Prospective Study of the Association between Serum Antibodies to Lipopolysaccharide O Antigen and the Attack Rate of Shigellosis

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A means for determining immune status against shigellosis would significantly improve the design and evaluation of interventional and other epidemiologic studies. Previous case-control studies have indicated the potential role of humoral antilipopolysaccharide antibodies. To test this proposition, 190 soldiers serving in a field unit were monitored prospectively for 2.5 months for shigellosis. Blood samples were taken at the beginning of the follow-up period and tested for serological evidence of prior exposure to Shigella sonnei and Shigella flexneri. The risk for acquiring S. sonnei shigellosis was 3.7 times higher for individuals lacking homologous antibodies (P < 0.02). The risk for acquiring S. flexneri shigellosis was 2.4 times higher for individuals lacking antibodies, although a low attack rate for S. flexneri resulted in numbers too small to achieve statistical significance. While the importance of the serum antilipopolysaccharide antibodies in protection against the disease remains unclear, these findings demonstrate that they are strong markers of acquired immunity. Serological markers should be incorporated in epidemiologic studies of shigellosis and in the design and evaluation of future trials of potential anti-Shigella vaccines.

Since there seems to be some role for acquired immunity in shigellosis (7, 8, 25), a readily measured indicator of immune status would greatly facilitate the design and analysis of interventional and other epidemiologic studies of the disease. The feasibility of using circulating antibodies for this purpose was mooted in a previous case-control study in which we showed that low or undetectable levels of anti-Shigella lipopolysaccharide (LPS) antibodies were associated with an increased risk of developing shigellosis (5). In particular, the presence of high levels of non-immunoglobulin M (IgM) anti-Shigella LPS antibodies in sera showed the strongest correlation with protection against shigellosis (5). In that study, however, levels of anti-Shigella LPS antibodies at the early acute stage of the disease were used as estimates of the preexposure levels of serum antibodies against Shigella spp., which could theoretically have introduced bias. For example, a rapid antibody response might spuriously result in the misclassification of specimens from either patients with the disease or controls as having high titers. This would tend to underestimate relative risk. On the other hand, if the rate of antibody appearance differs between cases and controls, either over- or underestimation of the risk may occur. To test the association while controlling for this possible bias, we conducted a prospective study of the relationship between low levels of anti-Shigella LPS antibodies and disease caused by homologous and heterologous Shigella organisms in a military population.

MATERIALS AND METHODS

Study population. During a surveillance program, three separate field units were observed for significant Shigella spp.-associated disease for 2.5 months during summer, 1986. A specially trained medical orderly, whose sole task was to process study subjects, was stationed at each unit. In these units, cooking facilities were provided by field kitchens, water supply was chlorinated, and field trench latrines served as toilets. An outbreak of shigellosis due to Shigella sonnei occurred in one of the units, which had a population of 190 subjects. The attack rate of shigellosis in those who had preexisting antibodies was compared with that in those without antibodies. In addition, a case-control study nested in the follow-up cohort was carried out in order to compare the preexposure levels of anti-Shigella LPS antibodies in bacteriologically proven cases of shigellosis and those in healthy controls from the same base.

At the start of the surveillance period, a questionnaire was administered to all soldiers regarding recent diarrhea history, and baseline blood samples were collected. Stool samples, data on presence or absence of symptoms and signs of disease, date of onset, and a description of the feces were obtained from each subject complaining of diarrhea at the time of his visit to the unit clinic. Additional paired serum samples were obtained from patients with diarrhea at the acute and convalescent stages of the disease. Blood samples were taken from all soldiers at the end of the study. In addition to diarrhea resulting in clinic visits, all soldiers completed a final questionnaire at the end of the study period in which all diarrheal incidents recalled were recorded. It is acknowledged that such a questionnaire might result in both a degree of underestimation of true diarrhea rates and some bias in favor of more easily remembered (more severe) disease. However, it is felt that such problems are mitigated by the fact that no bias for preexisting antibodies is introduced in this way.

A case of shigellosis was defined as diarrhea (the passage of more than two liquid stools in a 24-h period) with either isolation of Shigella organisms in the stool culture or significant antibody response against Shigella LPS as measured by the enzyme-linked immunosorbent assay (ELISA) or both.

Bacteriology. Stool samples were obtained at the time of the visit to the unit medical clinic. Specimens were inoculated immediately on MacConkey agar and salmonella-shigella agar. In parallel, swabs with fecal matter were introduced into buffered glycerol transport medium and gram-
negative enrichment broth. At the central laboratory, additional culturing of samples from transport and enrichment media was done on MacConkey and salmonella-shigella agars. Identification of Shigella spp. was performed by routine morphological, biochemical, and serological testing, and isolates were submitted to the Reference Laboratory of the Ministry of Health for serotyping.

**Seroepidemiology.** Sera obtained from the subjects in the study were separated, and aliquots were frozen at −20°C until tested. Serological tests were carried out in duplicate by using both a passive hemagglutination (HA) test and an ELISA. The microtiter adaptation of the passive HA technique for detection of shigella-specific antibodies was used (18). LPS extracted by the method of Westphal and Jann (26) from single strains of S. sonnei (form 1) and Shigella flexneri 2a isolated from outbreaks was used as antigen. The HA titers of sera preincubated with 0.1 M 2-mercaptoethanol for 1 h at 37°C, to inactivate immunoglobulin M (IgM), were determined in parallel. ELISA was performed in microtiter plates (Nunc, Roskilde, Denmark) as previously described (4). Goat anti-human IgM, IgG, or IgA, conjugated to alkaline phosphatase (Kirkegaard & Perry Laboratories, Gaithersburg, Md.), was used as a second antibody. A significant antibody response of either IgM or IgA fractions was defined as a threefold or greater increase in the corresponding IgA or IgM ELISA optical density measured at a 1:100 dilution of sera in which the posttraining specimen showed an optical density of at least 0.25.

We defined preexisting anti-LPS antibodies as a passive HA titer of 1:10 or more after treatment of sera with 0.1 M 2-mercaptoethanol. Compared with various immunoglobulin fractions, including anti-Shigella LPS ELISA IgM, ELISA IgA, ELISA IgG, and HA total antibodies, the HA non-IgM fraction showed the strongest association with the risk to develop shigellosis (5).

**Statistical analysis.** The differences between the attack rates in the groups with and without preexisting antibodies were assessed by means of relative risk (or the odds ratio for the nested case-control study). The two-tailed Fisher's exact test was used to evaluate the significance of the differences, and test-based confidence intervals were computed.

**RESULTS**

The occurrence of diarrhea and shigellosis (determined on the basis of both bacteriologic and serologic data) is shown in Table 1. Of 21 stool samples obtained from 23 soldiers who visited the unit medical clinic with symptoms of diarrhea, 8 were positive for S. sonnei and 1 was positive for S. flexneri 2a. Together these represent 42.9% of cultured cases. Of the soldiers with diarrhea during the training period, 18.4% showed a significant antibody response against S. sonnei LPS and/or had positive stool cultures for S. sonnei. Only 3.7% of the soldiers with diarrhea were diagnosed by the same criteria as having S. flexneri shigellosis (Table 1). Among asymptomatic subjects, 6.7% showed an immune response against S. sonnei and 2.1% showed a response against S. flexneri LPS. The attack rates of diarrhea and of S. sonnei shigellosis were 65.8 and 18.4%, respectively, during the follow-up period (Table 1).

The attack rate of shigellosis due to S. sonnei was significantly higher in subjects who lacked preexisting circulating antibodies to S. sonnei LPS (Table 2). The risk of developing symptomatic S. sonnei infection was 3.7 times higher among subjects without preexisting specific anti-LPS HA non-IgM antibodies than among subjects who had preexisting antibodies of the non-IgM fraction (Table 2). In contrast, no significant difference was found in the attack rate of shigellosis due to S. sonnei between subjects with and without preexisting heterologous antibodies (against S. flexneri 2a) of the HA non-IgM fraction (28 of 135 versus 7 of 55, respectively; P = 0.13). The attack rate of S. flexneri shigellosis (3.7%) was much lower than that of S. sonnei during the follow-up period (18.4%) and did not yield a significant association with anti-S. sonnei (Table 2). The attack rate of S. flexneri shigellosis was higher (2.4 times) in subjects with preexisting homologous antibodies than in those without (6 times).

**TABLE 1. Incidence rates of diarrhea and shigellosisa**

<table>
<thead>
<tr>
<th>Diagnostic criteria and illness</th>
<th>No. of subjects (%) with illnessa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recalled diarrhea ..................</td>
<td>125 (65.8)</td>
</tr>
<tr>
<td>Clinic visits for diarrhea ........</td>
<td>23 (12.1)</td>
</tr>
<tr>
<td>Culture positive&quot; for S. sonnei</td>
<td>8 (4.2)</td>
</tr>
<tr>
<td>Culture positive&quot; for other Shigella groups</td>
<td>1 (0.5)</td>
</tr>
<tr>
<td>S. sonnei† shigellosis ............</td>
<td>35 (18.4)</td>
</tr>
<tr>
<td>S. flexneri† shigellosis ..........</td>
<td>7 (3.7)</td>
</tr>
</tbody>
</table>

a Over a 2.5-month period.

" From a total of 190 subjects.

† Twenty-one stool samples were submitted from 23 clinic visits. The positive cultures therefore represent a 42.9% positivity rate among cultured episodes of diarrhea.

<table>
<thead>
<tr>
<th>Serum antibody titer</th>
<th>Shigellosis</th>
<th>Other diarrhea</th>
<th>No diarrhea</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. sonnei</td>
<td>31/129 (24.0)</td>
<td>5/129 (3.9)</td>
<td>60/934 (64.5)</td>
</tr>
<tr>
<td>S. flexneri</td>
<td>4/61 (6.5)</td>
<td>2/61 (3.2)</td>
<td>31/554 (56.4)</td>
</tr>
</tbody>
</table>

Relative risk 3.7 1.2 1.1 0.6

P value 0.002 0.6 0.3 0.04

a Number of cases observed among 190 soldiers over a 2.5-month period.

b Diarrhea with positive stool culture for S. sonnei or S. flexneri or with significant antibody response of IgM or IgA fractions against homologous LPS or recalled incidence of diarrhea with significant antibody response.

c Diarrhea caused by organisms other than Shigella spp.

d The denominator used does not include cases of S. sonnei or S. flexneri shigellosis.

**TABLE 2. Occurrence of shigellosis and diarrhea not caused by Shigella spp. by baseline HA levels of non-IgM antibody against S. sonnei LPSa**
of 135 versus 1 of 55), but this difference did not reach statistical significance ($P = 0.34$). No correlation was found between presence of preexisting anti-\textit{S. sonnei} LPS antibodies of the non-IgM fraction and the attack rate of diarrhea not caused by \textit{Shigella} spp. (Table 2).

A small case-control study, nested in the follow-up cohort, was carried out in order to compare the preexisting levels of anti-\textit{Shigella} LPS antibodies in bacteriologically proven cases of shigellosis and in subjects from the same base that did not complain of diarrhea during the whole follow-up period (healthy controls). None of the 8 patients with stool cultures positive for \textit{S. sonnei}, compared with 33 of the 57 healthy controls, had preexisting antibodies against \textit{S. sonnei} LPS ($P = 0.02$) (Table 3). No significant difference was found between the prevalence of preexisting antibodies against \textit{S. flexneri} LPS in the two groups (odds ratio, 1.2; $P = 0.6$).

**DISCUSSION**

The evidence on the role played by serum anti-\textit{Shigella} spp. antibodies in protection against shigellosis is controversial. In challenge studies, no association was found between prechallenge hemagglutinins and protection of volunteers against disease due to virulent \textit{S. flexneri} 2a (8). Similar findings were generated by studies of \textit{Shigella} vaccines, which showed that circulating antibodies elicited by killed \textit{Shigella} organisms were not associated with protection against natural or experimental infection (10–12). On the other hand, in animal models, it has been shown that immunity against shigellosis can be induced in mice and rabbits after passive immunization with antitoxin or antibacterial antibodies elicited in rabbits or horses by virulent strains of \textit{S. flexneri} or \textit{Shigella dysenteriae} type 1 (Shiga) (1, 3).

To the best of our knowledge, the present study is the first study to demonstrate prospectively that preexisting anti-\textit{Shigella} LPS antibodies of the non-IgM fraction, as determined by HA, are associated with significant protection against shigellosis under natural conditions of exposure, and it strengthens and extends the results of our previous case-control study. Preexisting levels of anti-LPS antibodies were associated with protection against disease caused by the homologous but not by the heterologous \textit{Shigella} group, supporting previous data on occurrence of serogroup-specific immunity after shigellosis (9, 21). The immunity was also found to be specific to shigellosis and was not associated with protection against diarrhea not caused by \textit{Shigella} spp. These findings are supported by data reported by Black et al. (2), in which the presence of serum IgA and IgG antibodies prior to experimental challenge with virulent \textit{S. sonnei} was correlated with protection against illness.

It is possible that anti-\textit{Shigella} serum antibodies may participate actively in the immune mechanisms or simply be markers of other mechanisms of immunity directed against \textit{Shigella} spp. at the local level of the intestinal mucosa. It has been reported that serum antibodies against the O-polysaccharide antigen of \textit{Shigella} spp. mediate opsonization and complement-mediated killing of the organism (20, 24). Serum-derived antibodies to \textit{Shigella} LPS may operate at the local level in conjunction with various phagocytic cells (in the presence or absence of the complement system) (13). Antibody-dependent cell-mediated immunity or antibody-dependent cellular cytotoxicity may be the mechanisms responsible for the antibacterial activity of the circulating anti-\textit{Shigella} antibodies in cooperation with various effector cells (13, 19).

The protective immune mechanism may involve secretion of IgA by intestinal secretory cells (6, 15) and/or activation of the cellular arm of the immune system (16, 22, 23). A good correlation has recently been found between serum and secretory antibody levels elicited in monkeys challenged with \textit{S. flexneri} 2a (6). Other studies revealed that secretion of local antibodies to various antigens after natural infection with enteropathogens including \textit{Shigella} spp., \textit{Salmonella} spp., and \textit{Vibrio cholerae} was accompanied by a parallel rise in levels of serum antibody to the same antigens (14, 17).

In conclusion, the results of the present study demonstrate a strong group-specific association between preexisting levels of serum anti-\textit{Shigella} LPS antibodies and protection against shigellosis. While it is not possible from this study to determine whether these antibodies play an active role in protection against the disease, it can at least be stated that they are strong markers of acquired immunity. These serological markers may be of great value in epidemiologic studies of shigellosis.

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