatening complications in young children. Three aspects of peripheral blood neutrophil function, polarization, attachment to yeast particles, and locomotion, were therefore studied in 111 children with S. dysenteriae type 1 infection and 57 children without any infection. All children were aged 12 to 60 months. Of the children with S. dysenteriae type 1 infection, 42 had leukemoid reaction, hemolytic-uremic syndrome, or septicemia (complicated shigellosis), while the others did not (uncomplicated shigellosis).

Polarization and locomotion in the absence of chemoattractants and in response to N-formylmethionyl-leucyl-phenylalanine (FMLP) and the lipopolysaccharide (LPS) of S. dysenteriae type 1 were determined. Attachment to unopsonized and opsonized yeast particles was also determined. Children with shigellosis (uncomplicated or complicated) had more polarized neutrophils with and without chemoattractants than uninfected children (P < 0.05). Children with complicated shigellosis had more polarized neutrophils with FMLP at 10^{-7} and 10^{-6} M (P < 0.05) and with LPS than children with uncomplicated shigellosis (P < 0.05). At 3 to 5 days after enrollment, the numbers of polarized neutrophils with 10^{-8}, 10^{-7}, and 10^{-6} M FMLP declined in children with uncomplicated shigellosis but not in those with complicated shigellosis. Attachment to yeast particles was similar in all three groups of children. Locomotion was inhibited by LPS in children with shigellosis (P < 0.05), whether it was uncomplicated or complicated, compared with locomotion in uninfected children. Finally, neutrophil polarization in uninfected children was negatively influenced by nutritional status. Thus, poorly nourished uninfected children had more polarized neutrophils with FMLP at 10^{-9} M (P = 0.020) and 10^{-5} M (P = 0.043) than their better-nourished counterparts. In summary, altered neutrophil responses are associated with both uncomplicated and complicated shigellosis.

Invasion of the gut epithelium is a key event in the pathogenesis of shigellosis. Shigellae invade gut epithelial cells via the M cells overlying the Peyer’s patches in the small intestine (20) and via the basolateral poles of colonic epithelial cells (18). Neutrophils play a pivotal role in this invasion process. Initial entry via M cells leads to an intense inflammatory reaction with dense infiltration of the lamina propria with neutrophils and mononuclear cells. The shigellae present in the gut lumen presumably either secrete or induce secretion by epithelial cells of neutrophil chemotactic factors (19). Neutrophils then transmigrate through the epithelium, allowing bacteria to invade the cells through the paracellular pathway (19).

Severe, often fatal complications are associated with shigellosis, particularly that caused by Shigella dysenteriae type 1, in children younger than 5 years of age. These complications include leukemoid reaction, in which there is an increase in the total leukocyte (WBC) count (≥40,000/mm³ of blood), granulocytosis, and an increase in the number of immature granulocytes (5); hemolytic-uremic syndrome (HUS), which consists of a triad of hemolytic anemia, thrombocytopenia, and acute renal failure (21); and septicemia (23) and intestinal complications, such as toxic megacolon (3). Leukemoid reaction in patients with shigellosis is a bad prognostic indicator (5), and when it accompanies other complications such as HUS, the prognosis is worse (24). The pathogenesis of these complications is unknown, but damage to the vascular endothelium may be one of the etiological events, particularly in patients with HUS (10) and intestinal perforation following toxic megacolon (3). One of the factors inducing vascular endothelial cell injury is activated neutrophils (25, 26). High levels of elastase in plasma (17), increased adherence of neutrophils to endothelium, and neutrophil-mediated damage of endothelial cell fibronectin have been reported in patients with HUS associated with diarrhea from enterohemorrhagic Escherichia coli (10).

Other inflammatory conditions of the gut, such as ulcerative colitis and Crohn’s disease, are associated with alterations in peripheral blood neutrophil function (8, 13, 16) which are thought to contribute to intestinal and extraintestinal nonspecific injury. Neutrophil function has not been measured in patients with shigellosis, particularly that caused by S. dysenteriae type 1, in which there is extensive colitis. It is also not known whether complicated shigellosis is associated with altered neutrophil function as is seen with HUS caused by enterohemorrhagic E. coli. In the present study, we therefore investigated three aspects of peripheral blood neutrophil function, polarization or shape change (22) (an early activation event), locomotion, and attachment to yeast particles (later activation events), in children with S. dysenteriae type 1 infec-

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tion with and without leukemoid reaction, HUS, or septicemia and in children without any infection. In addition, the effect of nutritional status on these functional parameters was also investigated.

MATERIALS AND METHODS

Study population. Children aged 12 to 60 months attending the Clinical Research Centre of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), with acute dysenteric (visible blood in stool with or without fever) were initially enrolled in the study. Stools were examined microscopically and were cultured for enteric bacteria (29). Only children who were negative for S. dysenteriae type 1 infection were included in the study. Informed consent was obtained from the guardians of each child before enrollment. Clinical evaluation of the patients included daily physical examination and determination of vital signs. Laboratory investigations included determination of hematocrit, total and differential WBC (white blood cell) counts (including bands, metamyelocytes, and myelocytes), platelet counts, fragmented erythrocytes (RBCs), serum electrolytes, and creatinine concentration. All patients were treated with ampicillin (pivoxil) or ciprofloxacin. Some children received additional antibiotics for concomitant infections, such as respiratory tract infection, meningitis, and septicemia.

Cells were then fixed with 2% glutaraldehyde (Sigma) in HBSS-MOPS and were centrifuged at 800×g for 10 min. A few drops of unopsonized or opsonized (in fresh autologous plasma) yeast particles were added to the cells, and the cell suspension was then incubated at 37°C for 10 min. After washing twice with HBSS-MOPS, the percentage of yeast particles attached to the cells was determined by counting at least 100 cells.

RESULTS

Study groups. Table 1 provides the clinical characteristics of the children studied. The children in the three study groups were comparable for age (P = 0.338), nutritional status (P = 0.372), and sex (P = 0.469). Stool frequency on the day of enrollment and the duration of diarrhea before enrollment were comparable between children with uncomplicated shigellosis and those with complicated shigellosis (P = 0.670 and 0.823, respectively). However, on the day of enrollment, the numbers of WBCs per high-power microscopic field (HPF) (using a ×40 objective of a light microscope; BH-2 Olympus) and RBCs per HPF in stools were lower (P < 0.001 for both WBCs and RBCs), while total WBC counts per microliter of blood and the percentage of polymorphonuclear cells were higher (P < 0.001 for both WBC counts and percent polymorphonuclear cells) in children with uncomplicated shigellosis compared with children with uncomplicated shigellosis.

Neutrophil polarization on enrollment. In the absence of any chemoattractant, children with S. dysenteriae type 1 infection (whether uncomplicated or complicated) had a higher percentage of polarized neutrophils than uninfected children (P < 0.05) (Fig. 1). The percentages of polarized neutrophils were similar in children with uncomplicated and complicated shigellosis. In response to FMLP, the percentage of polarized neutrophils was higher in children with S. dysenteriae type 1 infection (whether complicated or uncomplicated) than in uninfected children (P < 0.05) (Fig. 1). There were also more polarized neutrophils in children with uncomplicated shigellosis than those with uncomplicated shigellosis with FMLP at 10−7 M and 10−6 M (P < 0.05).

In response to the LPS of S. dysenteriae type 1 (1 μg/ml), the percentages of polarized neutrophils were significantly different among the three groups of children (P < 0.001) (Fig. 1). There were more polarized neutrophils in children with shigellosis (uncomplicated and complicated) than in uninfected children (P < 0.05) as well as in children with complicated shigellosis (P < 0.05) (Fig. 1).
Further comparisons between children with leukemoid reaction and HUS showed no difference in polarization, with or without chemoattractants, between children with these two types of complications (data not shown).

**Neutrophil polarization 3 to 5 days after enrollment.** Comparison of paired samples between the day of enrollment and 3 to 5 days later in children with uncomplicated shigellosis (Fig. 2A) showed a significant decline in the percentages of polarized neutrophils with $10^{-8}$, $10^{-6}$, and $10^{-5}$ M FMLP. Between the two study periods, no statistically significant decline was observed in the absence of any chemoattractant (Fig. 2A) or with the LPS of *S. dysenteriae* type 1. In children with complicated shigellosis (Fig. 2B), there were no differences in the percentages of polarized neutrophils in the absence of any chemoattractant, with FMLP, or with the LPS of *S. dysenteriae* type 1 between the two study periods.

**Neutrophil attachment to yeast particles.** On enrollment, the percentage of neutrophils attached to unopsonized yeast particles and the numbers of yeast particles (both opsonized and unopsonized) attached were similar for the three study groups (Table 2). Also, 3 to 5 days after enrollment, neutrophil attachment to yeast particles in children with uncomplicated and complicated shigellosis was similar (data not shown).

**Neutrophil locomotion.** Locomotion of neutrophils in the absence of any chemoattractant or with FMLP at $10^{-8}$ M was similar for the three study groups (Table 3). With the LPS of *S. dysenteriae* type 1, neutrophils from children with shigellosis, whether it was uncomplicated or complicated, showed less locomotion ($P < 0.05$) than those from uninfected children; the locomotion of neutrophils from children with uncomplicated and complicated shigellosis was similar. Comparison within study groups showed that locomotion of neutrophils from uninfected children was similar with or without chemoattractants. However, in children with shigellosis, whether it was uncomplicated or complicated, the response with FMLP was more ($P < 0.05$) and that with the LPS of *S. dysenteriae* type 1 was less ($P < 0.05$) than that in the absence of chemoattractants. At 3 to 5 days after enrollment, not enough samples were available for statistical comparison.

**Effects of clinical parameters on neutrophil function.** Multiple regression analysis revealed that neutrophil polarization, attachment to yeast particles, or locomotion was not affected by sex, concomitant infections, stool frequency, or duration of diarrhea before enrollment. However, nutritional status had an inverse relationship with polarization (but not phagocytosis or locomotion). Reanalyses of the polarization data, after dividing the children into poorly nourished and better-nourished subgroups, revealed that there were differences in polarization between the two nutritional subgroups, but only in uninfected children. Poorly nourished, uninfected children had more polarized neutrophils with FMLP at $10^{-5}$ M ($P = 0.01$, standard deviation [SD] = 14.2, $n = 24$) and $10^{-4}$ M ($mean = 36.4, SD = 16.2, n = 25$) than better-nourished, uninfected children (for $10^{-5}$ M, $mean = 41.1, SD = 14.4, n = 13$; for $10^{-4}$ M, $mean = 25.7, SD = 11.2, n = 23$) ($P = 0.004$ and $0.020$ for $10^{-5}$ and $10^{-4}$ M FMLP data, respectively, for children of the two nutritional subgroups). There were no differences at other FMLP concentrations or with the LPS of *S. dysenteriae* type 1. In children with shigellosis, whether it was uncomplicated or complicated, there were no differences between the two nutritional subgroups.

**DISCUSSION**

Experimental colitis has been shown to alter neutrophil function in the peripheral circulation (6), with enhancement of superoxide generation, leukotriene B4 synthesis, and phagocytosis and inhibition of chemotaxis (6). In patients with inflammatory bowel diseases, peripheral neutrophil functions are altered (8, 13, 16), and it is thought that these alterations may lead to nonspecific injury. In patients with shigellosis there is extensive colitis with a predominant neutrophil infiltration (14).
so that, as in patients with inflammatory bowel disease, neutrophil activation could have a role in precipitating nonspecific injury in patients with shigellosis. This is particularly pertinent for patients with infection with \textit{S. dysenteriae} type 1 infection, in whom colitis is more severe, and children often develop life-threatening complications such as leukemoid reaction and HUS. Although the pathophysiology of these complications is not well understood, it has been shown that in patients with diarrhea-associated HUS, neutrophils are more activated (10, 17) and that the products of the activated neutrophils may lead to vascular endothelial cell damage, which in turn may precipitate HUS (10, 17). This report describes, for the first time, alterations in the early and later events of neutrophil function in children with \textit{S. dysenteriae} type 1 infection, with and without complications.

We found that polarization, an early event in neutrophil activation, was higher in the presence and absence of chemotactants (FMLP and LPS of \textit{S. dysenteriae} type 1) in children with shigellosis than in uninfected children. Increased numbers of polarized neutrophils in the absence of any chemotactrant probably represent cells that were activated in vivo. Similar in vivo activation of neutrophils has been demonstrated in patients with ulcerative colitis and Crohn’s disease (16). In vivo activation may be a result of several factors, one of which is bacterial products. The LPSs of gram-negative bacteria are known to prime neutrophils, so that they become more responsive to other stimuli (30). \textit{Shigella} LPS may be similar in this respect. Enhanced polarization in response to low doses of FMLP reflects the already activated state of cells (30). The reason for the increased polarization with higher doses of FMLP may suggest that a previously unresponsive population of cells are activated or that FMLP receptors are modulated. The LPSs of gram-negative bacteria enhance the responsiveness of neutrophils to other stimuli by increasing the number of FMLP receptors (30). In patients with Crohn’s disease, the number of FMLP receptors expressed on neutrophils is increased, and these neutrophils show increased chemiluminescence with high doses of FMLP (2). These results were interpreted to suggest that neutrophil changes were secondary to exposure to bacterial products through a leaky gut or inflammatory cytokines. It may be hypothesized that a similar situation occurs in patients with shigellosis. Increased polarization with LPS may again be due to alterations in the receptor for LPS on neutrophils. CD14, which is the receptor for LPS (12), can be upregulated by LPS itself (30).

Neutrophil locomotion with FMLP was higher than that without chemotactants in children with shigellosis but not in uninfected children. Exposure to LPS is known to enhance f-actin formation (30), which may be related to chemotaxis. Therefore, it is possible that in children with shigellosis, exposure to LPS in vivo leads to enhanced neutrophil locomotion with subsequent exposure to FMLP in vitro. However, why exposure to the LPS of \textit{S. dysenteriae} type 1 in vitro inhibited
neutrophil locomotion in children with shigellosis and not in uninfected children is not clear.

Another important aim of the present study was to compare peripheral neutrophil responses in children with complicated shigellosis versus the responses in those with uncomplicated shigellosis. Children with complicated shigellosis did not have more polarized cells in the absence of chemoattractants or with low doses of FMLP than children with uncomplicated shigellosis, suggesting that their cells were similarly activated in vivo. However, neutrophils from children with complicated shigellosis were more polarized in the presence of high FMLP concentrations ($10^{-7}$ and $10^{-6}$ M) and the LPS of S. dysenteriae type 1. Although it is not clear why this happens, a further increase in FMLP receptor or CD14 expression may take place in neutrophils from children with complicated infection. This could be due to exposure to more bacterial antigens such as LPS and other inflammatory products, including cytokines such as tumor necrosis factor alpha, the levels of which are known to be raised in the serum of children with complicated shigellosis (9). However, there is no evidence to support this hypothesis.

From the time of enrollment to 3 to 5 days later, there were

![FIG. 2. Percentage of polarized neutrophils on the day of enrollment (day 0 [■]) and 3 to 5 days later (□) in response to no chemoattractant (0), log dilutions of FMLP from $10^{-7}$ to $10^{-6}$ M, and the LPS of S. dysenteriae type 1 (1 mg/ml). Datum points represent means, and vertical bars represent SDs. The Wilcoxon matched-pair signed rank sum test was used for comparison. (A) Children with uncomplicated shigellosis. (B) Children with complicated shigellosis. * $P = 0.008$; ** $P = 0.006$; *** $P = 0.015$.](image)

<table>
<thead>
<tr>
<th>TABLE 2. Attachment of peripheral blood neutrophils to yeast particles on enrollment</th>
<th>Neutrophils attaching yeast particles (%)(\text{a})</th>
<th>No. of yeast particles attached/100 neutrophiles(\text{a})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Opsonized</td>
<td>Unopsonized</td>
</tr>
<tr>
<td>Uninfected children (n = 57)</td>
<td>85.2 (11.9)</td>
<td>14.0 (9.5)</td>
</tr>
<tr>
<td>Children with shigellosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uncomplicated (n = 69)</td>
<td>87.5 (12.4)</td>
<td>15.7 (14.5)</td>
</tr>
<tr>
<td>Complicated (n = 42)</td>
<td>89.5 (12.0)</td>
<td>19.8 (16.8)</td>
</tr>
<tr>
<td>(P^b)</td>
<td>NS(^c)</td>
<td>NS</td>
</tr>
</tbody>
</table>

\(^a\) All values are means (SDs).

\(^b\) Kruskal-Wallis test comparing the three study groups.

\(^c\) NS, not significant.
no changes in polarization of neutrophils from children with uncomplicated or complicated shigellosis in the absence of chemoattractants or with 10^{-6} M FMLP, suggesting that the cells were still activated in vivo. A decline in polarization was observed with high concentrations of FMLP in children with uncomplicated shigellosis but not in children with complicated shigellosis. At 3 to 5 days after enrollment, children with uncomplicated shigellosis had improved clinically so that their stool frequency, stool WBC counts, stool RBC counts, and peripheral blood total WBC count were all lower than those at the time of enrollment (Table 1). In contrast, the clinical condition of children with complicated shigellosis was unchanged 3 to 5 days after enrollment (Table 1). Therefore, responses to high concentrations of FMLP appear to be more short-lived and related to the clinical condition of the children.

We also found that nutritional status had a profound effect on neutrophil polarization. Poorly nourished, uninfected children had more primed cells (as suggested by increased polarization in the presence of 10^{-9} M FMLP) than their better-nourished counterparts. This confirms the previous findings of Anderson et al. (1), who showed that peripheral neutrophils from malnourished children have a more activated morphology. The reason for increased polarization with FMLP at 10^{-3} M is not clear, but FMLP receptors may be susceptible to nutritional deprivation, resulting in alterations in receptor numbers or the type of receptors expressed (high- or low-affinity receptors). However, no evidence supports this hypothesis, although it is recognized that cell surface glycoproteins are altered in malnourished children (7). No effect of nutrition was observed in children with shigellosis. Nutrition also did not influence the outcome of infection in patients with shigellosis in terms of the development of complications, because the numbers of children ≤70% weight-for-age and >70% weight-for-age were similar in the two groups of children with shigellosis.

All children with shigellosis (whether it was uncomplicated or complicated) were treated with antimicrobial agents; most children received amdinocillin pivoxil and four children received ciprofloxacin. The effects of amdinocillin pivoxil on neutrophil responses are not known, but penicillin G and methicillin are known to inhibit murine neutrophil cytokinesis (15). Ciprofloxacin, on the other hand, may act synergistically with the immune system (11). It is not possible from the results of the present study to rule out the influence of drugs on neutrophil responses. In addition, since antimicrobial therapy forms the cornerstone of treatment in patients with shigellosis, it is not possible to carry out immune function studies in patients without antibiotic treatment.

In summary, children with S. dysenteriae type 1 infection have more polarized (activated) neutrophils in their peripheral circulation than uninfected children. In patients with complicated infection, further polarization with high doses of FMLP and the LPS of S. dysenteriae type 1 may reflect responses to the inflammatory milieu. The relationship of this increased response to the development of complications remains to be elucidated.

ACKNOWLEDGMENTS

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REFERENCES


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**TABLE 3. Locomotion by peripheral blood neutrophils of children on enrollment**

<table>
<thead>
<tr>
<th>Study group</th>
<th>Leading front values (μm) with the following chemoattractants:</th>
<th>P&lt;sup&gt;x&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>FMLP (10^{-6} M)</td>
</tr>
<tr>
<td>Uninfected children (group a)</td>
<td>61.8 (27.3) (n = 11)</td>
<td>74.3 (27.4) (n = 11)</td>
</tr>
<tr>
<td>Children with shigellosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uncomplicated (group b)</td>
<td>65.1 (17.3) (n = 24)</td>
<td>77.0 (15.7) (n = 24)</td>
</tr>
<tr>
<td>Complicated (group c)</td>
<td>61.7 (28.3) (n = 21)</td>
<td>79.0 (27.3) (n = 21)</td>
</tr>
</tbody>
</table>

<sup>x</sup> All values are means (SDs) (n is number of children).
<sup>y</sup> Analysis of variance between three chemoattractants within each group of children. When P < 0.05, the Student-Newman-Keuls method was used to compare any two groups.
<sup>z</sup> NS, not significant.


