Characterization and Quantitative Analysis of Serum IgG Class and Subclass Response to *Shigella sonnei* and *Shigella flexneri* 2a Lipopolysaccharide following Natural *Shigella* Infection

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The IgG subclass response to *Shigella sonnei* and *Shigella flexneri* 2a lipopolysaccharide (LPS) was examined in subjects naturally exposed to these organisms. Affinity-purified LPS antibodies obtained using a column of *Shigella* LPS bound to epoxy-activated Sepharose 6B were used as standards to calibrate the serum antibody response to natural *Shigella* infection. The geometric mean concentrations of specific IgG in sera from those not exposed to *Shigella* organisms were 7.9 μg/mL against *S. sonnei* LPS and 18.6 μg/mL against *S. flexneri* 2a LPS. After natural exposure to *S. sonnei* or *S. flexneri* 2a, the concentrations rose to 30.3 and 127.9 μg/mL, respectively. IgG2 was the major component in the anti-*S. flexneri* subclass response, while the anti-*S. sonnei* response was dominated by IgG1. High levels of IgG1 antibodies before exposure to organisms from either *Shigella* serogroup correlated with a lower risk of developing symptomatic infection.

Dysentery and diarrhea caused by *Shigella* organisms are major public health problems, especially in developing countries. It has been estimated that there are >200 million cases of shigellosis or bacillary dysentery, with ~650,000 deaths per year [1]. The members of the genus *Shigella* cross several lines of host defense mechanisms: They invade epithelial cells of the distal small intestine and the colonic mucosa; multiply therein; spread intra- and intercellularly, causing dysfunction, which may eventually lead to apoptosis [2] that kills the host cell; and cause the dysenteric syndrome [3, 4]. The relative importance of locally secreted antibodies, serum antibodies, and cell-mediated immunity in protection against shigellosis is still not clear.

*Shigella* infection confers serotype-specific protection against recurrent infection [5, 6]. It is accompanied by a rise in serum antibodies of the three immunoglobulin classes (IgG, IgM, and IgA) against *Shigella* lipopolysaccharides (LPSs) [7]. Earlier seroepidemiologic studies have demonstrated that preexisting high levels of serum IgG anti-*Shigella* LPS antibodies are strongly associated with a lower risk of developing shigellosis due to infection with the homologous serogroup [5].

Human IgG is known to be composed of four structurally distinct subclasses with different biologic and physiochemical properties. The distribution of IgG subclass antibodies in normal human serum reveals predominance of IgG1 (66%), followed by IgG2 (23%), IgG3 (8%), and IgG4 (4%) [8]. IgG1 and IgG3 were found to be superior to other subclasses in complement activation, attachment to cell membranes through Fc receptors, which subsequently enhances phagocytosis, and mediation of antibody-dependent cytotoxicity [9].

In the present study, we purified human anti-*Shigella sonnei* and anti-*Shigella flexneri* 2a LPS antibodies to determine the different IgG concentrations before and after infection. We examined the distribution of the IgG anti-*S. sonnei* and anti-*S. flexneri* LPS subclasses following natural infection with these organisms and evaluated the association between preexposure levels of the four IgG subclasses to *Shigella* LPS and the risk of developing shigellosis.

Materials and Methods

**Study Population**

The study subjects were men between the ages of 18 and 22 years serving in field units of the Israeli Defence Force. They belonged to 3 different cohorts maintained under surveillance for episodes of diarrhea during 2.5-month training cycles in the field in the summers of 1988, 1989, and 1990. One or more outbreaks of *S. sonnei* and of *S. flexneri* 2a shigellosis occurred in each of these cohorts during the surveillance periods. Sera were obtained from volunteers at the beginning and end of follow-up. Additional serum samples were collected from subjects with diarrhea at the convalescent stage (10–14 days after the onset of disease). Serum samples were kept frozen at −20°C until tested. Subjects with symptomatic *Shigella* infection were defined as those who visited the unit clinic with complaints of diarrhea and had either a stool culture positive for *Shigella* organisms or a significant rise (4-fold) in the total IgG anti-*Shigella* LPS antibodies in paired sera collected before exposure and at convalescence. Diarrhea was defined as passage of three or more loose stools in 24 h. Asymp-
omatic subjects were defined as soldiers who did not complain of diarrhea or related symptoms during the follow-up period and shared food supplies, sanitation, and other living facilities with symptomatic subjects.

The attack rates of *S. sonnei* symptomatic infection were 150 (11.0%) of 1360, 185 (22.8%) of 810, and 26 (7.3%) of 356 in the 3 cohorts maintained under follow-up in the summers of 1988, 1989, and 1990, respectively. The corresponding figures for *S. flexneri* 2a symptomatic infections were 98 (7.2%) of 1360, 310 (38.3%) of 810, and 103 (28.9%) of 356. Forty-four and 41 of the symptomatic subjects with the highest total IgG responses to *S. flexneri* 2a and *S. sonnei* LPSs, respectively, were selected from the cohorts for measurement of the IgG subclass distribution. The preexposure sera from subjects who developed *S. sonnei* or *S. flexneri* 2a symptomatic infection and from their asymptomatic controls were randomly chosen from sera collected from subjects belonging to the 3 cohorts and were examined for IgG subclass antibodies to *Shigella* LPS.

**LPS Preparation**

*S. sonnei* (form 1) and *S. flexneri* serotype 2a isolated during outbreaks were the source for LPS extraction by the hot phenol–water method of Westphal and Jann [10]. The presence of potential cross-reactive protein contaminants, such as outer membrane proteins in the extracted LPS, was ruled out by the complete lack of antibody response to *Shigella* LPS of heterologous groups in paired sera from patients with culture-proven cases of shigellosis.

**O-Specific Polysaccharide Preparation**

LPS (10 mg/mL) was treated with DNase (D4527; Sigma, St. Louis), RNase (24686; Merck, Rahway, NJ), and then pronase (P0390; Sigma). Further purification was followed by acid hydrolysis and centrifugation as described previously [11].

**Purification of Anti–*Shigella* LPS Antibodies from Serum**

Coupling of *S. flexneri* and *S. sonnei* LPS to epoxy-activated Sepharose 6B. Via stable ether linkages to hydroxyl groups, epoxy-activated Sepharose 6B can be used to couple sugars and other carbohydrates. LPSs obtained from *S. flexneri* 2a and *S. sonnei* were coupled to the epoxy-activated Sepharose 6B (Pharmacia, Piscataway, NJ) at a ratio of 30 mg of ligand/8 mL of gel and reacted in a stoppered vessel using a water-bath shaker (Tuttnerau, Jerusalem) for 16 h at 40°C. The coupled gel was washed according to the Pharmacia protocol, and the active groups were blocked by overnight incubation at 40–50°C with 1 M ethanolamine (pH 8.0). Excess blocking agent was removed by washing the gel in at least three cycles of alternating pH consisting of a wash with 0.1 M acetate buffer (pH 4.0) containing 0.5 M NaCl, followed by 0.1 M TRIS-HCl buffer (pH 8.0) containing 0.5 M NaCl. The gel was suspended in saline and stored at 4°C.

Affinity chromatography using LPS coupled to epoxy-activated Sepharose 6B. Sera of volunteers maintained under natural exposure to *Shigella* species were used for purification of anti–*Shigella* LPS antibodies and passed over a 15- to 20-ml affinity column. The columns were washed with 100–120 mL of 0.15 M NaCl and 50 mM TRIS-HCl (pH 7.5), followed by 50 mL of 250 mM NaCl and 50 mM acetate (pH 5.0), until the OD at 280 nm of the flow-through was <0.05. LPS-specific antibodies were then eluted from the column with 40–60 mL of 3.5 M MgCl2. Eluted antibodies were immediately diazylated against PBS and concentrated, as needed, using Omegacell 30K (Filtron Technology, North Borough, MA). The test of Bradford [12] was used to analyze the concentration of protein in serum, washes, and elutions through all stages of the affinity chromatography.

**Specificity of samples.** Samples were then tested for their specificity by two methods.

Eluted samples were collected into a pool and analyzed by one-dimensional gel electrophoresis to provide information about the content and purity of proteins in the specimen. The electrophoresis was performed using 10% SDS-polyacrylamide minigels, and each test included purified human IgG and IgA (I4506, I1010; Sigma) as controls and a prestained molecular weight–SDS blue marker (SDS7B; Sigma). Proteins were then stained by the silver staining modified method of Wray et al. [13].

For ELISA, a previously described protocol [7] with some modifications (as described below) was used to examine different heterologous *Shigella* LPS and O-specific polysaccharides as antigens.

**Quantitation of Specific Antibodies**

Quantitation of IgG anti–*Shigella* LPS antibodies. The amount of IgG anti-LPS antibodies in our pooled samples was calculated using double antibody-sandwich ELISA. This was done according to a standard curve (optical density vs. concentration) involving 10 μg/mL goat anti-human IgG (I3382; Sigma) as antigen on the solid phase and human purified IgG (I4506; Sigma) with known concentration as standard.

Determination of IgG subclasses. The concentration of anti-*Shigella* IgG subclass antibodies (IgG1, IgG2, IgG3, and IgG4) in the eluted samples was measured by a radial immunodiffusion procedure with kits (RID008; Binding Site, Birmingham, UK).

**Distribution of Serum Anti-*Shigella* IgG and IgG Subclass Antibodies in Serum Samples**

Blood samples were taken before or at the onset of disease and 10–14 days later. Sera were separated and samples were frozen at −20°C until tested. Serologic tests were carried out in eight double dilutions using ELISA, performed in polystyrene microtiter plates (model 3590; Costar, Cambridge, MA), according to the method of Cohen et al. [7] with some minor modifications. Briefly, 100 μL of coating buffer (0.05 M carbonate buffer, pH 9.6) containing 10 μg/mL *S. sonnei* or *S. flexneri* 2a LPS was added to each of 96 wells, and the wells were incubated for 1 h at 37°C. After removal of the coating solution, the plates were incubated for 1 h at 37°C with 0.05 M PBS supplemented with casein and bovine serum albumin (both at 5 g/L) in order to block the remaining unbound plastic sites. The wells were then washed twice in PBS–Tween 20 washing solution. Sera were added to the wells, and wells were double diluted in PBS, starting with a 1:50 dilution, and incubated overnight at room temperature. After four further washings, goat anti-human IgG conjugated to alkaline phosphatase (075-1006; Kirkegaard & Perry, Gaithersburg, MD) or monoclonal mouse anti-human IgG subclasses (IgG1, IgG2, IgG3, IgG4) con-
jugated to alkaline phosphatase (Zymed, San Francisco), at 1:1500 and 1:500 dilutions, respectively, were added to the wells of the experiment. The plates were incubated overnight at room temperature and washed, and ELISA was completed sequentially by the addition of the enzyme-substrate solution containing p-nitrophenyl phosphate (1 mg/mL) in diethanolamine buffer at pH 9.8 and 3 M NaOH. Optical density was read at 405 nm with an automatic ELISA reader (Bio-kinetics EL340; Bio-Tek, Winooski, VT). Serum samples taken from each subject before and after exposure to bacteria were tested within the same assay; positive and negative control sera were included in every microtiter plate in each of the assays.

A calculated ELISA optical density based on a linear regression analysis of eight double dilutions was determined and, in order to convert ELISA results to micrograms per milliliter of specific antibody, was compared with similarly calculated values of standards with known antibody concentrations present on the same plate. Serum samples in which the total IgG anti–Shigella antibody concentration increased by 4-fold between the pre- and postexposure sera were tested for their anti–Shigella IgG subclass antibody distribution. Significant IgG subclass response was defined as a ≥4-fold increase in the subclass titers of the paired sera.

Results

In affinity chromatography purification studies, we found that the mean protein concentration loaded in the case of S. sonnei LPS–Sepharose 6B was 1400 mg/20 mL of serum sample, while in the case of S. flexneri 2a LPS–Sepharose 6B, it was 1560 mg/20 mL of serum sample. The mean total protein recovered from the column in the case of S. sonnei LPS–Sepharose 6B was 1133 mg and in the case of S. flexneri 2a LPS–Sepharose 6B was 1206 mg. From these results, we found that the yield of the column that produces anti–S. sonnei antibodies was 81.0% while the anti–S. flexneri 2a column yield was 77.3%.

Samples loaded onto and samples recovered from the columns were examined for the presence of antibodies against the homologous and heterologous bacteria. The geometric mean titer (GMT) of homologous antibodies decreased from 930.7 in the S. sonnei LPS–Sepharose 6B column to very low levels and up again to 201.1 in the elution samples (with the treatment of MgCl₂); in the S. flexneri 2a LPS–Sepharose 6B column, it decreased from 1099.6 to negligible amounts through the washes, and up to 320 in the elutions. The heterologous tests revealed that the GMTs decreased very fast, to negligible amounts in both cases.

The content, specificity, and purity of samples released from the column with the treatment of 3.5 M MgCl₂ were examined in several different ways.

By gel electrophoresis, there were no other proteins in the examined fractions than were found in purified IgG (I4506; Sigma) and IgA (I1010; Sigma), proving the purity of the samples (figure 1).

Specificity of antibodies was examined in the samples by ELISAs against heterologous LPS (samples from the S. flexneri 2a LPS–Sepharose 6B column were tested against LPS of S. sonnei and vice versa). The GMTs to S. flexneri 2a (homologous LPS) and S. sonnei (heterologous LPS) in the concentrated pools of S. flexneri 2a LPS antibodies were 1797.3 and <5, respectively, and the GMTs to S. sonnei (homologous LPS) and S. flexneri 2a (heterologous LPS) in the concentrated pools of S. sonnei LPS antibodies were 981.7 and <5, respectively. Measurement of antibodies to S. sonnei and S. flexneri 2a purified O-specific polysaccharide preparations was also used to exclude the possibility of binding to proteins or nucleic acids that may contaminate crude LPS preparations. It was found that the purified antibodies responded in the same pattern and magnitude against LPS extraction prepared by the hot phenol–water method or further purified O-specific polysaccharides.

Concentration of specific anti–Shigella species IgG antibodies in serum samples. The purified antibodies were used to determine the concentration of anti–Shigella IgG antibodies in paired serum samples collected from patients with symptomatic Shigella infection. Results presented in table 1 show that, in the case of S. sonnei infection, the preexposure mean concentration of anti–S. sonnei LPS antibodies was 7.9 μg/mL, which increased to 30.3 μg/mL after natural infection. In the case of S. flexneri 2a infection, the preexposure mean concentration was 18.6 μg/mL, and it increased to 127.9 μg/mL after natural infection.

IgG subclass response to S. sonnei and S. flexneri LPS after symptomatic infection. Figure 2 shows the pattern of response found against S. flexneri LPS. There was a significant rise in the concentration of anti–S. flexneri 2a IgG2 subclass antibodies in sera taken 10–14 days after onset of disease compared with the concentration in a preexposure sample (from 6.4 to 56.8 μg/mL). A less-pronounced but significant increase was found for IgG1 and IgG3 antibodies (from 1.7 to 11.4 μg/mL and from 1.1 to 2.86 μg/mL, respectively). Examination of the...
distribution of IgG subclasses elicited after natural exposure to S. sonnei (figure 3) revealed an increase in the level of IgG1 antibodies (from 0.48 to 20.06 µg/mL) and a lower, but significant, rise in the IgG2 and IgG3 subclass antibodies (from 1.2 to 5.78 µg/mL and 0.015 to 0.146 µg/mL, respectively). We did not detect any rise in the IgG4 subclass antibodies to either S. flexneri 2a or S. sonnei by ELISA. According to these results, it follows that in S. flexneri infections, the predominant subclass antibody was IgG2, whereas in S. sonnei infections, the main subclass antibody amplified was IgG1. When a significant response was defined as at least a 4-fold increase in the level of anti–Shigella LPS subclass antibodies, we found (table 2) that 95.1% of 41 patients with S. sonnei shigellosis showed a significant increase in anti–S. sonnei IgG1 subclass, whereas only 38.6% of the 44 who were exposed to S. flexneri showed a significant increase in the anti–S. flexneri 2a IgG1 antibodies. Also, 68.2% and 97.6% of the patients infected with S. flexneri 2a and S. sonnei, respectively, showed a significant increase in the IgG2 subclass. Antibodies of the IgG3 subclass increased predominantly in patients who encountered S. sonnei infection (70.7%).

Preexposure levels of IgG subclass antibodies in symptomatic and asymptomatic subjects. The possible association between the presence of anti–Shigella IgG subclass antibodies in serum samples obtained from subjects before natural exposure to Shigella organisms and the subsequent development of

### Table 1. Concentration of anti-Shigella IgG antibodies in patients infected by S. sonnei or S. flexneri 2a.

<table>
<thead>
<tr>
<th>Antibody, mean* (µg/mL) (95% confidence interval)</th>
<th>S. sonnei LPS</th>
<th>S. flexneri 2a LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before infection</td>
<td>7.9 (6.6–9.5) (n = 40)</td>
<td>18.6 (13.4–30.8) (n = 25)</td>
</tr>
<tr>
<td>After infection</td>
<td>30.3 (18.2–50.4) (n = 40)</td>
<td>127.9 (75.9–214.9) (n = 31)</td>
</tr>
</tbody>
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* Geometric mean titer of each serum sample was determined by linear regression analysis of 8 double dilutions. Conversion of results to µg/mL specific antibody was performed using standards with known antibody concentrations present on same plate.

### Table 2. IgG subclass antibody response in paired serum samples from persons exposed to S. sonnei or S. flexneri 2a infection.

<table>
<thead>
<tr>
<th>Human IgG subclass</th>
<th>S. flexneri–infected (n = 44)</th>
<th>S. sonnei–infected (n = 41)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG1</td>
<td>38.6</td>
<td>95.1</td>
<td>.001</td>
</tr>
<tr>
<td>IgG2</td>
<td>68.2</td>
<td>9.67</td>
<td>.001</td>
</tr>
<tr>
<td>IgG3</td>
<td>6.8</td>
<td>7.07</td>
<td>.001</td>
</tr>
</tbody>
</table>

NOTE. Levels of IgG subclasses were tested before and after exposure to either S. flexneri or S. sonnei. Results are % of subjects with significant humoral response, defined as >4-fold increase in geometric mean titer. Statistical significance was calculated using χ² test.
Analysis in this study of the IgG subclass distribution of anti–Shigella LPS antibodies showed distinct patterns of IgG subclass response after S. sonnei or S. flexneri infections. In the IgG response against S. sonnei, IgG1 was the major component produced, and an increase in IgG2 and IgG3 antibodies was also evident. In contrast, the anti–S. flexneri 2a response was marked by a predominant increase in IgG2 and, to a lesser extent, in IgG1 and IgG3. This study is the first to report differences in the IgG subclass response against the two species of Shigella. Using relative titer as an estimate of subclass concentration, Islam et al. [14] reported that the response to Shigella dysenteriae 1 and S. flexneri Y LPS is initially an increase in the order IgG2 > IgG1 > IgG3 > IgG4, which changes later to IgG2 > IgG3 > IgG1 > IgG4. In our study, we evaluated the subclass concentration of each specific anti-Shigella antibody and found that the first pattern is consistent with our findings on the distribution of anti–S. flexneri 2a IgG subclasses, but we did not detect the late pattern in our cohort in spite of measurements taken 10–14 days after exposure. In contrast, it seems that the pattern of IgG subclass response to S. sonnei infection is unique among the genus Shigella, being predominantly composed of the IgG1 subclass. For another study, Persson et al. [15] reported that the major IgG subclass to appear after S. flexneri infections was IgG1, although substantial amounts of IgG2 were also present. In that study, 8 of 13 patients were children <2 years of age, who produced predominantly IgG1 subclass antibodies against polysaccharide antigens, thus differing from adults in the production of antibodies [16, 17].

The immune response to bacterial LPS and carbohydrate antigens is considered to be T cell–independent, and the IgG antibodies elicited are primarily restricted to the IgG2 subclass (reviewed in [9, 18]). This phenomenon is not strict and seems to be dependent also on the type of bacteria. Humans vaccinated with group A meningococcal polysaccharides produced both IgG1 and IgG2, although IgG1 was predominant in some sera and IgG2 in others [19]. Human antibodies to streptococcal and pneumococcal carbohydrates in adults were found to be mainly IgG2 [17, 20, 21], while immunization with Haemophilus influenzae type b (Hib) capsular polysaccharides triggered the production of both IgG1 and IgG2 antibodies [22]. It should be stressed, however, that vaccination with a conjugate of (Hib) polysaccharide and tetanus toxoid resulted in mainly IgG antibodies, with the highest increase in the IgG1 subclass [23]. The difference in the immune responses could be explained by the observation that (Hib) polysaccharide is a T-independent antigen, whereas glycoconjugates elicit a T-dependent response. A whole bacterium, such as Vibrio cholerae, was found to effectively stimulate antitoxin antibody production of all IgG subclasses during natural infection [24].

It is thus evident from the above-mentioned studies that the pattern of antipolysaccharide IgG subclass response depends on the bacterial polysaccharide involved; in this study, we found that it may also vary within a given bacterial species, as is the case with S. sonnei and S. flexneri 2a. One implication...
of our findings is that for purposes of evaluation of previous exposure, it is preferable to measure IgG1 antibodies in cases of suspected *S. sonnei* infections, while for *S. flexneri*, IgG2 anti-LPS antibodies should be measured.

The present study focused on the description of the pattern of IgG subclass response in subjects exposed to *S. sonnei* and *S. flexneri* 2a and the association of these parameters with the risk of developing symptomatic *Shigella* infection. This study was carried out as a further step following our previous findings pointing out that preexisting total IgG anti-LPS antibodies are good markers of acquired natural immunity against shigellosis [5]. We demonstrated in the present study that persons who showed resistance to *S. sonnei* had elevated IgG1 anti-LPS antibodies, while those resistant to *S. flexneri* 2a showed elevated levels of both IgG1 and IgG2 anti-*S. flexneri* LPS. The correlation between high levels of IgG1 anti-LPS antibodies and resistance to both *S. sonnei* and *S. flexneri* may suggest that this IgG subclass is of importance in the antibacterial protective response. In complement-mediated bactericidal assays, it was demonstrated by other investigators that IgG1 antibodies against capsular polysaccharides of (Hib) were more efficient than IgG2 antibodies and required lower concentrations of complement for comparable bacteriolytic activity. IgG1 anti–capsular polysaccharides were also more efficient than IgG2 in protecting rats from bacteremia with *H. influenzae* [25, 26]. The anti-*Shigella* IgG1 subclass may be involved in protection against shigellosis by exerting similar functions. This does not exclude the potential role played by other arms of the immune response, such as production of specific secretory IgA at the mucosal level or cell-mediated immune mechanisms in protection against shigellosis.

In conclusion, the results presented in this study indicate that in sera of naturally infected subjects, the patterns of IgG subclass anti–*Shigella* LPS response may vary among different *Shigella* species and that the level of preexisting *Shigella* IgG subclass antibodies correlates with resistance to symptomatic homologous infection.

References