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Protection against Experimental *Pseudomonas aeruginosa* Infection by Recombinant *P. aeruginosa* Lipoprotein I Expressed in *Escherichia coli*

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Lipoprotein I (OprI) is one of the major proteins of the outer membrane of *Pseudomonas aeruginosa*. OprI is a candidate for a vaccine against *P. aeruginosa*, because it cross-reacts antigenically in all serotype strains of the International Antigenic Typing Scheme. We recently cloned and expressed the gene coding for OprI in *Escherichia coli*. This heterologously expressed OprI was used successfully to immunize mice against *P. aeruginosa*. In addition, OprI from serogroup 12 of *P. aeruginosa* was highly purified by preparative isoelectric focusing and used for immunization of mice. Both vaccines protected the mice against a challenge with a four- to fivefold 50% lethal dose of *P. aeruginosa*.

Outer membrane proteins of *Pseudomonas aeruginosa* (OPRs) have been used successfully for the induction of in vivo protective antibodies in experimental animal models (5, 13, 19). However, the extent to which protection is due to contamination of OPRs by endotoxins is still a matter of debate (5). To exclude the influence of lipopolysaccharide (LPS)-directed antibodies in experimental protection studies with OPRs, several approaches can be used.

Gilleland et al. used lipoprotein F (OprF) that was extracted from the separated protein bands after polyacrylamide gel electrophoresis for immunization (5). However, even in this highly purified preparation, contamination with endotoxin could be demonstrated with the induction of LPS-specific antibodies. The problem of LPS contamination in OPR preparations is the high toxicity of the lipid A portion of the LPS molecule, which causes severe local and systemic side reactions such as fever, chills, and pain at the site of injection (14). Another approach was used recently in our laboratory. Mice were immunized with OPRs from serogroup 12 of *P. aeruginosa*, and protection experiments were performed with all 17 serogroups that have been identified by the International Antigenic Typing Scheme (19).

We have directed our attention towards cloning and heterologous expression of OPR genes and have cloned and characterized the genes coding for OprF and OprI (2, 3). The gene coding for OprI was successfully expressed in *Escherichia coli* (2). In this paper we present evidence that the antibody titers against *P. aeruginosa* which can be induced by OprI expressed in *E. coli* are comparable to those obtained after immunization with OprI isolated from *P. aeruginosa*. Furthermore, by using an infected-mouse model, we could prove that OprI which is free of *P. aeruginosa* LPS contamination can be used successfully as a protective vaccine.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains of *P. aeruginosa* serogroup 6 (ATCC 33354), serogroup 8 (ATCC 33355), and serogroup 12 (ATCC 33359) were obtained as a gift from A. Bauernfeind, Max von Pettenkofer-Institut, Munich, Federal Republic of Germany. *E. coli* K-12 strain JE5513 (7) was a gift of S. Mizushima, Nagoya University, Aichi, Japan. The cloning and expression of the *P. aeruginosa* OprI gene in *E. coli* have been described recently (2). Bacteria were grown on Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.) or in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) for 18 to 20 h with vigorous shaking at 37°C. Bacteria were harvested at late log phase and washed three times with phosphate-buffered saline. The density of the bacteria (in cells per milliliter) was calculated from the optical density at 546 nm. Additionally, serial 10-fold dilutions were performed, samples were plated on agar, and the colonies were counted. For protection experiments, bacteria were harvested at mid-log phase, and after the cells were washed with phosphate-buffered saline, they were adjusted to the required concentration with 0.9% saline.

Purification of OprI. *P. aeruginosa* OprI was purified from serogroup 12 of *P. aeruginosa* and from transformed *E. coli* JE5513 by the method of Inouye et al. (8). The *E. coli*-derived OprI was further purified by a 12% trichloroacetic acid precipitation step which removed mainly OmpF. After centrifugation, the supernatant was treated with an equal volume of acetone to precipitate OprI. The precipitated OprI was washed three times with H_2O and finally suspended in 0.9% saline. The OprI preparation that was purified from *P. aeruginosa* serogroup 12 was subjected to preparative isoelectric focusing as described by Görg and co-workers to reduce the amount of contaminating LPS (6).

Determination of OprI. The concentrations of *P. aeruginosa* OprI during purification and in the final sample used for immunization were determined by rocket electrophoresis by the method of Laurell (9) with the monoclonal antibody 2A1, which is specific for *P. aeruginosa* OprI (12). Isoelectric focusing-purified OprI was used as a standard. The protein content of the standard preparation was determined by a Lowry assay with bovine serum albumin as the standard (10).

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SDS-polyacrylamide gel electrophoresis and Western blotting (immunoblotting). Sodium dodecyl sulfate (SDS)-polyacrylamide slab gel electrophoresis was performed under reducing conditions, as described by Lugtenberg et al. (11). For Western blotting, proteins were transferred after SDS-polyacrylamide gel electrophoresis to nitrocellulose by the method of Towbin et al. (18). OPRs were visualized with specific polyclonal and monoclonal antibodies from mice, as described recently (12).

Enzyme-linked immunosorbent assay. Antibody titers against *P. aeruginosa* in the sera of mice were measured by enzyme-linked immunosorbent assay in microtiter plates coated with sonicated *P. aeruginosa* serogroup 12, 8, or 6 (0.45 μg per well), as described in detail in a previous paper (19). A polyclonal antiserum against OPRs induced in mice (enzyme-linked immunosorbent assay titer, 1:100,000) was used as a standard.

Protection experiments. Female BALB/c mice, 12 to 16 weeks old, received 100 μl (27 μg) of OprI suspended in 100 μl of 1.5% $Al(OH)_3$ intraperitoneally on days 0, 14, 35, and 63. Controls received $Al(OH)_3$ only. Animals were bled from the tail vein for serum collection to determine antibody titers in individual mice. Groups of 20 to 24 immunized or control mice were challenged intraperitoneally 10 days after the last immunization with 200 μl of a *P. aeruginosa* serogroup 6 suspension. Mice vaccinated with OprI that was isolated directly from *P. aeruginosa* received 2×10^7 to 5×10^8 living organisms each. Mice vaccinated with recombinant OprI received 6×10^6 to 1×10^8 living organisms each. Survival rates after challenge were monitored for 7 days.

Statistics. Fisher's exact test (20) was used to calculate two-tailed *P* values for significant levels of protection. Values of ≤0.5 were considered significant. The 50% lethal dose (LD_{50}) values were calculated by the method of Reed and Muench (16).

RESULTS

P. aeruginosa OprI was expressed in the lipoprotein-negative *E. coli* K-12 strain JE5513. Bacteria (4×10^{13}, 40 g [wet weight]) produced 3.5 mg of *P. aeruginosa* OprI, as measured by rocket electrophoresis and staining with the *P. aeruginosa* OprI-specific monoclonal antibody, 2A1 (12). After purification by the method of Inouye et al. (8) and a 12% trichloroacetic acid precipitation which removed residual contaminating outer membrane proteins of high molecular weight, such as OmpF, OmpG, and OmpH, the final yield of OprI contained 2 mg of protein, present in one double band in SDS-polyacrylamide gel electrophoresis (Fig. 1). Forty percent of this preparation was identified (by rocket electrophoresis) as *P. aeruginosa* OprI. After purification of OprI from *P. aeruginosa* serogroup 12, 9 mg of OprI was obtained from 40 g (wet weight) of bacteria. After preparative isoelectric focusing, 8 mg of electrophoretically pure OprI was isolated. The endotoxin contamination, as measured by the *Limulus* assay (4), was 10 μg/mg of protein. In order to test the ability of both preparations to induce antibodies against *P. aeruginosa*, BALB/c mice received four injections of 27 μg each of either *P. aeruginosa* OprI or *E. coli*-derived OprI suspended in $Al(OH)_3$ on days 0, 14, 35, and 63. Antibody titers against *P. aeruginosa* serogroup 6 were measured by enzyme-linked immunosorbent assay.

The median antibody titers in the groups immunized with the OprI derived from *E. coli* and the OprI purified from *P. aeruginosa* were 20 and 60.5%, respectively, of those obtained with polyclonal antiserum (data not shown). To test

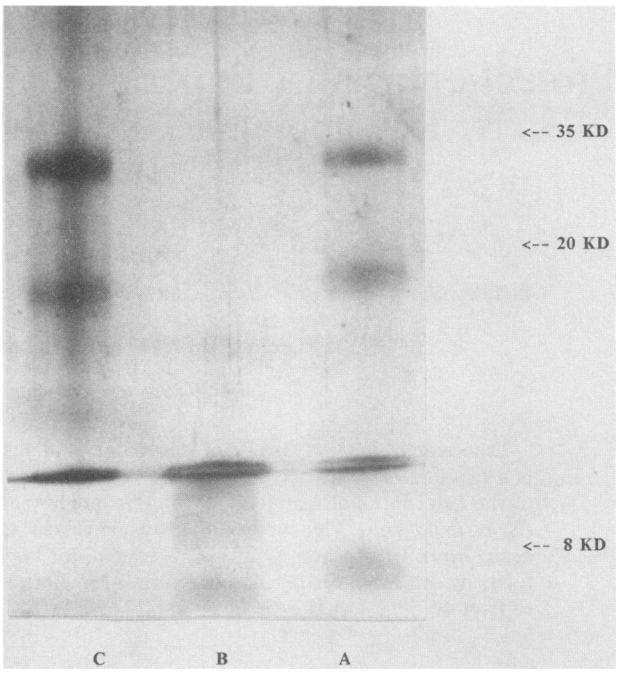

FIG. 1. SDS-polyacrylamide gel electrophoresis of purified OprI from *E. coli*. Lane A, Fraction after the last purification step by the method of Inouye et al. (8). Beside OprI, two bands of higher molecular weight (20 and 35 kilodaltons [KD]) can be seen. Lane B, Supernatant of fraction in lane A after precipitation with 12% trichloroacetic acid. Lane C, Fraction in lane A precipitated by 12% trichloroacetic acid.

the protective ability of the induced antibodies against infection with *P. aeruginosa* serogroup 6, groups of immunized and control mice ($n = 20$) received between 6×10^6 and 5×10^8 live *P. aeruginosa* organisms intraperitoneally on day 73. Survival was monitored for 7 days. An LD_{50} of 5×10^7 bacteria was observed for nonimmunized control mice (Fig. 2).

Immunization with OprI purified from *P. aeruginosa* increased the LD_{50} up to fivefold of that of the control (Fig. 2A), and immunization with the heterologously expressed OprI increased the LD_{50} up to fourfold of that of the control (Fig. 2B). The results of the group challenged with 10^8 bacteria were found to be significant, as analyzed in a Fisher exact test (Table 1).

DISCUSSION

LPS-based vaccines against *P. aeruginosa* have been used successfully in animals (15) as well as in humans (17). However, in spite of convincing results in burn patients, the clinical use of LPS-based vaccines has been questioned because of the toxicity of the lipid A portion of the LPS molecule (14).

In other studies, OprF or nonpurified OPRs were used successfully (5, 19). Until now, OprI has not been considered as a candidate for vaccine, since its surface accessibility to antibodies has been questioned (1). Our recent work with monoclonal antibodies against *P. aeruginosa* OprI (12) has clearly shown that epitopes of OprI are accessible for antibodies. This result was confirmed by this study. Since the isolation of LPS-free OPRs is extremely difficult, the contribution of antibodies against *P. aeruginosa* LPS to the

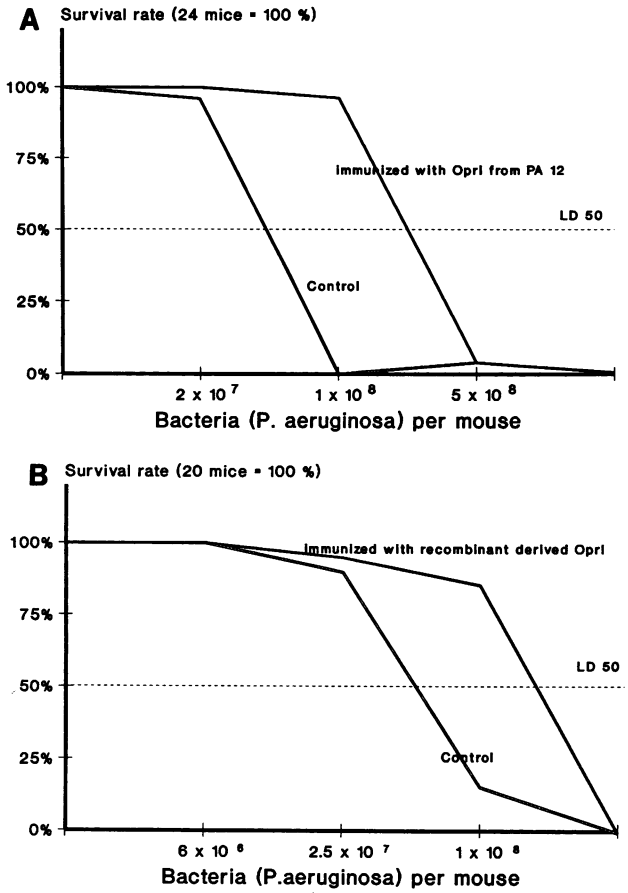

FIG. 2. Shift of LD_{50} and survival rate of BALB/c mice after immunization with OprI from *P. aeruginosa* serogroup 12 (PA 12) followed by challenge with *P. aeruginosa* serogroup 6 (A) and after immunization with recombinant derived OprI followed by challenge with *P. aeruginosa* serogroup 6 (B).

observed protection could not be excluded until now. We have used two different approaches to obtain OprI which is free of *P. aeruginosa* LPS: isoelectric focusing of *P. aeruginosa* OprI and heterologous expression of the *P. aeruginosa* OprI gene in *E. coli*.

The protection experiments carried out with mice showed that mice vaccinated with OprI that was purified by isoelectric focusing were protected against a challenge of *P. aeruginosa* that was five times the LD_{50}. Mice which were vaccinated with the *E. coli*-derived *P. aeruginosa* OprI were protected against challenge that was four times the LD_{50}. The observed slight differences in the degrees of protection after immunization with *E. coli*-derived OprI and *P. aeruginosa*-derived OprI could be explained by slight differences in the posttranslational observed acylation or by the different purification procedures. The results obtained with the heterologously expressed OprI are the first proof that an OPR which is free of *P. aeruginosa* LPS can provide significant protection against fatal *P. aeruginosa* infection.

TABLE 1. Statistical analysis of survival rates of mice[a]

Vaccine	No. of survivors/no. tested (% survival) in group	
	Immunized	Control
OprI from PA 12	23/24 (95.8)	0/24 (0)
Recombinant OprI	17/20 (85)	3/20 (15)

[a] Mice were vaccinated with OprI purified from *P. aeruginosa* serogroup 12 (PA 12) and with recombinant heterologously expressed OprI purified from *E. coli* K-12 JE5513. $P < 0.005$ for values in the same row.

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LITERATURE CITED

1. **Braun, V., V. Bosch, E. R. Klumpp, and I. Neff.** 1976. Antigenic determinants of murein lipoprotein and its exposure at the surface of enterobacteriaceae. Eur. J. Biochem. **62**:553–566.
2. **Duchêne, M., C. Barron, A. Schweizer, B.-U. von Specht, and H. Domdey.** 1989. *Pseudomonas aeruginosa* outer membrane lipoprotein I gene: molecular cloning, sequence, and expression in *Escherichia coli*. J. Bacteriol. **171**:4130–4137.
3. **Duchêne, M., A. Schweizer, F. Lottspeich, G. Krauss, M. Marget, K. Vogel, B.-U. von Specht, and H. Domdey.** 1988. Sequence and transcriptional start site of the *Pseudomonas aeruginosa* outer membrane porin protein F gene. J. Bacteriol. **170**:155–162.
4. **Friberger, P., M. Knös, and L. Mellstam.** 1982. A quantitative endotoxin assay utilizing LAL and a chromogenic substrate. Progr. Clin. Biol. Res. **93**:195–206.
5. **Gilleland, H. E., Jr., M. G. Parker, J. M. Matthews, and R. D. Berg.** 1984. Use of a purified outer membrane protein F (porin) preparation of *Pseudomonas aeruginosa* as a protective vaccine in mice. Infect. Immun. **44**:49–54.
6. **Görg, A., W. Postel, and R. Westermeier.** 1978. Ultrathin layer isoelectric focusing in polyacrylamide gels on cellophane. Anal. Biochem. **89**:60–70.
7. **Hirota, Y., H. Suzuki, Y. Nishimura, and S. Yashuda.** 1977. On the process of cellular division in *Escherichia coli*: a mutant of *E. coli* lacking a murein-lipoprotein. Proc. Natl. Acad. Sci. USA **74**:1417–1420.
8. **Inouye, S., K. Takeishi, N. Lee, M. DeMartini, A. Hirashima, and M. Inouye.** 1976. Lipoprotein from the outer membrane of *Escherichia coli*: purification, paracrystallization, and some properties of its free form. J. Bacteriol. **127**:555–563.
9. **Laurell, C. B.** 1966. Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. Anal. Biochem. **15**:45–52.
10. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurements with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
11. **Lugtenberg, B., J. Meijers, R. Peters, P. van der Hoek, and L. van Alphen.** 1975. Electrophoretic resolution of the major outer membrane proteins of *E. coli* K 12 into 4 bands. FEBS Lett. **58**:245–258.
12. **Marget, M., A. Eckhardt, W. Ehret, B.-U. von Specht, M. Duchêne, and H. Domdey.** 1988. Cloning and characterization of cDNAs coding for the heavy and light chains of a monoclonal antibody specific for *Pseudomonas aeruginosa* outer membrane protein I. Gene **74**:335–345.
13. **Matthews-Greer, J. M., and H. E. Gilleland, Jr.** 1987. Outer membrane protein F (porin) as a protective vaccine against heterologous immunotype strains in a burned mouse model. J. Infect. Dis. **155**:1282–1291.
14. **Morrison, D. C.** 1983. Bacterial endotoxins and pathogenesis. Rev. Infect. Dis. **5**(Suppl. 4):733–747.
15. **Pennington, J. E., W. F. Hickey, F. Blackwood, and M. Arnaut.** 1981. Active immunization with lipopolysaccharide *Pseudomonas* antigen for chronic *Pseudomonas* bronchopneumonia in guinea pigs. J. Clin. Invest. **68**:1140–1148.

16. **Reed, L. J., and H. Muench.** 1938. A simple method for estimating fifty per cent endpoints. Am. J. Hyg. **27**:493–497.
17. **Roe, E. A., and R. J. Jones.** 1983. Immunization of burned patients against *Pseudomonas aeruginosa* infection at Safdarjang Hospital, New Delhi. Rev. Infect. Dis. **5**(Suppl. 5):922–930.
18. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
19. **von Specht, B.-U., G. Strigl, W. Ehret, and W. Brendel.** 1987. Protective effect of an outer membrane vaccine against *Pseudomonas aeruginosa* infection. Infection **15**:408–412.
20. **Zar, J. H.** 1974. The binomial distribution, p. 281–300. *In* J. H. Zar (ed.), Biostatistical analysis. Prentice-Hall, Inc., Englewood Cliffs, N.J.