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

GRAŻYNA ADAMUS,¹ MARIAN MULCZYK,^{2*} DANUTA WITKOWSKA,² AND ELŻBIETA ROMANOWSKA¹

Laboratories of Microbial Immunochemistry¹ and General Microbiology,² Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, 53-114 Wrocław, Poland

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 lipopolysaccharide 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 resuspended in saline. Guinea pigs and rabbits' eyes were inoculated (conjunctival infection) with 5×10^8 and 1×10^9 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 washed with 10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.4) containing 10 mM MgCl₂ were suspended 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 $7,000 \times g$ to remove unbroken cells. The supernatant was centrifuged at $200,000 \times g$ 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 $200,000 \times g$, 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 $200,000 \times g$ 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. Mulczyk, 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 μ g) 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 μ g.

(iii) **With BSA.** Guinea pigs were immunized subcutaneously at 1-week intervals with two doses (100 and 150 μ g) 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 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.*

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

Animal species	Immunized with OMP from:	No. of animals	No. of animals developing keratoconjunctivitis after 48 h		
			None	Mild	Severe
Guinea pigs	<i>S. sonnei</i> phase I	7	1	5	1
	Nonimmunized	7	0	0	7
Rabbits	<i>S. sonnei</i> phase I	3	0	0	0
	Nonimmunized	3	0	0	3

TABLE 1. Protection against keratoconjunctivitis in homologous systems

Animal species	Immunized with OMP from:	Challenged with virulent:	No. of animals	No. of animals developing keratoconjunctivitis after 48 h		
				None	Mild	Severe
Guinea pigs	<i>S. flexneri</i> 3a virulent	<i>S. flexneri</i> 3a	30	22	8	0
	<i>S. flexneri</i> 3a avirulent variant		30	24	6	0
	Nonimmunized		30	0	0	30
	<i>S. sonnei</i> phase I virulent	<i>S. sonnei</i> phase I	8	5	3	0
	<i>S. sonnei</i> phase I avirulent variant		8	6	2	0
	Nonimmunized		8	0	0	8
Rabbits	<i>S. flexneri</i> 3a virulent	<i>S. flexneri</i> 3a	3	3	0	0
	Nonimmunized		3	0	0	3

TABLE 3. Lack of protection in animals immunized with LPS and BSA and challenged with virulent *S. flexneri* 3a

Animal species	Immunized with:	No. of animals	No. of animals developing keratoconjunctivitis after 48 h		
Rabbits	<i>S. flexneri</i> 3a	3	0	0	3
	LPS				
	Nonimmunized	3	0	0	3
Guinea pigs	BSA	7	0	0	7
	Nonimmunized	7	0	0	7

TABLE 4. Protective effect of anti-OMP serum against keratoconjunctivitis in a group of three rabbits challenged with virulent *S. flexneri* 3a

Immunized with	No. of animals developing keratoconjunctivitis after 48 h		
	None	Mild	Severe
Anti- <i>S. flexneri</i> 3a OMP serum	3	0	0
Nonimmunized	0	0	3

sonnei 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 reinfected 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

This work was supported by the Polish Academy of Sciences under grant 10.5.

LITERATURE CITED

1. Formal, S. B., E. H. LaBrec, A. Palmer, and S. Falkow. 1965. Protection of monkeys against experimental shigellosis with attenuated vaccines. *J. Bacteriol.* **90**: 63-68.
2. Kowalewska, D., and M. Mulczyk. 1974. Immunity to keratoconjunctivitis shigellosa in rabbits after oral immunization with *Shigella sonnei* free endotoxin. *Acta Microbiol. Acad. Sci. Hung.* **21**:99-102.
3. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
4. Manolov, D. G. 1957. A study of some questions of pathogenicity and immunity in dysentery using experimental models of *Shigella* keratoconjunctivitis in guinea pigs. *J. Hyg. Epidemiol. Microbiol. Immunol.* **1**:322-328.
5. Mel, D. M., B. L. Arsic, B. D. Nikolic, and M. L. Radovanic. 1968. Studies on vaccination against bacillary dysentery. 4. Oral immunization with live monotypic and combined vaccines. *Bull. W.H.O.* **39**:375-380.
6. Mel, D. M., E. J. Gangarosa, M. L. Radovanovic, B. L. Arsic, and S. Litvinenko. 1971. Studies on vaccination against bacillary dysentery. 6. Protection of children by oral immunization with streptomycin-dependent *Shigella* strains. *Bull. W.H.O.* **45**:457-464.

7. Romanowska, E. 1970. Sepharose gel filtration method of purification of lipopolysaccharides. *Anal. Biochem.* **33**:382-389.
8. Romanowska, E., A. Pelczarska, W. Wnuk, M. Mulczyk, H. Godzińska, and S. Słopek. 1970. Isolation, purification and physico-chemical characteristics of *Shigella sonnei* phase I free endotoxin. *Eur. J. Biochem.* **12**:435-441.
9. Schnaitman, C. A. 1974. Outer membrane proteins of *Escherichia coli*. III. Evidence that the major protein of *E. coli* O111 outer membrane consists of four distinct polypeptide species. *J. Bacteriol.* **118**:442-453.
10. Sereny, B. 1957. Experimental keratoconjunctivitis shigellosa. *Acta Microbiol. Acad. Sci. Hung.* **4**:367-376.
11. Sereny, B. 1959. Acquired natural immunity following recovery from keratoconjunctivitis shigellosa. *J. Hyg. Epidemiol. Microbiol. Immunol.* **3**:292-305.
12. Sereny, B. 1962. A new method for the measurement of protective potency of dysentery vaccines. *Acta Microbiol. Acad. Sci. Hung.* **9**:55-60.
13. Truszczyński, M., and M. E. Baker. 1962. Inhibition with sample sugars of haemagglutination of red cells modified with Salmonella antigens. *J. Immunol.* **89**:154-159.
14. Westphal, O., and K. Jann. 1965. Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure, p. 83. In R. L. Whistler and M. L. Wolfrom (ed.), *Methods in carbohydrate chemistry*. Academic Press, Inc., New York.
15. Witkowska, D., and M. Mulczyk. 1978. Studies on virulence of *Shigella flexneri*. *Arch. Immunol. Ther. Exp.* **26**:603-610.