Protection Against Keratoconjunctivitis Shigellosa Induced by Immunization with Outer Membrane Proteins of *Shigella* spp.

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Active immunization of guinea pigs and rabbits with outer membrane proteins (OMP) isolated from *Shigella flexneri* 3a and *Shigella sonnei* phase I protected the animals against keratoconjunctivitis shigellosa induced with the homologous or heterologous strain. Protection was also achieved in rabbits after passive immunization with anti-OMP immune serum. Active immunization with lipo-polysaccharide of *S. flexneri* 3a did not protect rabbits against keratoconjunctivitis shigellosa.

The role of the outer membrane proteins (OMP) of *Shigella* in inducing immunity against this organism has not been investigated. The aim of the present study was to examine whether OMP isolated from *Shigella* are protective. The keratoconjunctivitis shigellosa test (10) was used for demonstrating acquired immunity. Our results showed that both active immunization with OMP isolated from *Shigella* and passive immunization with anti-OMP serum protected rabbits and guinea pigs against keratoconjunctivitis shigellosa.

**MATERIALS AND METHODS**

**Bacterial strains.** A virulent strain of *Shigella flexneri* 3a, its avirulent variant, and a virulent strain of *S. sonnei* phase I were used. An avirulent variant of *S. flexneri* 3a was obtained from the virulent parent strain after serial passage on artificial medium (15). The virulent strains consistently caused keratoconjunctivitis in guinea pigs and rabbits.

**Keratoconjunctivitis test.** The keratoconjunctivitis test was carried out according to the method described by Sereny (10). An overnight broth culture was centrifuged, and the bacterial sediment was suspended in saline. Guinea pigs and rabbits’ eyes were inoculated (conjunctival infection) with 5 x 10⁶ and 1 x 10⁸ organisms, respectively, and after 48 h the animals were inspected for the development of keratoconjunctivitis. The disease was classified on the basis of intensity of clinical symptoms (severe or mild). The severe form was characterized by blepharoconjunctivitis associated with discharge of mucopurulent exudate from the infected eye and keratitis; the mild form was characterized by light conjunctivitis with excessive tearing but without keratitis.

**Animals.** Randomly bred guinea pigs weighing 250 to 300 g and albino rabbits weighing 2.5 to 3 kg were used.

**Isolation of OMP.** Bacteria were grown at 37°C in proteose broth containing 1% proteose peptone no. 3 (Difco Laboratories), 0.1% beef extract (Difco), and 0.5% NaCl for 7 h (exponential phase) and harvested by centrifugation. OMP were obtained by Triton X-100 extraction according to the method of Schnaitman (9) with slight modification as follows. The cells were washed with 10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.4) containing 10 mM MgCl₂ to suspend them in the same buffer containing 20 μg of ribonuclease and 20 μg of deoxyribonuclease per ml and then disrupted in an ultrasonic disintegrator for 10 min. The broken cell suspension was centrifuged at 7,000 × g to remove unbroken cells. The supernatant was centrifuged at 200,000 × g for 45 min to sediment the envelope fraction. The cell envelope sediment was extracted (10 mg/ml) twice with 10 mM Tris-hydrochloride buffer (pH 7.4) containing 2% Triton X-100 at room temperature to solubilize the cytoplasmic membrane. After centrifugation at 200,000 × g, the sediment was then extracted twice with the same buffer containing 2% Triton X-100 and 5 mM ethylenediaminetetraacetic acid. This resulted in the solubilization of OMP. The supernatant obtained after centrifugation at 200,000 × g contained OMP which were precipitated with 2 volumes of 95% ethanol. The OMP fraction was shown by sodium dodecyl sulfate-gel electrophoresis to contain 15 to 17 proteins; the major components were 33,000 and 36,000 daltons in size. OMP also contains about 5% lipopolysaccharide (LPS) as calculated from its 3-deoxyoctulosonic acid content (D. Witkowska, G. Adamus, M. Mulczyz, and E. Romanowska, FEMS Microbiol. Lett., in press).

**Preparation of LPS.** LPS was obtained by phenol-water extraction of the dry bacterial mass according to the method described by Westphal and Jann (14) and purified by gel filtration on Sepharose 2B according to Romanowska (7). The LPS preparation did not contain protein and was nearly ribonucleic acid-free (<1%), without absorbance maximum at 260 nm.

**Protein determination.** Protein content was estimated by the method of Lowry et al. (3), with bovine serum albumin (BSA) used as a standard.

**Immunization of animals.** (i) With OMP. Groups of guinea pigs were injected subcutaneously at 1-week
intervals with two protein doses (100 and 150 µg) in complete Freund adjuvant. Rabbits were subcutaneously given OMP in complete Freund adjuvant three times at 1-week intervals. The first dose was 2 mg of OMP; the second and third were 4 mg.

(ii) With LPS. Rabbits were injected intravenously at 3-day intervals, using a series of six injections with increasing LPS doses: 50, 100, 150, 200, 250, and 300 µg.

(iii) With BSA. Guinea pigs were immunized subcutaneously at 1-week intervals with two doses (100 and 150 µg) of standard BSA in complete Freund adjuvant.

The immune status of the immunized animals was checked by the keratoconjunctivitis test, which was performed 10 days after the last injection.

Assay for antibodies. The anti-LPS titer was determined by passive a hemagglutination test (13).

Passive immunization. Rabbits were injected intravenously at 3-day intervals with five 2-ml doses of rabbit immune serum containing antibodies against OMP isolated from the virulent strain of S. flexneri 3a.

RESULTS

Active immunization with OMP. (i) In a homologous system. Most of the animals immunized with OMP from S. flexneri and S. sonnei and challenged with virulent homologous bacteria did not develop keratoconjunctivitis; those of this group that did so showed a mild form of the disease (Table 1). In contrast, each animal of the nonimmunized group developed keratoconjunctivitis in a severe form. OMP isolated from virulent as well as avirulent variants were similarly effective.

(ii) In a heterologous system. To show protection, groups of rabbits and guinea pigs were immunized with OMP from S. sonnei and challenged with virulent S. flexneri 3a. Each immunized rabbit was completely protected against keratoconjunctivitis (Table 2). The lower protection was found for the group of immunized guinea pigs, a high proportion of which developed a mild form of keratoconjunctivitis, whereas all control animals showed a severe form of the disease.

Immunization with LPS and BSA. Rabbits were injected with LPS from the virulent strain of S. flexneri 3a, and before challenge with the homologous strain, their sera were tested for the presence of hemagglutinins. In all immunized animals, an anti-LPS titer ranging from 512 to 1,024 was detected. The rabbits and guinea pigs immunized with LPS and BSA, respectively, were not protected against keratoconjunctivitis induced by S. flexneri (Table 3).

Passive immunization with anti-OMP serum. All three passively immunized rabbits were protected against infection with virulent S. flexneri 3a; none of the animals developed keratoconjunctivitis (Table 4).

DISCUSSION

The experiments reported herein have shown that OMP of Shigella have protective properties. Immunization of rabbits and guinea pigs with OMP isolated from S. flexneri 3a and S. sonnei phase I protects against challenge with virulent S. flexneri 3a.

### TABLE 2. Protection against keratoconjunctivitis in heterologous systems after challenge with virulent S. flexneri 3a

<table>
<thead>
<tr>
<th>Animal species</th>
<th>Immunized with OMP from:</th>
<th>No. of animals developing keratoconjunctivitis after 48 h</th>
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<tbody>
<tr>
<td>Guinea pigs</td>
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<tr>
<td>S. sonnei phase I</td>
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<td>Immunized</td>
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<td>Nonimmunized</td>
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<tr>
<td>Immunized</td>
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<tr>
<td>Nonimmunized</td>
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### TABLE 1. Protection against keratoconjunctivitis in homologous systems

<table>
<thead>
<tr>
<th>Animal species</th>
<th>Immunized with OMP from:</th>
<th>Challenged with virulent:</th>
<th>No. of animals developing keratoconjunctivitis after 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea pigs</td>
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<tr>
<td>S. flexneri 3a virulent</td>
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<td>S. flexneri 3a avirulent variant</td>
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<td>S. flexneri 3a virulent</td>
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<tr>
<td>Nonimmunized</td>
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- **None**: animals with no evidence of keratoconjunctivitis
- **Mild**: animals with mild keratoconjunctivitis
- **Severe**: animals with severe keratoconjunctivitis
gave protection against keratoconjunctivitis induced by the homologous or heterologous strain, indicating that protection afforded by OMP is nonspecific. Such protection also was observed in mice immunized with OMP isolated from a variety of somewhat related or even unrelated strains of Enterobacteriaceae against challenge with S. flexneri 3a (M. Mulczyk, G. Adamus, D. Witkowska, and E. Romanowska, Arch. Immunol. Ther. Exp., in press). Nonspecific protection was also reported in guinea pigs by Manolov and Sereny; clinical resistance of eyes recovered from keratoconjunctivitis shigellosa and reinfe\cent with the heterologous Shigella strain was shown (4, 11). In contrast, specific protection against experimental challenge with Shigella in monkeys (1) and against natural disease in humans (5, 6) was found when living oral vaccines were used for immunization. It should be emphasized, however, that the latter results were obtained on entirely different models.

Although the OMP fraction of S. flexneri 3a or S. sonnei phase I contained low amount of LPS, it is not likely that the admixture of LPS was responsible for the observed protection. It was shown that a pure LPS preparation of S. flexneri 3a did not protect rabbits against challenge with the homologous strain. Moreover, the protective activity of OMP as was mentioned above was not specific; it also was observed when the immunization was performed with OMP derived from the strain serologically unrelated to that used for challenge. LPS present in the OMP fraction may act as adjuvant in the immunization process. Further studies will be done to define the protein components of OMP that are active in protection against keratoconjunctivitis shigellosa.

Another problem arises: how to explain failures of other investigators (4, 12) who were unable to protect animals against keratoconjunctivitis shigellosa by using a variety of vaccine preparations containing proteins such as corpuscular vaccines made of living avirulent, attenuated, or killed Shigella bacteria. Extracts obtained by treating Shigella with trichloroacetic acid, urea, diethylene glycol, and pyridine also proved ineffective. These failures may have been caused by (i) administering vaccine preparations containing amounts of protein insufficient for inducing immunity to keratoconjunctivitis shigellosa or by (ii) the denaturation of proteins during the extraction procedure. Only adsorbed dysentery vaccine and free endotoxin of S. sonnei phase I, which is an LPS-protein complex (6), were found to give protection (2, 12). Both of these preparations however, contained proteins in a relatively native state because a mild extraction procedure was used in their preparation.

It is conceivable that a vaccine preparation containing OMP may also protect humans against natural infection with Shigella.

ACKNOWLEDGMENTS

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LITERATURE CITED


