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Recombinant protein composed of *Pseudomonas* exotoxin A, outer membrane proteins I and F as vaccine against *P. aeruginosa* infection

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Abstract We have constructed a chimeric protein composed of the receptor binding and membrane translocation domains of *Pseudomonas* exotoxin A (PE) with the outer membrane proteins I and F, together designated as PEIF. The potential of PEIF as a vaccine against *Pseudomonas* infection was evaluated in BALB/c mice and New Zealand white rabbits. We examined titers of anti-PE and anti-OprF antibodies, and the ability both to neutralize PE cytotoxicity and to increase opsonophagocytic uptake of *Pseudomonas aeruginosa* strain PAO1, serogroups 2 and 6. The results showed that PEIF can induce antibodies not only to neutralize the PE cytotoxicity but also to promote the uptake of various strains of *P. aeruginosa* by murine peritoneal macrophages. In a burned mouse model, PEIF afforded significant protection against infection by the homologous *P. aeruginosa* strain PAO1, heterologous serogroup 2, and the PE hyperproducing strain PA103. These observations thus indicate that PEIF may be used as a novel vaccine against *P. aeruginosa* infection.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that has become a major cause of nosocomial infections in immunocompromised patients, such as individuals with cystic fibrosis, severe burns or neoplasias (Bodey et al. 1983). Therapy for *P. aeruginosa* infection is hindered by the well-known antibiotic resistance intrinsic to the organism (Hancock 1986). Thus, an effective immunotherapy is desirable to supplement conventional antibiotic chemotherapy (Cryz 1984).

P. aeruginosa produces a large number of extracellular products contributing to its virulence, such as *Pseudomonas* exotoxin A (PE), exoenzyme S, elastase, and alkaline proteases. However, the relative importance of each virulent factor may vary depending on the stage of infection (Nicas and Iglewski 1985). PE is the most toxic pathogenic factor (Liu 1974), inhibiting protein synthesis through ADP-ribosylation of elongation factor 2, as does diphtheria toxin (Iglewski and Kabat 1975). Previously, we constructed a deletion of 38 amino acids from the carboxyl terminus, designated as PE(Δ 576-613), which was shown to be nontoxic (Chow et al. 1989). This nontoxic PE retains most of its antigenicity and can induce protective immunity against PE intoxication. However, PE(Δ 576-613) cannot block the colonization of *P. aeruginosa* infection in mice (unpublished data). We therefore attempted to develop an effective vaccine that can induce antibodies not only to neutralize the PE cytotoxicity but also to block bacterial colonization and invasiveness.

The lipopolysaccharides (LPSs) and outer membrane proteins (Oprs) are two major antigenic cell surface components of *P. aeruginosa*. *P. aeruginosa* vaccines based on these two bacterial surface antigens have been developed and tested in various animal models (Cryz et al. 1984; Gilleland et al. 1984; Pier et al. 1978) and clinical trials (Cryz et al. 1987a). The LPS-based vaccines are processed as T-lymphocyte-independent antigens and have a toxic side effect due to the lipid A

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portion of the LPS molecule. In contrast, Oprs are nontoxic and antigenically conserved in all 17 known serogroups of the International Antigenic Typing Scheme (IATS) (Mutharia et al. 1982). Porin protein F (OprF), and lipoprotein I (OprI) are two major outer membrane proteins. Protection against heterologous serogroups of *P. aeruginosa* infection by immunization with purified native or recombinant *Escherichia coli* derived Oprs (LPS-free) has been demonstrated in several animal models (Clement et al. 1994; Finke et al. 1991; von Specht et al. 1987). In order to increase the efficacy of the Oprs-based vaccine, von Specht et al. (1995) fused two different Oprs (OprF and OprI) into a single molecule, and demonstrated that mice immunized with GST-OprF₁₉₀₋₃₄₂-OprI were protected against a lethal *P. aeruginosa* infection, while mice immunized with GST-OprI-OprF₁₉₀₋₃₅₀ were not protected. It has been thought that the different positions of OprI and OprF in the recombinant hybrid antigens may have different effects on the formation and presentation of different epitopes, thus leading to the controversial results obtained with the two recombinant hybrid proteins GST-OprF₁₉₀₋₃₄₂-OprI and GST-OprI-OprF₁₉₀₋₃₅₀ (Specht et al. 1995). However, Finnen et al. (1992) and Hughes et al. (1992) have demonstrated that surface-exposed B-cell epitopes of OprF are located at the C-terminal between amino acid 190 to 350. This observation thus suggests that the most C-terminal part of OprF may be a suitable candidate antigen for conferring protection (Rawling et al. 1995). In this study, we replaced the GST component of GST-OprI-OprF₁₉₀₋₃₅₀ with the receptor binding and membrane translocation domains of PE. The tripartite chimeric protein was designated as PEIF, and the potential of PEIF as vaccine against *P. aeruginosa* infection was examined in a burned mouse model.

Materials and methods

Bacterial strains

The *P. aeruginosa* strain PAO1 corresponding to an IATS serogroup 5 (ATCC 15692) was chosen for OprI and OprF gene cloning. *P. aeruginosa* strain PA103 produces high levels of exotoxin A and low levels of protease (ATCC 29260), and *P. aeruginosa* serogroup 2 (ATCC 33349), serogroup 6 (ATCC 33354), serogroup 8 (ATCC 33355), and serogroup 12 (ATCC 33359) were used to examine the potency of PEIF as a vaccine against such heterologous immunotype strains of *P. aeruginosa*. *E. coli* strain HB101 was used as the host for propagating plasmids. *E. coli* strain BL21(DE3), containing the T7 RNA polymerase gene under the control of the *lac* promoter in a lysogenic form, was used for expressing the chimeric proteins (Studier and Moffatt 1986).

Construction of plasmids encoding PEIF, OprF, and PE(ΔIII)

Plasmid pJH4, which encoded the full length of PE (Hwang et al. 1987), was used as the starting material to construct a plasmid expressing various fusion proteins. There are two *AatII* sites, one located at the boundary region of domain Ib and domain III of the PE gene, and the other at the downstream of the PE gene near the

EcoRI site beyond the 3' end. As shown in Fig. 1, we destroyed the second *AatII* site by partially cleaving the plasmid pJH4 with *AatII*, followed by removing the single strand end with mung bean nuclease and religation with T4 DNA ligase to construct the plasmid pJMx. The *AatII* site immediately upstream of domain III of the PE was retained, while the other *AatII* site downstream of the PE gene was removed. The strategy used to construct the plasmid expressing PEIF was to cleave the PE gene with *AatII* and *EcoRI*, and then replace domain III of PE with the Opr gene. The expression vector pET-15b (Novagen), containing the T7 promoter followed by a hexahistidine-tag sequence and polylinker, was used for expressing all the recombinant proteins in this study. A *NdeI*-*PstI* fragment, containing the full length PE structural gene obtained from pJMx, was subcloned into the corresponding restriction sites of pET-15b. The resulting plasmid, designated as pET-15bJMx, encodes the full length of PE with a N-terminal (His)₆ tag.

The *OprI* and *OprF* genes were amplified by polymerase chain reaction (PCR) from the genomic DNA of *P. aeruginosa* PAO1 strain as described by Maniatis et al. (1989). The oligonucleotide I-1 (5'-GCTGTTCTGGACGTCGGTTGCAGCAGCCAC-3') with an *AatII* site at the 5' end corresponding to nucleotide residues 40-69 of the *OprI* gene (Duchêne et al. 1989), and the reverse primer I-2 (5'-CAGGTTCGGAATTCCTATTACTTGGCGCT-3'), with an *EcoRI* site at the 5' end complementary to nucleotide residues 241-269 of the coding region of the *OprI* gene, were used for PCR to amplify a 229-bp *OprI* DNA fragment. The amplified *OprI* DNA fragment was then used as the template for a second PCR. The oligonucleotide I-1, and the reverse primer I-3 (5'-CGGAATTCCTATTACTCGAGGCTG-3'), with a *XhoI* site before the stop codon of the *OprI* gene and an *EcoRI* site at the 5' end complementary to nucleotide residues 240-264 of the *OprI* gene, were used in the second PCR to obtain a 224 modified *OprI* DNA fragment. The modified *OprI* DNA fragments were then digested with *AatII* and *EcoRI*, and subcloned in-frame into the *AatII*/*EcoRI* site of pET-15bJMx. The resulting plasmid was designated as pET-15bJlx. The oligonucleotide F-1 (5'-GCAATGAACGCCTCGAGCAGGGCCAG-3'), with a *XhoI* restriction site at the 5' end corresponding to nucleotide residues 55-81 of the *OprF* gene (Duchêne et al. 1988), and the reverse primer F-2 (5'-TTTCTT-TGAATTCTCAGCCGATTACTTGGC-3'), with an *EcoRI* restriction site at the 5' end complementary to nucleotide residues 1045-1075 of the coding region of the *OprF* gene, were used in PCR to amplify a 1020-bp *OprF* DNA fragment with a *XhoI* site at the 5' end and an *EcoRI* site at the 3' end. The *XhoI* and *EcoRI* fragment from the *OprF* gene was then subcloned in-frame into the corresponding site of the plasmid pET15bJlx to obtain the plasmid pET15bJIF, which was then used to express the tripartite hybrid protein (His)₆-PE(1-405)-OprI(19-83)-LE-OprF(24-350). This protein was abbreviated as PEIF. Numbers in parenthesis in PEIF indicate the beginning and end of the amino acid residues of each original protein.

Plasmid pET15bF, encoding OprF (24-350) with a N-terminal (His)₆ tag, was constructed by subcloning the *XhoI* and *EcoRI* fragment of OprF DNA into the corresponding sites of the expression vector pET-15b. PE(Δ398-613) with an N-terminal (His)₆ tag was obtained by subcloning a *NdeI* and *EcoRI* fragment from plasmid pJJ10, which encoded PE with a deletion of domain III as described previously (Chow et al. 1989), into the corresponding sites of the plasmid pET-15b to obtain the plasmid pET-15bJJ10. The protein expressed by plasmid pET-15bJJ10 was named (His)₆PE(Δ398-613), abbreviated as PE(ΔIII). All plasmids were sequenced to ensure that no mutation was introduced by PCR.

Expression, purification, and characterization of the recombinant proteins PEIF, OprF, and PE(ΔIII)

For large-scale production of recombinant fusion proteins, the resulting plasmids were transformed into *E. coli* strain BL21(DE3). BL21(DE3) containing appropriate plasmids for expressing the (His)₆-tag proteins were cultured in LB broth at 37 °C. When

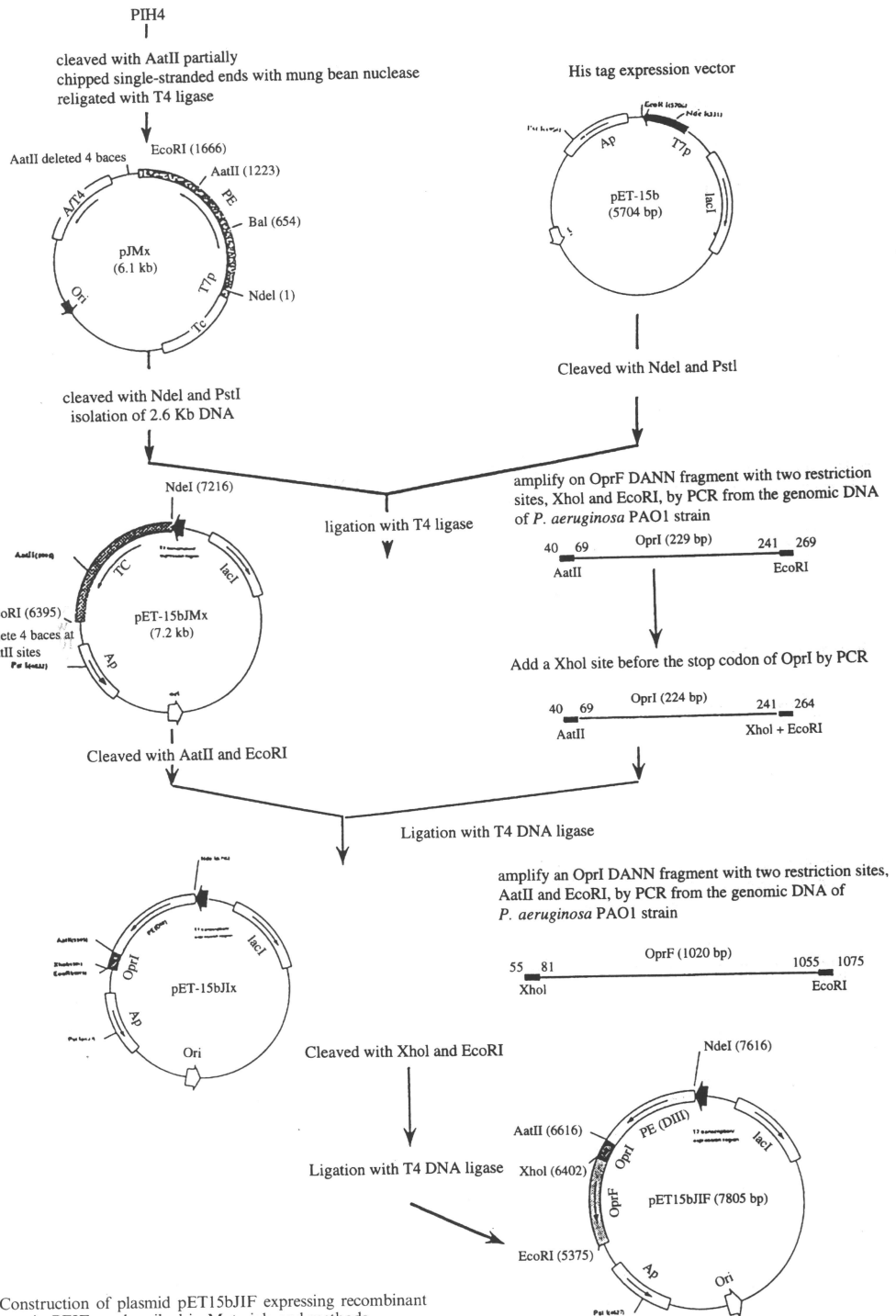


Fig. 1 Construction of plasmid pET15bJIF expressing recombinant fusion protein PEIF, as described in Materials and methods

absorbance at 600 nm reached 0.3, isopropyl-1-thio- β -D-galactopyranoside (IPTG) was added to a final concentration of 0.5 mM for 90 min. Since all three (His)₆-tag proteins were highly expressed in inclusion bodies, they could be purified from crude bacterial lysates under denaturing conditions through the Ni-charged His-Bind column following procedures recommended by the supplier (Novagen). The purified proteins were analyzed by 12% SDS PAGE and stained with Coomassie blue after electrophoresis as previously described (Hwang and Chen 1989). The PE identities of the fusion proteins were confirmed by immunoblotting with polyclonal rabbit anti-PE antisera (Hwang and Chen 1989). In addition, the OprI and OprF identities of the recombinant fusion proteins were confirmed by immunoblotting with murine antisera against OprF or PEIF, which can recognize the native OprF or OprI from the total cell lysates of all six heterologous *P. aeruginosa* strains.

Active immunization of mice and rabbits

Three groups of BALB/c mice (6 weeks old, 30 per group) were immunized with PEIF, OprF, and PE(Δ III), which were adsorbed to AlPO₄ (2.5 mg/ml) as adjuvant. The mice were immunized intraperitoneally on day 1, 21, and 35 at a dose (0.2 ml) of 0.5 μ M, 0.75 μ M, and 1 μ M, respectively. Antisera to different recombinant proteins were also generated in New Zealand white rabbits (two rabbits per group) by immunizing subcutaneously at six different locations near lymph nodes with 200 μ g of purified proteins emulsified with Freund's complete adjuvant on day 1, and boosted with 300 μ g and 400 μ g of purified proteins emulsified with Freund's incomplete adjuvant on day 21 and 35, respectively.

Monitoring the immune responses

The immunized BALB/c mice and rabbits were bled on day 42 before bacterial challenge. Their respective sera were pooled and stored at -70 °C. To compare the immune responses induced by different vaccine preparations, sera were thawed immediately before the following assays.

ELISA

As a first step, the immune responses of mice and rabbits after three doses of immunization were monitored by ELISA performed as described previously (Voller et al. 1979). The pooled antisera from each group were diluted two-fold (ranging from 1:50 to 1:12 800) with 0.5% BSA-PBS. Purified PE (ICN Biomedicals) and recombinant OprF were used to coat polyvinylchloride, flat-bottom, 96-well Falcon microtiter plates at a protein concentration of 3 μ g/ml. The antisera were also titrated against whole bacterial cells from the six heterologous immunotype strains of *P. aeruginosa*. PBS washed bacteria (10⁸ cells in 0.1 ml PBS) were coated on plates precoated with poly-L-lysine (1 μ g in 0.1 ml PBS) overnight at 4 °C. Peroxidase conjugated anti-mouse or anti-rabbit immunoglobulin was used as the secondary antibody. The substrate solution contained 0.54 mg/ml 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) and 0.03% H₂O₂ in 0.1 M citric acid. A negative/positive cutoff value was defined as the value higher than 2 SD above the mean A₄₀₅ readings obtained from five nonimmune rabbit or mice control antisera. The titer was defined as the reciprocal of the highest dilution of serum yielding a positive value. Some results were also expressed as optical density (OD₄₀₅) values obtained for a given serum dilution.

Pseudomonas exotoxin A neutralization assay

The ability of various rabbit antisera to neutralize the cytotoxicity of PE was determined in NIH 3T3 cells as described previously, with slight modification (Cryz et al. 1986; Pollack et al. 1976). NIH 3T3 cells were seeded 24 h prior to the cytotoxicity assay in a 96-well tissue culture plate at a density of 10⁴ cells per well in 200 μ l.

PE (1 μ g/ml) was mixed with an equal volume of various dilutions of antisera for 30 min at 37 °C, and then 50 μ l was added to the cultured cells in each well. The final concentration of PE was 100 ng/ml, which contained ten times the minimal dose required for $\geq$ 95% cell death. After incubation for 72 h, the monolayers were washed once with PBS and then stained with 0.05% crystal violet in 20% ethanol for 10 min at room temperature to detect surviving cells. The stained cells were lysed with 100% methanol. Absorbance was determined with an automated microplate reader at a wavelength of 595 nm. Each diluted antiserum was determined in replicates of eight. Toxin neutralization was calculated by the following formula:

% neutralization of cytotoxicity

$$= \frac{\left[\frac{\text{average absorbance of eight wells containing antiserum with toxin}}{\text{average absorbance of eight wells containing antiserum without toxin}} \right] - B}{-B}$$

B is the average background of stained wells without cells. The neutralizing titer was defined as the reciprocal of the highest dilution of serum eliciting 50% neutralization of cytotoxicity.

Opsonophagocytic uptake by murine peritoneal macrophages

The ability of PEIF vaccinated antisera to promote the uptake of *P. aeruginosa* by murine peritoneal macrophages was determined by the visual phagocytosis assay as described previously, with a slight modification (Gilleland et al. 1992). The bacterial cultures used in this assay were *P. aeruginosa* strains PAO1, PA103, and *P. aeruginosa* serogroup 2. The bacteria were grown to mid-log phase, washed twice in cold PBS and suspended in RPMI 1640 at a concentration of 10⁸ CFU/ml. Opsonization was performed by tumbling the bacteria at 37 °C for 30 min in 10% (vol/vol) complement-inactivated murine antisera obtained from various vaccinated groups on day 42. Opsonized bacterial suspensions were then washed with PBS and resuspended in serum-free RPMI.

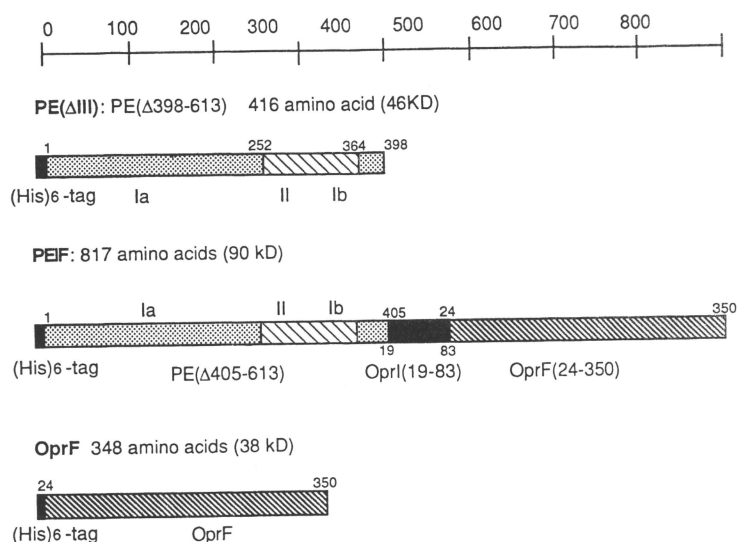
Macrophages were elicited from 6- to 8-week old BALB/c mice by intraperitoneal injection of 2 ml thioglycolate broth (Difco) 4 days before cell harvesting. Peritoneal exudate cells were collected by washing the peritoneal cavity with 5 ml of PBS. After washing with cold RPMI 1640, the pelleted macrophages were resuspended in RPMI 1640 containing 10% FCS at a concentration of 2 $\times$ 10⁵ cells/ml. Macrophages (1 ml) were added to individual wells of 24-well plates containing 11-mm-diameter round glass coverslips, and incubated for 2 h at 37 °C in 5% CO₂. After incubation, nonadherent cells were washed off and the medium was replaced with 1 ml of pre-opsonized bacterial suspension (10⁷ CFU/ml) in serum-free RPMI 1640. Phagocytosis was allowed to proceed for 30 min at 37 °C in 5% CO₂. After ingestion, extracellular bacteria were removed by washing the monolayer macrophages three times with cold PBS. Fixed coverslips were stained with 1% crystal violet, and intracellular bacteria within each of 100 cells were counted by oil immersion light microscopy. Phagocytosis of *P. aeruginosa* was expressed as the percentage of macrophages with < 6, 6-19, or > 19 bacteria ingested. All experiments were repeated at least two times with macrophages from different mice.

Burned mouse model

A modification of the burned mouse procedure was used (Holder 1985). BALB/c mice were anesthetized with sodium pentobarbital (0.71 μ g/g body weight) and shaved dorsally prior to burning. A Teflon template with a precisely cut window (35 $\times$ 25 mm) was firmly placed over the shaved backs and 0.5 ml 95% (v/v) ethanol was pipetted onto the surface and ignited. Burns were precisely timed for 15 s. Approximately 30% of the total body surface was burned. The challenged bacteria (in 0.2 ml cold PBS with about 1-5 $\times$ 10⁴ bacteria - about two times the 50% lethal dose of each strain) were immediately injected subcutaneously into the burned

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Fig. 2 Simplified map of the recombinant fusion proteins. All three recombinant fusion proteins with a (His)₆-tag at the N-terminal



area. Survival of the ethanol-burned mice was recorded for 10 days. The 50% lethal dose (LD₅₀) values were calculated separately on nonvaccinated ethanol-burned mice for different serotypes by the method of Reed and Muench (1938). Then two times the LD₅₀ was used as the challenge dose to examine the protection afforded by different vaccines.

Statistics

Fisher's exact test was used to calculate two-tailed *P* values for significant levels of protection. *P* values ≤0.05 were considered significant.

Results

Expression, purification, and characterization of recombinant proteins PEIF, PE(ΔIII), and OprF

The constructed recombinant fusion proteins PEIF, PE(ΔIII), and OprF are diagrammatically shown in Fig. 2. All three (His)₆-tag proteins were highly expressed in *E. coli* and could be one-step purified by metal-affinity chromatography. As shown in Fig. 3A, most of these proteins had been purified to at least 90% homogeneity when analyzed by SDS-PAGE. Western blot analysis of the proteins showed that PEIF, native PE, and PE(ΔIII), but not OprF, could be recognized by rabbit anti-PE polyclonal antibodies (Fig. 3B). However, the OprF and OprI identities of the recombinant fusion proteins were confirmed by Western blotting with murine or rabbit polyclonal anti-PEIF antisera. This can recognize both native OprF (band 36 kDa) and OprI (band 7 kDa) from the total bacterial lysate of homologous and six heterologous *P. aeruginosa* strains, but not *E. coli* BL21(DE3)

(Fig. 4A), while the antisera against OprF can only specifically recognize OprF (Fig. 4B).

Monitoring immune responses by ELISA

The immune responses of BALB/c mice after three doses of immunization with different recombinant fusion proteins were determined by ELISA on day 42, the day of bacterial challenge in experiments for active immu-

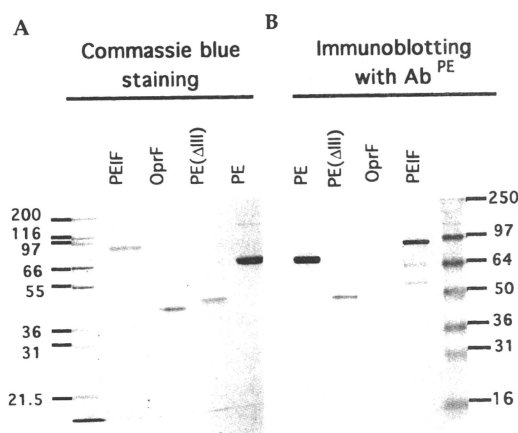


Fig. 3A, B Characterization of recombinant fusion proteins PEIF, PE(ΔIII), and OprF. **A** Purified fusion proteins were analyzed by 12% SDS-PAGE and stained by Coomassie blue after electrophoresis. **B** Western immunoblot of purified recombinant proteins with rabbit anti-PE polyclonal antisera

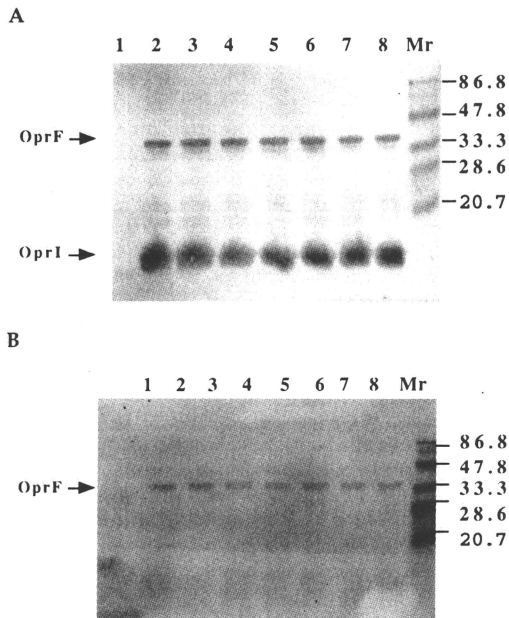
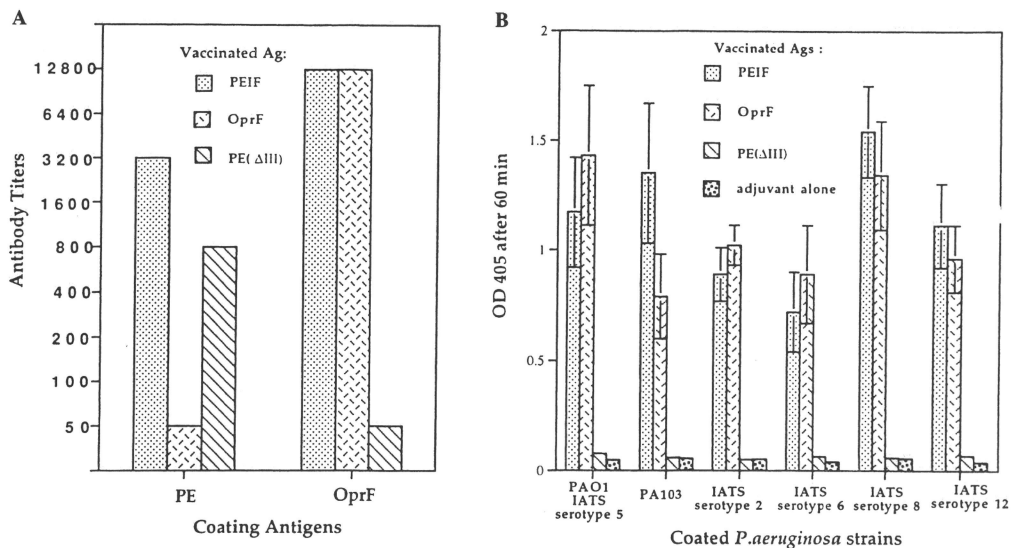


Fig. 4A, B Identification of the native OprF and OprI of the homologous PAO1 strain and six heterologous *Pseudomonas aeruginosa* immunotype strains by Western blot analysis with pooled murine antisera against PEIF (A) and OprF (B). Bacterial cell extracts were prepared from *E. coli* BL21(DE3) (lane 1), *P. aeruginosa* PAO1 strain (lane 2), PA103 strain (lane 3), ATCC 33349 (lane 4), ATCC 33354 (lane 5), ATCC 33355 (lane 6), ATCC 33359 (lane 7), and ATCC 33359 (lane 8). The antisera against PEIF reacted with both OprF (36 kDa) and OprI (7 kDa) of *P. aeruginosa*, whereas the antisera against OprF only reacted with OprF

nization protection. The antibody titers against purified PE and OprF, each from pooled sera of 30 mice per group, are summarized in Fig. 5A. As predicted, the fusion proteins containing a PE moiety [such as PE(Δ III) and PEIF] can elicit antibodies specifically against the native PE. However, the antibody titers against PE in the PEIF vaccinated antisera were higher than those for PE(Δ III) alone (1:3200 vs 1:800). On day 28 (7 days after the second vaccination), the average optical densities of antisera against PE from PEIF- and PE(Δ III)-vaccinated groups (1:500 dilution) at 405 nm after 1 h incubation at 25 °C with substrate ABTS were 1.583 ± 0.095 and 0.241 ± 0.072 , respectively. Both PEIF and PE(Δ III) contained the same receptor binding and membrane translocation domains of PE. The different antibody titers against native PE might be explained by the following reasons. First, as the OprF has been demonstrated to be a strong antigen, it might function as a T-helper lymphocyte carrier protein to increase the immunogenicity of PEIF against PE. Second, the exposed B-cell epitopes of PE in PEIF may be much more similar to the native PE than those of the PE(Δ III) alone. On the other hand, both PEIF and OprF vaccinated mice had high titers (1:12 800) of antibodies against purified OprF. The average optical densities of anti-PEIF and anti-OprF antisera against OprF (1:3200 dilution, 7 days after the second vaccination) at 405 nm after 1 h incubation at 25 °C with substrate ABTS were

Fig. 5A, B Antibody titer determination by ELISA against purified PE and OprF (A), and whole bacterial cells of six immunotype strains of *P. aeruginosa* (B). BALB/c mice were vaccinated three times with the fusion proteins PEIF, PE(Δ III), and OprF, and antisera pooled for ELISA on day 42 as described in Materials and methods



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0.963 $\pm$ 0.012 and 1.204 $\pm$ 0.078, respectively. These results demonstrated that the position of OprF at the C terminus of the hybrid protein PEIF did not reduce its immunogenicity against OprF.

As the molecular weight of OprI was only 7 kDa, it was difficult to obtain recombinant OprI in an unfused form. We therefore did not determine the antibody titers against purified OprI directly by ELISA. However, the antibody specificity against OprI induced by PEIF can still be observed in Western blotting. Both native OprF and OprI in the bacterial lysate can be recognized by the same diluted (1:5000) anti-PEIF antisera (Fig. 4A). In addition, we immunized rabbits with the purified antigens. The rabbit anti-PE or anti-OprF antibody titers on day 42 were much higher than those of murine sera (data not shown). As expected, the rabbit anti-PE antibody titer elicited by PEIF was also higher than that elicited by PE(Δ III) (9000 vs 3000). These results further confirm that the fusion of PE(Δ III) with OprI and OprF may increase the antigenicity of PE(Δ III).

In order to determine whether the anti-PEIF antibodies could recognize surface-exposed antigens on intact whole bacteria, whole cells of various strains of *P. aeruginosa* were coated on the plate for ELISA tests. The mean titer from triplicate determinations of antisera induced by PEIF or OprF against whole cells of the various *P. aeruginosa* strains was 1:3200. The average optical density of each antiserum (1:1000 dilution) at 405 nm after 1 h incubation with substrate ABTS is shown in Fig. 5B. Compared to normal mice sera, the antisera from mice immunized with PE(Δ III) did not react to an appreciable degree with whole cells ($OD_{405} < 0.08$), while fusion proteins containing the OprF moiety (such as PEIF and OprF) were capable of eliciting antibodies at high titers reactive with the whole cells of not only the homologous PAO1 strain, which had been chosen for OprI and OprF gene cloning, but also the other five heterologous strains. There was no significant difference in the antibody titers between anti-PEIF and anti-OprF antisera against whole bacteria of various *P. aeruginosa* strains. This is consistent with previous results that OprF is surface exposed and present in very high copy numbers (estimated 200 000 copies per cell) on the cell surface (Mutharia and Hancock 1983), while OprI is anchored in the outer membrane and its surface accessibility to antibodies has been questioned (Braun et al. 1976).

PE cytotoxicity neutralization assay and opsonophagocytosis by murine peritoneal macrophages

According to the ELISA results, it was demonstrated that the recombinant fusion protein PEIF could elicit antibodies reactive to the native PE and also to the cell surface of various strains of *P. aeruginosa*. Hence, we further studied the functional activities of these antibodies by evaluating their PE cytotoxic neutralizing titers and the opsonophagocytic activity. As shown in

Fig. 6, antibodies elicited by PEIF and PE(Δ III) were capable of neutralizing the cytotoxicity of PE on NIH3T3 cells. Both anti-PEIF and anti-PE(Δ III) antisera (diluted 1:10) were able to completely inhibit PE cytotoxicity, probably by inhibition of PE binding to target cells. The cytotoxicity neutralizing titers of antisera elicited by PEIF and PE(Δ III) were 561 and 374, respectively. The cytotoxic neutralizing capacity correlated well with the ELISA titer against PE.

The ability of PEIF or OprF to elicit antibody capable of promoting the uptake of *P. aeruginosa* by murine peritoneal macrophages is shown in Table 1. Most of the macrophages (59–66%) phagocytosed few nonopsonized bacteria (less than 6 bacteria per cell). Both anti-PEIF and anti-OprF antisera led to a significant increase in opsonophagocytic uptake of either homologous or heterologous *P. aeruginosa* by peritoneal macrophages. As expected, anti-PE(Δ III) antisera exhibited no opsonic activity against *P. aeruginosa*.

Protection afforded by PEIF against the infection of *P. aeruginosa* in ethanol burned mice

The *P. aeruginosa* infection in burned mouse model developed by Stieritz and Holder (1975) was used to evaluate the vaccine efficacy of PEIF. The LD_{50} of *P. aeruginosa* PAO1, PA103, and IATS serogroup 2 (ATCC 33349) in ethanol burned mice were 5.36×10^3 , 3.87×10^3 , and 2.15×10^4 CFU/mouse, respectively. Most *P. aeruginosa* infected burned mice died 2–5 days

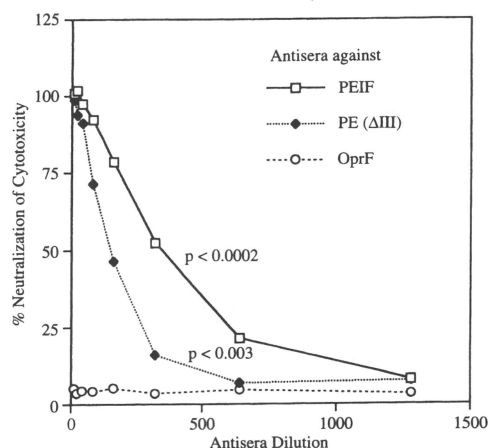


Fig. 6 Cytotoxicity neutralizing activity of antisera elicited by recombinant fusion proteins PEIF, PE(Δ III), and OprF. The indicated serum dilution was mixed with equal volume of 1 μ g/ml of PE for 30 min at 37 $^{\circ}$ C, and then 50 μ l was added to NIH 3T3 cells (4×10^4 cells per well). Each dilution was performed in replicates of eight. The cell viability was counted after 72 h by staining with 0.05% crystal violet. Neutralizing activity of antisera was expressed as a percentage of cytotoxicity, calculated as described in Materials and methods

Table 1 Opsonophagocytosis of different strains of *P. aeruginosa* by murine peritoneal macrophages. Bacteria were opsonized with 10% pooled murine antisera as indicated and then mixed 50:1 with

thioglycolate-elicited murine peritoneal macrophages for 30 min at 37 °C. Intracellular bacteria were counted by light microscopy as described in Materials and methods

Opsonized murine antisera	Phagocytosis								
	Homologous strain IATS type 5 (PAO1) (ATCC 15692)			Heterologous strain LATS type 2 (ATCC 33349)			Heterologous strain PA103 (ATCC 29260)		
	< 6 ^a	6–19	> 19	< 6	6–19	> 19	< 6	6–19	> 19
Normal	66	29	5	61	27	12	59	33	8
Anti-PEIF	10	52	38	6	65	29	17	49	34
Anti-OprF	16	47	37	18	51	31	22	45	33
Anti-PE(ΔIII)	65	22	13	56	37	7	61	36	3

^a Data represents the percentage of macrophages with the indicated number of bacteria ingested

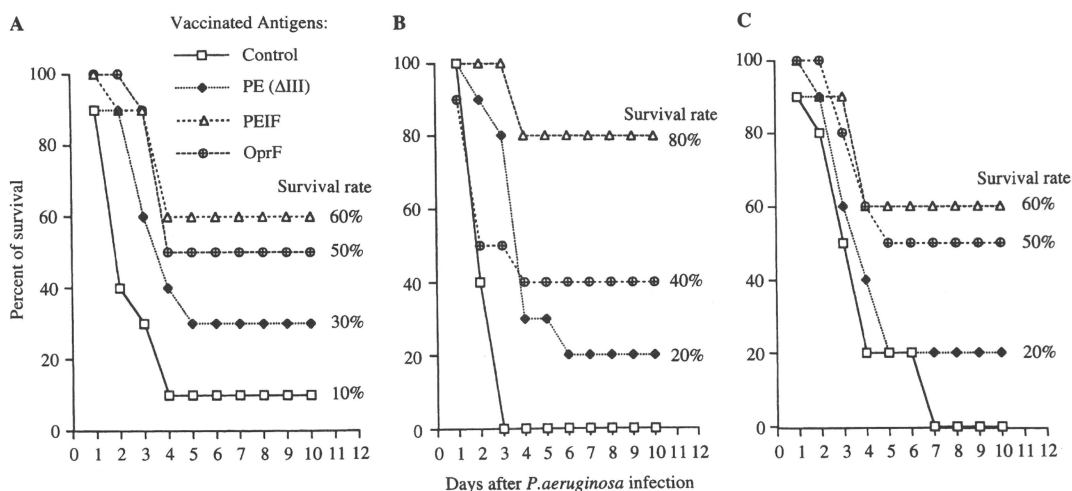
after infection. Compared to the non-vaccinated control mice, the burned mice immunized with PEIF or OprF were afforded significant protection against challenge with homologous or heterologous *P. aeruginosa*, whereas PE(ΔIII) alone was less effective in this model system (survival rate about 50–60% vs 20–30%, $P < 0.01$), even though it was shown to elicit antibodies capable of neutralizing the lethal activity of native PE. As shown in Fig. 7, PEIF did not enhance the protection afforded by OprF alone against the infection by homologous PAO1 or heterologous *P. aeruginosa* (survival rate about 60% vs 50%, $P < 0.5$). However, PEIF afforded significantly higher protection against infection

of PE-hyperproducing strain PA103 than did OprF alone (survival rate about 80% vs 40%, $P < 0.001$).

Discussion

Bacterial infection involves the adhesion and multiplication of the microbe in the host's target organ. Usually, the pathogenesis of bacterial infection is accompanied by the production of toxic or aggressive metabolites by the microorganism. The host's immune response against pathogenic bacteria includes the activation of phagocytic cells to ingest and kill the microorganism, as well as the activation of B cells to secrete antibodies to neutralize the toxicity of the microbial toxins and aggressins. Several potential *P. aeruginosa* vaccine candidates have been reported, including a PE toxoid (Pollack and Prescott 1982), elastase and alkaline protease toxoids (Homma et al. 1978), detoxified LPS-protein conjugates (Seid and Sadoff 1981), polysaccharide-protein conjugates (Cryz et al. 1987b, c; Tsay and Collins 1984), and outer membrane proteins (Finke et al. 1991; Gilleland

Fig. 7A–C Effects of vaccination with recombinant fusion proteins on the survival of burned mice challenged with homologous *P. aeruginosa* IATS serogroup 5 PAO1 (A), toxin hyperproducing strain PA103 (B), and heterologous IATS serogroup 2, ATCC 33349 (C). BALB/c mice, in groups of 10, were immunized with the indicated recombinant fusion proteins three times. Control mice were immunized with BSA, as described in Materials and methods



et al. 1984). Since there are several stages in *P. aeruginosa* infection adherence, colonization, invasion, dissemination, and systemic effects of toxemia the relative importance of each stage in different types of *P. aeruginosa* infection is variable (Nicas and Iglewski 1985). It is unlikely that antibodies to a single cell-surface determinant or secreted protein will protect a susceptible host. In order to have an effective vaccine which may elicit protective antibodies against different bacterial antigens, Homma et al. (1978) developed a multicomponent vaccine consisting of a common protective antigen (designated OEP) which resides in the cell wall of *P. aeruginosa* (Abe et al. 1975), plus toxoids of PE, elastase and alkaline protease (Homma et al. 1978; Yamamoto et al. 1986). The rationale for using multicomponent vaccine for *P. aeruginosa* immunoprophylaxis is to generate antibodies to OEP which would help the infected host reduce the microbial load by providing opsonins to aid phagocytosis and intracellular killing, while at the same time provides antibodies to neutralize the harmful effects of some of the organism's toxins and aggressins. However, the preparation and quality control of the multicomponent vaccine have many inherent limitation.

In this study, we used a recombinant DNA technique to construct a novel vaccine PEIF against *P. aeruginosa* infection. The ADP-ribosylation domain of PE was replaced with the outer membrane proteins I and F. It has been reported that the expression of OprF of *P. aeruginosa* in *E. coli* was toxic to the host (Duchêne et al. 1988). However, in our case DNA encoding OprF or PEIF could be subcloned into an expression vector pET-15b and highly expressed in *E. coli*. The data presented in this study indicate that the recombinant fusion protein PEIF preserved critical epitopes of PE, OprI, and OprF, which can induce antibodies against all three antigens in rabbits and mice. In addition, the moiety of OprF in PEIF might function as a T-helper lymphocyte carrier protein to increase the immunogenicity of the PE portion of PEIF. Our results also showed that the antibodies induced by PEIF can neutralize the PE cytotoxicity and recognize the conserved bacterial surface antigens OprI and OprF. As a result, they enhance macrophage phagocytosis of various strains of *P. aeruginosa*. Immunization with PEIF or OprF alone conferred statistically significant protection against the fatal experimental burn wound sepsis caused by the homologous *P. aeruginosa* strain PAO1 and heterologous serogroup 2, whereas PE(Δ III) alone did not. However, PEIF-immunized mice have better protection against infection by the PE hyperproducing strain PA103 than did the OprF immunized group.

PE is the most toxic pathogenic factor of *P. aeruginosa* and contributes to mortality in experimental animals and patients. It therefore appears that optimal protection against *P. aeruginosa* infection could be obtained by using a vaccine capable of eliciting PE-neutralizing antibodies. In conclusion, the fusion protein PEIF described in the present study possesses those

characteristics desired of a candidate vaccine: it is a single-molecule, nontoxic, nonpyrogenic vaccine against *P. aeruginosa* infection.

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