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Pseudomonas aeruginosa Outer Membrane Protein F Produced in *Escherichia coli* Retains Vaccine Efficacy

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Abstract. *Pseudomonas aeruginosa* outer membrane protein F was purified by extraction from polyacrylamide gels of cell envelope proteins of an *Escherichia coli* strain expressing the cloned gene for protein F. Antisera directed against protein F purified from *P. aeruginosa* PAO1 reacted with this *E. coli* strain by immunofluorescence assay and immunoblotting, whereas these antisera were nonreactive with *E. coli* strains lacking the *Pseudomonas* protein F gene. The protein F purified from this *E. coli* strain was used to immunize mice by intramuscular injection of 10 μg of protein F preparation on days 1 and 14, followed by burn and challenge of the mice on day 28. As compared with control mice immunized with *E. coli* K-12 lipopolysaccharide, immunization with the *E. coli*-derived protein F afforded significant protection against subsequent challenge with heterologous Fisher-Devlin immunotype 5 and 6 strains of *P. aeruginosa*. Antisera from mice immunized with the *E. coli*-derived protein F reacted at bands corresponding to protein F and 2-mercaptoethanol-modified protein F upon immunoblotting against cell envelope proteins of the PAO1, immunotype 5, and immunotype 6 strains of *P. aeruginosa* and the *E. coli* strain containing the cloned F gene, but failed to react at these sites in an *E. coli* strain lacking the F gene. These data demonstrate that *P. aeruginosa* protein F produced in *E. coli* through genetic engineering techniques retains its vaccine efficacy in the complete absence of any *P. aeruginosa* lipopolysaccharide.

We have previously shown that an outer membrane (OM) protein F (porin) preparation purified from *Pseudomonas aeruginosa* PAO1 can function as an effective vaccine to protect against subsequent challenge with heterologous immunotype strains of *P. aeruginosa* in a murine acute infection model [7], in a burned mouse model [16], and in a rat model of chronic pulmonary infection [8]. However, since it is not possible to purify from *P. aeruginosa* OM proteins completely free from contamination with trace amounts of lipopolysaccharide (LPS), the possible role of *P. aeruginosa* LPS in affording the protection provided by the protein F preparation has to be considered. In our previous studies we addressed this issue by comparing control mice immunized with *P. aeruginosa* PAO1 LPS versus test mice immunized with protein F purified from the PAO1 strain upon challenge with various heterologous LPS immunotype strains of *P. aeruginosa*. LPS afforded protection only against challenge with the homologous LPS immunotype strain, whereas protein F protected against challenge with all the heterologous LPS immunotype strains. In the present study we provide more definitive evidence that the protein F in our vaccine preparation is the protective immunogen by purifying *P. aeruginosa* protein F from an *Escherichia coli* strain containing the cloned protein F gene [26]. This *E. coli*-produced protein F, which completely lacks *P. aeruginosa* LPS, retains its vaccine efficacy against challenge with heterologous immunotype strains of *P. aeruginosa* in a burned mouse model.

Materials and Methods

Bacterial strains and growth conditions. The strains of *P. aeruginosa* used included the following: the PAO1 strain (Difco 0-5; Difco Laboratories, Detroit, Michigan) that corresponds to a Fisher-Devlin immunotype 7, ATCC 27584 (Difco 0-6, Fisher-Devlin immunotype 1), ATCC 27313 (Difco 0-11, Fisher-Devlin immunotype 2), ATCC 27314 (Difco 0-2, Fisher-Devlin immuno-

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type 3), ATCC 27315 (Difco 0-1, Fisher-Devlin immunotype 4), ATCC 27585 (Difco 0-10, Fisher-Devlin immunotype 5), ATCC 27579 (Difco 0-7, 8, Fisher-Devlin immunotype 6), and ATCC 27318 (which upon receipt was typed as a Difco 0–untypable strain). All ATCC strains were obtained from the American Type Culture Collection (Rockville, Maryland). The *Pseudomonas cepacia* and *Pseudomonas fluorescens* are College of American Pathologists strains obtained from the Clinical Microbiology Laboratory at Louisiana State University Medical Center (Shreveport, Louisiana) (LSUMC-S). The strains of *Escherichia coli* used included a urine isolate obtained from the LSUMC-S Clinical Microbiology Laboratory and two K-12 strains, strain HB101 (a gift from J. Staczek, LSUMC-S) and strain JF733 (pHN4), which is a porin-deficient strain of *E. coli* containing a protein F-expressing cosmid [26]. The original source of the *E. coli* strain JF733 was J. Foulds, but our laboratory received the JF733(pHN4) strain from Wendy Woodruff and R.E.W. Hancock (Vancouver, British Columbia).

All strains were grown at 30°C with stirring and, except for use in the immunofluorescence assay (see below), all *Pseudomonas* strains and the *E. coli* urine isolate were grown in nutrient broth (Difco). The *E. coli* strain HB101 and all strains used in the immunofluorescence assay, except for the *E. coli* JF733(pHN4), were grown in LB-glucose broth (1% Bacto-tryptone (Difco), 0.5% yeast extract (Difco), 0.5% NaCl, and 0.1% glucose). Growth of the *E. coli* JF733(pHN4) was in LB-glucose broth containing increased salt (1.7%) supplemented with 25 μg/ml tetracycline (Sigma Chemical Co., St. Louis, Missouri).

Purification of protein F. Protein F was purified either from the *P. aeruginosa* strain PAO1 (designated as PsF) or the *E. coli* K-12 strain JF733(pHN4) (designated as EcF) by the gel extraction method of Kabir [12], modified as previously described [7]. Protein determinations were performed by using the Lowry method as modified by Markwell et al. [14]. The 2-keto-3-deoxyoctulosonic acid (KDO) content was assayed by the method of Dröge et al. [3]. PsF preparations used for immunization contained 2.5% protein with 1 μg of *P. aeruginosa* PAO1 LPS per 10 μg of protein. EcF preparations contained 3.5% protein with 2 μg of *E. coli* K-12 JF733(pHN4) LPS per 10 μg protein.

LPS extraction. LPS was extracted from *P. aeruginosa* strain PAO1 and from *E. coli* strain K-12 HB101 by the phenol–water extraction method of Westphal and Jann [24].

Active immunization of mice. Five-week-old specific pathogen-free CD-1 mice (Charles River Breeding Laboratories, Wilmington, Massachusetts) were routinely immunized on days 1 and 14 by i.m. injection into alternate hips. Mice designated as PsF-immunized received 0.2 ml of sterile saline containing 10 μg of PsF. Mice designated as EcF-immunized received 0.2 ml of sterile saline containing 10 μg of EcF. Mice designated as LPS-immunized received 0.2 ml of sterile saline containing 7 μg of *E. coli* K-12 HB101 LPS.

Burn model. Control and experimental mice were immunized as specified above. The scald-burn model of Walker et al. [23] was used, modified as previously described [16]. After burning, the mice were challenged with a 1-2 LD_{50} dose of live *P. aeruginosa*, Fisher-Devlin immunotype 5 or 6, injected subcutaneously at the burn site, and observed for death over a 10-day period. Challenge doses were prepared from 18-h, midlogarithmic-phase-growth cultures grown in nutrient broth.

Statistics. Fisher's exact test was used to calculate P values according to the IBM EpiStat Basic Statistics Program. Values ≤ 0.05 were considered significant.

Western blotting. Western blotting was performed as described by Towbin et al. [22] by use of pooled antisera from two groups of 10–30 mice immunized as described above, either with EcF or PsF.

Immunofluorescence. For the immunofluorescence assays, overnight cultures of bacteria were harvested for 10 min at 7000 *g* at 4°C. The pellets were vortexed and washed twice with 0.05 *M* sodium phosphate buffer, pH 7.0, and resuspended to an absorbance at 660 nm of 0.4. For each bacterial strain, drops containing 200 μl of resuspended organisms were added to three separate microscope slides. These were then subsequently heat- or methanol-fixed and kept at 4°C in a moisture chamber throughout the remainder of the assay. All slides were first rinsed with phosphate-buffered saline containing 1% Tween 20 (Tw-PBS) and then washed twice for 10 min with phosphate-buffered saline (PBS). One slide per bacterial strain was then incubated with either 200 μl of 1 : 10 dilution of murine PsF antisera, a 1 : 10 dilution of murine nonimmune antisera, or PBS for 60 min with shaking. All slides were rinsed with Tw-PBS and washed twice with PBS before adding 200 μl of a 1 : 50 dilution of goat anti-mouse FITC-conjugated IgG (Boehringer Mannheim Biochemicals, Indianapolis, Indiana). The slides were incubated for 30 min before being rinsed with Tw-PBS and washed twice with PBS. Immediately prior to examination, 20 μl of PBS and a coverslip were added to each slide. Micrographs were recorded with an Olympus BH-2 fluorescence microscope equipped with an Olympus 35-mm camera; phase contrast micrographs of the same field were also recorded. Rabbit antisera were used as the source of primary antisera in some fluorescent assays (data not shown). To quantify the intensity of fluorescence, we compared micrographs taken at 2-min exposure times with the micrographs of the homologous LPS immunotype PAO1 strain and graded them on a 0–3+ scale, with the *P. aeruginosa* PAO1 strain assigned a 3+ positive reaction. Negative reactions indicated no fluorescence. These assays were performed on each strain two to five times.

Results and Discussion

The potential exists for the development of clinically useful vaccines based upon OM proteins from a variety of medically important Gram-negative bacteria, such as *Haemophilus influenzae*, the pathogenic *Neisseria*, and *Pseudomonas aeruginosa* [6]. Protein F of *P. aeruginosa* would appear to be an ideal candidate for an OM protein vaccine. Protein F has been considered to be the major porin of the OM of *P. aeruginosa* [27], although recent evidence [9, 10, 25] now suggests a more structural role for protein F analogous to OmpA in *Escherichia coli*. Protein F is found in all strains of *P. aeruginosa* [2, 19], is antigenically conserved in all strains of *P. aeruginosa* [2], and has been shown to be surface-exposed in wild-type strains [13, 20]. Antibodies directed against protein F have been asso-

Table 1. Reactivity of antisera against protein F purified from *Pseudomonas aeruginosa* PAO1 with various strains

Organism	Immunofluorescence
E. coli urine isolate	–
E. coli K-12 HB101	–
E. coli K-12 JF733(pHN4)	++
P. aeruginosa PAO1	+++
P. aeruginosa 27584 (FD1)[a]	++
P. aeruginosa 27313 (FD2)	++
P. aeruginosa 27314 (FD3)	++
P. aeruginosa 27315 (FD4)	++
P. aeruginosa 27585 (FD5)	+
P. aeruginosa 27579 (FD6)	++
P. aeruginosa untypable	+++
P. cepacia	–
P. fluorescens	+

[a] Fisher-Devlin immunotype.

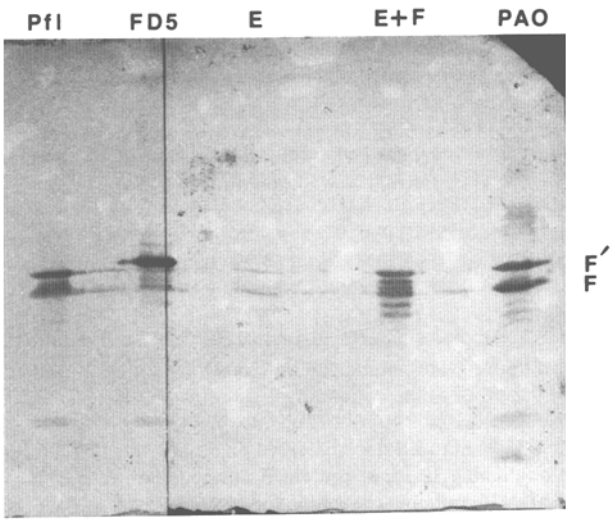

Fig. 1. Western immunoblot of pooled antisera from mice immunized with protein F purified from *Pseudomonas aeruginosa* PAO1 reacted with the cell envelope proteins of the PAO1 strain (PAO), the Fisher-Devlin immunotype 5 strain (FD5), *P. fluorescens* (Pfl), *Escherichia coli* K-12 HB101 lacking the cloned F gene (E), and the *E. coli* K-12 JF733(pHN4) strain containing the cloned F gene (E + F). The locations of unmodified protein F (F) and of 2-mercaptoethanol-modified protein F (F′) are indicated.

ciated with protection in both animal models [11, 18] and human infection [15]. Protein F purified from *P. aeruginosa* has been shown by us [7, 8, 16] to be an effective vaccine in a variety of rodent models of infection. Furthermore, immunization with purified OM protein H [16], E, or G could not afford protection against challenge with any of three heterologous immunotype strains of *P. aeruginosa* [17]. Immunization with purified OM protein D2 did afford protection against two heterologous immunotype strains, but failed to protect against a third strain [17]. There is speculation [1, 5, 21] that OM iron-regulated proteins may possess vaccine potential, but at present protein F remains the leading candidate for further development as an OM protein vaccine for *P. aeruginosa*.

The gene for *P. aeruginosa* protein F has been cloned into *E. coli* by several laboratories [4, 26]. We obtained one of these *E. coli* strains to determine whether protein F produced in *E. coli* would retain its vaccine efficacy in the complete absence of any *P. aeruginosa* LPS. Furthermore, in order to obtain protein F in sufficient quantity to be feasible for commercial vaccine production, such a gene cloning approach might be necessary, provided that the cloned protein retains vaccine efficacy.

Our PsF vaccine elicits no antibodies capable of cross-reacting with normal cells of *E. coli*. We confirmed in an active immunization experiment that PsF immunization offers no protection against *E. coli* challenge. In a control group immunized with *P. aeruginosa* PAO1 LPS, 10 of 25 mice died versus 12 of 25 mice dead in the PsF-immunized group following challenge with the urine isolate of *E. coli*.

Furthermore, there was no evidence in our immunological assays of any antibodies in the murine PsF antisera cross-reacting with the *E. coli* strains lacking the *Pseudomonas* gene insert. In the immunofluorescence assay (Table 1) the PsF antisera failed to give a detectable reaction with either the *E. coli* urine isolate or the *E. coli* K-12 HB101 strain. A Western blot of cell envelope proteins performed with the murine PsF antisera revealed no reaction with the *E. coli* K-12 HB101 strain (E in Fig. 1). In contrast, the *E. coli* K-12 JF733(pHN4) strain, which contains the cloned protein F gene, reacted with the PsF antisera by both immunofluorescence assay (Table 1) and Western blot (E + F in Fig. 1). As previously reported [16], the PsF antisera reacted with all strains of *P. aeruginosa* (Table 1 and Fig. 1, PAO and FD5). These antisera did not cross-react with *P. cepacia* (Table 1), but did with *P. fluorescens* (Table 1 and Pfl in Fig. 1). Note that the expression of the gene for protein F in the JF733 (pNH4) strain of *E. coli* converted its cells from a nonreactive state with PsF antisera to cells that fluoresced to the same degree as the heterologous immunotype strains of *P. aeruginosa* and that fluoresced more intensely than other species of *Pseudomonas*. This confirmed that this strain of *E. coli* indeed was producing a gene product that was recognized by antibodies elicited by PsF.

Table 2. Active immunization of mice with protein F purified from *Escherichia coli*, followed by challenge with strains representing two heterologous Fisher-Devlin immunotypes of *Pseudomonas aeruginosa*

Group	Challenge strain	No. live/ total no.	Survival (%)	P value
E. coli LPS[a]	27585(FD5)[b]	9/25	36	
E. coli-F[c]	27585(FD5)	15/23	65	.04[d]
E. coli LPS	27579(FD6)[e]	15/25	60	
E. coli-F	27579(FD6)	23/25	92	.009

[a] Control mice were immunized on days 1 and 14 by i.m. injection of 0.2 ml of saline containing 7 μg of *E. coli* K-12 LPS.
[b] Challenge dose of 5×10^5 organisms.
[c] Protein F-immunized mice received on days 1 and 14 0.2 ml of saline containing 10 μg of protein F purified from *E. coli* K-12 JF733(pHN4) containing the gene for protein F.
[d] P values were determined by Fisher's exact test.
[e] Challenge dose of 1×10^7 organisms.

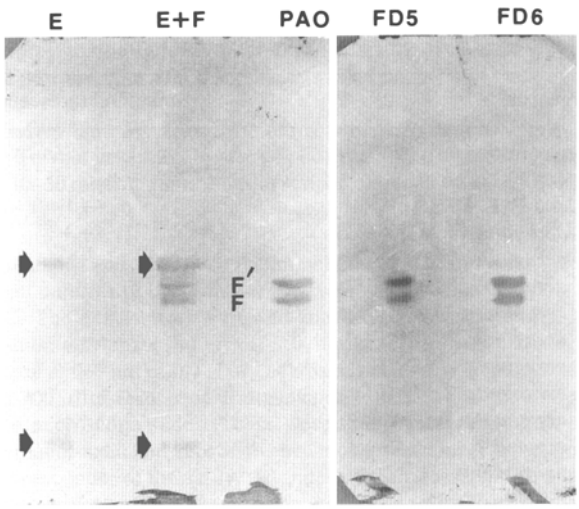

Fig. 2. Western immunoblot of pooled antisera from mice immunized with protein F purified from the *Escherichia coli* K-12 JF733(pHN4) strain containing the cloned F gene reacted with cell envelope proteins from the *E. coli* K-12 HB101 lacking the cloned F gene (E), the *E. coli* K-12 JF733(pHN4) strain containing the cloned F gene (E + F), the *Pseudomonas aeruginosa* strains PAO1 (PAO), Fisher-Devlin immunotype 5 (FD5), and Fisher-Devlin immunotype 6 (FD6). Two bands seen in E and E + F thought to be due to the presence of *E. coli* K-12 LPS in the vaccine preparation are denoted by arrows. The bands corresponding to unmodified protein F (F) and 2-mercaptoethanol-modified protein F (F') are labeled.

Protein F was purified from the *E. coli* K-12 JF733(pHN4) strain and used to actively immunize mice for challenge experiments in the burned mouse model. As compared with controls immunized with *E. coli* K-12 LPS, immunization with the EcF provided significant protection against subsequent challenge with either the heterologous Fisher-Devlin immunotype 5 or immunotype 6 strain of *P. aeruginosa* (Table 2). These data show that OM protein F retains its vaccine efficacy in the complete absence of *P. aeruginosa* LPS. PsF has been shown previously [7, 16] to afford protection to immunized mice against a 2-3 LD_{50} challenge dose. Thus, the EcF appears to afford immunized mice approximately the same level of protection as does PsF.

Desired challenge experiments with the *E. coli* K-12 HB101 strain, the *E. coli* K-12 JF733(pHN4) strain, the *P. fluorescens* strain, or a protein F-deficient mutant of *P. aeruginosa* [16] could not be successfully performed owing to the avirulence of these strains in our model system. The inability to determine whether our protein F vaccine would fail to provide protection against the protein F-deficient mutant is particularly vexing, but this mutant strain completely lacks virulence in our animal model.

Western blots were performed to analyze the antibody response of mice immunized with EcF. Bands corresponding to protein F and to a 2-mercaptoethanol-modified form of F (F') resulted upon immunoblotting against the cell envelope proteins of the *P. aeruginosa* PAO1 strain (PAO in Fig. 2), the Fisher-Devlin immunotype 5 (FD5 in Fig. 2) and immunotype 6 (FD6 in Fig. 2) strains of *P. aeruginosa*, and the *E. coli* K-12 JF733(pHN4) strain (E + F in Fig. 2). Protection in the challenge experiments is believed to be due to these antibodies, which react with the F and F' forms of protein F. Bands corresponding to F and F' were not encountered upon immunoblotting EcF antisera against cell envelope proteins of the *E. coli* strain HB101, which lacks the gene for protein F (E in Fig. 2). The reaction seen in both the HB101 and JF733(pNH4) strains of *E. coli* K-12 (arrows in Fig. 2), but absent in the three *P. aeruginosa* strains, may be owing to antibodies against *E. coli* K-12 LPS contaminating the EcF vaccine preparation.

In conclusion, the data presented here demonstrate that OM protein F of *P. aeruginosa* purified from an *E. coli* strain into which the gene for protein F had been cloned retains its vaccine efficacy in the absence of *P. aeruginosa* LPS in the burned mouse model. These results indicate more conclusively that the protective immunogen in our protein F vaccine preparation is indeed the protein F.

All animals used in this research were handled in accordance with the Animal Care & Use Committee guidelines of LSU School of Medicine in Shreveport, LA.

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