Demonstration of Antibodies Against *Yersinia enterocolitica* Lipopolysaccharide in Human Sera by Enzyme-Linked Immunosorbent Assay

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Antibodies against *Yersinia enterocolitica* serotype O:3 lipopolysaccharide present in sera from patients with *Yersinia* infection were studied by using an enzyme-linked immunosorbent assay. Of the sera with significant bacterial agglutination titers against *Y. enterocolitica* type O:3, 86% contained anti-lipopolysaccharide antibodies of the immunoglobulin G class. With the sera of some patients, we demonstrated increasing anti-lipopolysaccharide antibody levels of immunoglobulin G class in spite of decreasing bacterial agglutination titers. The assay was specific for lipopolysaccharide from *Y. enterocolitica* type O:3, and in inhibition experiments lipopolysaccharide could be detected in amounts of ≥0.5 μg/ml.

Infections caused by *Yersinia enterocolitica* serotypes O:3 and O:9 are associated with a strong antibody response. Isolation of bacteria from feces is not always achieved, and consequently the diagnosis often depends on serology (1), the antibodies being demonstrated by bacterial agglutination (21). The antibody titers usually rise a few days after the onset of clinical symptoms and decline within 2 to 6 months, but sometimes high titers are seen for up to 2 years (1).

The symptoms caused by *Yersinia* infections are mainly abdominal and usually are not serious. Occasionally, however, the infection is associated with extraintestinal symptoms such as "reactive" arthritis, carditis, iritis, and erythema nodosum (2, 3). In such complicated cases of yersiniosis, the bacterial agglutination titers tend to be high (1, 2). A further evaluation of the immune response in *Yersinia* infection could thus help toward the understanding of the pathogenesis of the complications.

The enzyme-linked immunosorbent assay (ELISA) (8, 9) offers a sensitive and specific method for assaying various class-specific antibodies (18). Experimentally induced antibodies against bacterial lipopolysaccharide (LPS) can be detected by ELISA (6), and the method has been used for the demonstration of antibodies against LPS from *Brucella abortus* and *Y. enterocolitica* type O:9 in rabbit antisera (5). ELISA has also found clinical application in the serological diagnosis of salmonella infections (7).

We have used the ELISA to study the occurrence of antibodies of immunoglobulin G (IgG) class against *Y. enterocolitica* LPS in the sera of patients with *Yersinia* infection.

**MATERIALS AND METHODS**

**Bacterial antigens.** *Y. enterocolitica* serotype O:3 and *Y. enterocolitica* serotype O:9 were cultivated on blood agar plates for 48 h at 22°C (21). For agglutination tests, bacteria were harvested and washed in saline and used in a suspension giving a Klett turbidity value of 90 at 620 nm (Klett-Summmerson photoelectric colorimeter; Klett Mfg. Co., Inc., New York, N.Y.) corresponding to a concentration of approximately 4 × 10¹⁰ bacteria per ml.

For the isolation of LPS, *Y. enterocolitica* type O:3 were harvested in distilled water, washed, and killed by adding formalin to a final concentration of 1%. LPS was extracted by the hot phenol-water method (20). The purity of the LPS preparation was checked by measuring the ribonucleic acid (RNA) (20) and protein (14) contents. The LPS preparation prepared in this way and used in the subsequent studies contained 15.6% RNA and no demonstrable protein.

LPS from *Escherichia coli* and *S. typhi* (*E. coli* O111:B4 LPS and *S. typhi* 0901 LPS) were purchased from Difco Laboratories, Detroit, Mich. A suspension of heat-killed *B. abortus* was obtained from the Public Health Laboratory, Helsinki, Finland.

**Bacterial agglutination.** Bacterial agglutination tests were performed on standard microtiter trays by using serial twofold dilutions of sera in 25 μl of saline and equal volumes of the bacterial suspension. Agglutination was read after incubation overnight at room temperature, and a titer (defined as the reciprocal of the highest serum dilution showing a definite agglutination) above 160 was regarded as positive.

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Sera. A total of 94 sera, each showing a bacterial agglutination titer ≥160 against Y. enterocolitica type O:3, were used in this study. They were obtained from 88 patients, including 17 males and 51 females (mean age, 33; range, 3 to 75 years), with clinically suspected yersiniosis. One serum sample was obtained from each of 53 patients, whereas serial serum samples were collected from 15 patients. In addition, three sera with agglutinating antibodies against Y. enterocolitica type O:9 were studied.

A pool of nine sera with a bacterial agglutination titer of 1,280 was prepared and used as a positive reference serum. Sera from healthy blood donors were used as controls, and a negative reference pool was prepared from these sera. A further control group consisted of 22 sera from patients with systemic lupus erythematosus (kindly provided by B. Skrifvars IV, Department of Medicine, University Central Hospital, Helsinki, Finland).

Ig fractions of sera. IgG and IgM fractions of five individual patient sera were separated by using diethylaminoethyl-cellulose (Pharmacia Fine Chemicals AB, Uppsala, Sweden) (19). The IgG and IgM concentrations in whole sera and in the concentrated Ig fractions were determined by single radial immunodiffusion on Tri-Partigen immunodiffusion plates (Behringwerke AG, Marburg/Lahn, W. Germany). The sera and the separated fractions were then tested in dilutions containing the same Ig concentrations.

Reduction of IgM antibodies was performed by mixing 100 μl of 0.2 M 2-mercaptoethanol with 100 μl of sera diluted 1:100 and incubating the mixture overnight at room temperature. Anti-Y. enterocolitica LPS ELISA. The ELISA was performed essentially as described earlier (5, 10). Disposable 1-ml polystyrene microcuvettes (Finnpipette, Labsystems, Helsinki, Finland) were incubated with 200 μl of LPS solution (0.1 to 10 μg of LPS/ml of 0.05 M carbonate buffer, pH 9.6) for 3 h at 37°C. After washing with phosphate-buffered saline (pH 7.2), containing 0.05% Tween 20 (PBS-T), the cuvettes were incubated for 5 h at room temperature with 175 μl of sera diluted in PBS-T. The cuvettes were then washed again, and 150 μl of a sheep anti-human IgG-alkaline phosphatase conjugate (10) diluted in PBS-T was added to the cuvettes and left at room temperature overnight. The cuvettes were then washed as described above and rinsed again with distilled water before the addition of the substrate solution (1 mg of Sigma 104 phosphatase substrate; Sigma Chemical Co., St. Louis, Mo., per ml of a 1 M diethanolamine buffer, pH 9.7, containing 0.5 mM MgCl2). The enzymatic reaction was allowed to proceed for 60 min at 37°C, after which the reaction was stopped by 250 μl of 0.16 M NaOH. The absorbance was measured at a wavelength of 404.7 nm vertically through the cuvettes by using an automated nine-channel photometer (Finnpipette analyzer FP-9, Labsystems, Helsinki, Finland). PBS-T in LPS-coated cuvettes and sera in noncoated cuvettes were run as technical controls.

Inhibition studies. Inhibition studies with LPS were performed by mixing equal volumes of sera diluted 1:50 and solutions containing LPS (0.001 to 100 μg/ml). After incubation overnight at 4°C, the sera were tested in the ELISA in a final dilution of 1:500. Absorptions with whole bacteria were performed by using equal volumes of sera diluted 1:500 and dilutions of the bacterial suspensions. After overnight incubation and centrifugation, the supernatants were tested.

RESULTS

Initial studies using the pooled positive anti-Yersinia reference serum. To find the optimal LPS concentration, the pooled positive reference serum was tested in the ELISA after coating the cuvettes with various amounts of LPS. The amount of antibody bound, as reflected by the shape of the titration curve, was dependent on the antigen concentration used (Fig. 1). Based on these experiments, a concentration of 1 μg of LPS per ml was chosen for coating in the subsequent studies.

Specificity of the assay. Complete inhibition of antibody binding to Y. enterocolitica type O:3 LPS-coated cuvettes was seen after absorption of sera with Y. enterocolitica type O:3 bacteria, whereas no effect was seen after absorption with Y. enterocolitica type O:9 or B. abortus (Table 1). Likewise, LPS extracted from Y. enterocolitica type O:3 inhibited the antibody activity, whereas the addition of up to 0.1 mg of E. coli LPS or S. typhi LPS to the sera did not interfere with the results of the assay (Table 2).

![Fig. 1. The pooled positive anti-Y. enterocolitica type O:3 reference serum titrated in the anti-LPS ELISA by using the following concentrations of antigen in the coating process (micrograms of LPS extracted from Y. enterocolitica type O:3 per milliliter of carbonate buffer): 5 (■); 1 (□); 0.5 (▲); and 0.1 (△).](http://jcm.asm.org/Downloadedfromhttp://jcm.asm.org/)
The inhibition experiments were also used to quantitate free LPS. The sensitivity of the assay was found to be greatest when using an initial serum dilution of 1:100. In this way, 0.5 μg of Y. enterocolitica type O:3 LPS per ml still caused a significant decrease of the absorbance in the ELISA (Fig. 2).

**Testing of individual sera in the anti-LPS ELISA.** Representative titration curves of individual sera from patients with *Yersinia* infection are shown in Fig. 3. The slopes of the individual titration curves vary, and a remarkable prozone effect was seen with many sera. To study this phenomenon, the effect of IgM antibodies on the assay was evaluated. When the isolated IgG fractions of five individual sera were assayed, no prozone was seen, and the slopes of the titration curves ran almost in parallel (Fig. 4). The bacterial agglutination titers of the IgG fractions were 3 to 5 titer steps below the titers of the IgM fractions which corresponded to the titers of the unFractionated sera. A total of 20 sera were treated with 2-mercaptoethanol; this reduction did not affect the results obtained in the ELISA but resulted in a significant decrease in the bacterial agglutination titers (*P* < 0.01 by the Wilcoxon signed-rank test).

To minimize the day-to-day variation in absorbance values (10 to 20%), results were expressed in relation to the pooled positive reference serum run simultaneously. A dilution of 1:5,000 was chosen as standard (Fig. 1 and 3). When the absorbance value of the pooled reference serum was expressed as 100 ELISA units, the individual sera investigated gave results of relative binding ranging from 3 to 220 U. The 59 sera from blood donors and the 22 systemic lupus erythematosus sera tested gave a mean value of 3 ± 1 standard deviation. Values below 3 equaled the technical controls. The three sera with agglutinating antibodies against *Y. enterocolitica* type O:3 gave results comparable to those for the normal controls.

**Correlation between bacterial agglutination titers and results obtained in the anti-LPS ELISA.** Antibodies of IgG class against LPS extracted from *Y. enterocolitica* type O:3 were detected in 86% of the sera from the patients with yersiniosis. Figure 5 shows the anti-LPS ELISA units plotted against the corresponding bacterial agglutination titers. Although considerable divergence of results from the two assays was evident with many individual

### Table 1. Specificity of the assay for antibodies against *Y. enterocolitica* type O:3

<table>
<thead>
<tr>
<th>Organism</th>
<th>Absorbance with the following amt of bacteria added/ml*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 × 10⁶ 1 × 10⁶</td>
</tr>
<tr>
<td><em>Y. enterocolitica</em> type O:3</td>
<td>0.020 0.360</td>
</tr>
<tr>
<td><em>Y. enterocolitica</em> type O:9</td>
<td>1.130 1.120</td>
</tr>
<tr>
<td><em>B. abortus</em></td>
<td>1.280 1.060</td>
</tr>
</tbody>
</table>

* Equal volumes of the pooled positive anti-*Y. enterocolitica* type O:3 serum (diluted 1:500) and the bacterial suspensions were mixed and incubated overnight at 4°C. After centrifugation, the supernatants were tested in the ELISA. Absorbance values were obtained at the final serum dilution of 1:1,000.

### Table 2. Specificity of the assay for antibodies against LPS extracted from *Y. enterocolitica* type O:3

<table>
<thead>
<tr>
<th>Source of LPS</th>
<th>Absorbance with the following amt of LPS added (mg/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1 0.01 0.001</td>
</tr>
<tr>
<td><em>Y. enterocolitica</em> type O:3</td>
<td>0.820 1.680 1.890</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>2.000 1.930 1.950</td>
</tr>
<tr>
<td><em>S. typhi</em></td>
<td>2.040 1.930 1.980</td>
</tr>
</tbody>
</table>

* Equal volumes of the pooled positive anti-*Y. enterocolitica* type O:3 serum (diluted 1:500) and the LPS solutions were mixed, incubated overnight at 4°C, and then tested in the ELISA. Values were obtained at a final serum dilution of 1:500.

![Fig. 2. Quantitation of *Y. enterocolitica* type O:3 LPS by absorption of the pooled positive anti-*Y. enterocolitica* type O:3 reference serum with *Y. enterocolitica* serotype O:3 LPS. Symbols: □, pooled positive reference serum; ○, pooled negative reference serum, final serum dilution 1:1,000.](http://jcm.asm.org/Downloadedfrom)
was obtained 2 to 4 weeks after the onset of clinical symptoms. Three characteristic types of results were obtained (Fig. 6). In some cases, the curves for the agglutinating antibodies and the ones for the IgG anti-LPS antibodies run in parallel (Fig. 6a). In some cases, there was an initial increase in the IgG anti-LPS antibodies when the agglutination titer already was decreasing (Fig. 6b and c), and in some cases this increase in anti-LPS antibodies of IgG class was very late (Fig. 6d, e, and f).

**DISCUSSION**

Although antibody affinity affects the results of ELISA (4), the method can be regarded as quantitative. Being sensitive, specific, and easy to perform, ELISA offers many advantages compared with other serological methods. We have developed an ELISA for antibodies against *Y. enterocolitica* type O:3 LPS and used it to quantify antibodies of IgG class in patient sera.

The assay was specific for *Y. enterocolitica* type O:3 LPS, because the inhibition experiments performed with *Y. enterocolitica* type O:9 or with LPS from *E. coli* and *S. typhi* did not affect the results. Since the LPS preparation used for coating the cuvettes had some RNA contamination, we further tested the specificity...
by assaying a group of sera from patients with systemic lupus erythematosus which are known to contain anti-RNA antibodies (17). These sera gave results similar to those of the normal controls, indicating that the RNA contamination did not affect the results.

The titration curves of some sera in this study exhibited a prozone. In an assay such as ELISA, antibodies of a different class present in the same serum sample may compete for the antigenic determinants (11). Such competition, apparently principally by IgM antibodies, appeared to be the probable cause of the prozone effect in this study, because no prozone was seen when isolated IgG fractions of individual sera were studied (Fig. 4). Competition between antibodies of different classes, as well as differences in avidity, could also explain the variations in the shape of the titration curves of whole sera.

The serum dilution of 1:5,000 chosen for expression of the results as units was in each case well beyond a possible prozone and should not cause any false-negative results.

The development of an assay specific for antibodies of IgG class against Y. enterocolitica LPS was motivated by our interest in the extraintestinal "reactive" complications occasionally seen in patients with yersiniosis (2, 3). The pathogenesis of these conditions has not been clarified, nor is it known whether the prolonged presence of bacteria or bacterial products is a prerequisite for the development of these complications. It is generally assumed that immunological mechanisms in combination with host factors are of importance (12, 15). Bacterial LPS is a potent mediator of diverse biological effects (16), but its possible role, if any, in the pathogenesis of the complications in yersiniosis is not
known. In this study, some patients exhibited elevated IgG anti-LPS values for long periods of time. The rise in anti-Y. enterocolitica LPS antibodies of IgG class despite a decline of bacterial agglutination titers observed in some cases with joint symptoms was interesting and encourages further studies in patients with reactive conditions.

Our ELISA was primarily designed to detect antibodies against LPS, but the inhibition studies indicate that the assay may be modified for the quantitation of LPS. In terms of sensitivity, such an inhibition assay cannot compete with, for instance, the Littorus amoebocyte lysate test (13) which can detect picogram quantities of LPS (22). A modification of the anti-LPS ELISA offers, however, a method for detecting specific LPS in concentrations down to 0.5 µg/ml and may be useful for some purposes.

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