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An Outer Membrane Protein Preparation as a Vaccine against *Pseudomonas aeruginosa* Infection

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We developed a simple and efficient method to prepare a *Pseudomonas* vaccine of outer membrane (OM) proteins free from lipopolysaccharide (LPS). A three step purification process including extraction, ultrafiltration and ultracentrifugation effectively removed LPS from the OM protein fraction. Approximately 2 mg of the OM proteins was obtained from 1 g of wet cell. LPS contaminant in the vaccine preparation was less than 0.003% (w/w) of protein and protease activity was not detectable. To achieve a wide range of protection, OM proteins prepared from four attenuated *P. aeruginosa* strains were mixed in equal amounts and used as a vaccine, which elicited in rabbits a high titer of antibody reactive to all of the seven Fisher types. The antisera from the immunized rabbit had a strong reactivity to vaccine proteins larger than 25 kDa. In a burned mouse infection model, immunization with the vaccine significantly enhanced bacterial clearance in the *Pseudomonas* infected skin. The vaccination also provided mice an excellent protection against *Pseudomonas* infection (11, 16). Data on antigenicity, mutagenicity, acute, subacute toxicity and pharmacological tests confirmed the safety of the vaccine (1, 3, 10, 12, 17). These data demonstrate that this method can be applied to manufacture a bacterial vaccine of OM proteins with safety and prophylactic efficacy at a practical low cost.

P. aeruginosa is an opportunistic pathogen which infects people with a defective immune system due to severe burns, cystic fibrosis, immunosuppressive or cancer therapy (7, 13, 15, 18). More than 90% of cystic fibrosis patients die of lung damage resulting from recurrent lung infections with *P. aeruginosa* (9). In patients with severe burn wounds or immunosuppressive therapy, a regional colonization with *P. aeruginosa* leads to systemic infection, causing septic shock. *P. aeruginosa* is naturally resistant to most antibiotics, and only a few antibiotics such as fluoroquinolones and gentamicin are effective (8). A more serious problem is that *P. aeruginosa* quickly develops resistance to these antibiotics and no effective means to treat these patients are available. Accordingly, development of an effective prophylactic and/or therapeutic vaccine for *Pseudomonas* is being urgently sought. Several attempts have been made to develop effective and safe vaccines against *Pseudomonas* (5, 6, 19, 21, 22). One major cell surface component, lipopolysaccharide (LPS) is highly immunogenic and an antibody to LPS is protective against *Pseudomonas* infection, but its toxicity limits its use as a vaccine. In addition, serotype specificity of LPS also makes it difficult to develop a vaccine with a wide protection range. A purified OM protein fraction was shown to give protection against *Pseudomonas* infection in an animal study and to be effective for antibody induction in human volunteers but side effects due to contaminating LPS has been a major drawback to further development (21, 22).

In this paper, we report a novel method for the preparation of an OM protein vaccine against *Pseudomonas* infection. Data on immunogenicity and efficacy of the vaccine in an animal model system also will be described.

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MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

P. aeruginosa strains isolated from hospital patients in Korea and other countries were classified into 7 types according to a Fisher-Devlin immunotype and examined for their pathogenicity in mouse model systems (11).

Among them, four strains were attenuated by repeated passages *in vitro* and designated CFCPA10142 (Fisher immunotype 1), CFCPA20215 (Fisher immunotype 2), CFCPA30720 (Fisher immunotype 3) and CFCPA60534 (Fisher immunotype 6), to prepare a vaccine. GN11189 strain (IATS type 8, Fisher immunotype 6) (14) was used to challenge animals after immunization. Seven wild type Fisher strains used for cross-reactivity were purchased from American Tissue Culture Collection (U. S.A.).

All *P. aeruginosa* strains were grown at 37°C in a 150 liter-jar fermenter in 100 liters of tryptic soy broth (TSB, Difco, U.S.A.) or enhanced medium (EM), which was developed by our laboratory and composed of 3% glucose, 1.5% peptone, 0.05% $MgSO_4$, 0.5% $CaCO_3$, 0.1% KH_2PO_4, and 0.0005% each of $FeSO_4$, $CuSO_4$ and $ZnSO_4$. The culture was stirred at 200 rpm for 12~16 h, while being aerated at 1 v/v/m.

Preparation and Analysis of a *Pseudomonas* Vaccine

After cultivation, cells were concentrated up to 20 times of original culture volume in the Sartocon II system (Sartorius, Germany) and centrifuged at 6,000×g for 20 min at 4°C. Three volumes of acetone was slowly added to the pellet and the mixture was kept for 12 h or longer at 4°C. Acetone was removed and two volumes of fresh acetone was added again to the residue. The mixture was stirred intermittently for 2 h and then allowed to stand for 12~16 h. This step was repeated once with one volume of acetone. The cell pellet was resuspended in an equal volume of 10 mM phosphate-buffered saline (PBS, pH 7.2) and the OM proteins were extracted with a homogenizer (EYELA, Japan) by stirring at 50~200 rpm at 4°C. The OM proteins were fractionized by ultrafiltration to obtain proteins with molecular weight (MW) between 10~100 kDa, which was followed by ultracentrifugation at 180,000×g for 3 h or longer at 4°C. The recovered supernatant was sterilized by filtration through 0.22 μm filter, mixed with mannitol as a stabilizer and lyophilized.

Lactate dehydrogenase (LDH) activity, which serves as a cytoplasmic enzyme marker, was measured using a kit, LDL10, according to the manufacturer's instructions (Sigma, U.S.A.). LPS content was determined by the chromogenic Limulus Amebocyte Lysate (LAL) Test using the QCL-1000 kit (Biowhittaker, U.S.A.). Protease activity of *Pseudomonas* strains and in the OM protein preparation was measured by an agar diffusion method. Briefly, 10 μl of *Pseudomonas* suspension, which was grown in TSB at 37°C overnight and adjusted to OD_{600} 1.0, was dropped on a filter disc on a TSB agar plate containing 3% skim milk. The plate was incubated at 37°C overnight and the size of halo around the disc was measured the next day. For the vaccine, 10 μl of vaccine solution (1 mg/ml) was directly applied to a filter disc.

Immunization and Antibody Analysis

Three month-old male Japanese white rabbits were immunized subcutaneously with 200 μg of the vaccine three times at 7-day intervals (days 0, 7, 14). For a control group, rabbits were injected with saline. Blood was drawn from ear veins on days 0, 31, 38, 45, 84, 104, 148 and 270 and centrifuged. The sera were kept frozen at -70°C until used.

Antibody titer in the rabbit serum was measured by enzyme-linked immunosorbent assay (ELISA) as previously described (16). Wells of a microtiter plate were coated with 2 μg of the vaccine. Goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (Accurate, U.S.A.) was used as a secondary antibody. To determine reactivity of the antibody to various Fisher types, microtiter plates were coated with 2×10^7 cfu of each strain and incubated with serially diluted antiserum. The absorbance at 490 nm with preimmune serum was subtracted from each value.

Antibody reactivity to the vaccine component was confirmed by western immunoblot analysis. The vaccine proteins were separated on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was incubated with the antisera from rabbits immunized with the vaccine, and after washing, treated with goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (Accurate, U.S.A.), followed by incubation with a chromogenic substrate.

Burned-mouse Infection Model

Five week-old ICR male mice were immunized intraperitoneally with 10 μg of the vaccine three times at 2 day intervals. Five days after the final injection, mice were anesthetized, the hair on the mouse back skin removed, and burn was induced on the skin by exposing to hot water (71°C). Four hours later mice were challenged with 9×10^7 cfu of GN11189 strain by a subcutaneous injection into the burned area. Mice were sacrificed 2 days after infection, and the skin was excised and homogenized in 10 mM PBS (pH 7.2). The supernatant was spread on a nutrient agar plate, which was incubated at 37°C overnight and colonies were counted the next day.

RESULTS

Preparation of the *Pseudomonas* Vaccine

We investigated the prevalence of *Pseudomonas* infection in hospitals in Korea and other countries and chose four Fisher immunotypes with high frequency among patients, which covered approximately 65% of *Pseudomonas* infection (2). Before vaccine preparation, we attenuated the strains to reduce virulence. In the attenuated strains, the ability to produce proteases was significantly reduced as determined by agar diffusion assay. While clinical isolates actively secreted protease (Fig. 1),

there was no visual sign of protease activity in the *Pseudomonas* vaccine.

To optimize culture conditions before starting the main culture, we investigated the growth characteristics of the selected *P. aeruginosa* strains. *P. aeruginosa* strains showed identical or similar growth rates under medium conditions at pH 5.0 to pH 9.0. The growth pattern of the attenuated strains on various carbon sources was not very different from that of wild type strains. To prepare OM proteins, *P. aeruginosa* strains were grown at 37°C in TSB or EM in a Chemap fermenter (Switzerland) with a working volume of 100 liters. The yield of bacterial mass was at least 30% higher in EM than in TSB.

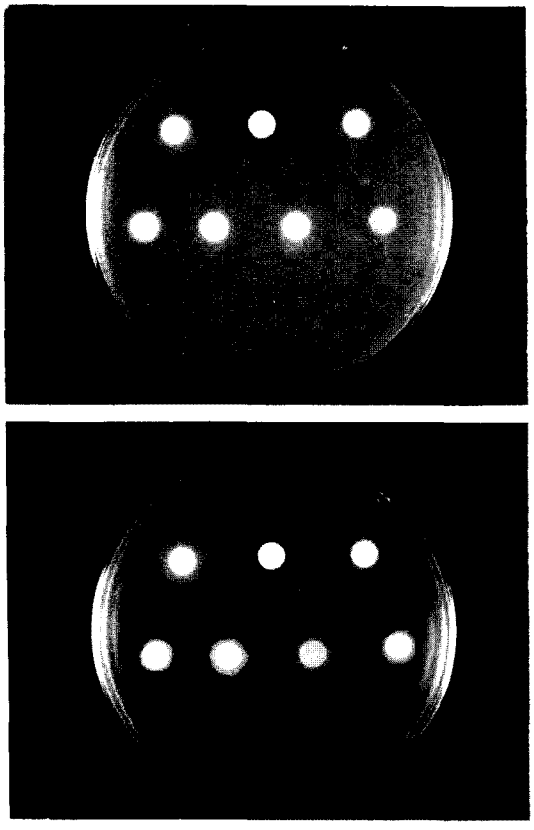

Fig. 1. Protease activity of wild and attenuated *P. aeruginosa* strains and in the *Pseudomonas* vaccine preparation.
Protease activity was determined by an agar diffusion assay as described in Materials and Methods. For both plates, the top row is, from the left, saline, trypsin (0.5 mg/ml), and vaccine preparation (1.0 mg/ml). The second row of plate A shows vaccine strains; CFCPA10142, 20215, 30720 and 60534, respectively. The second row of plate B shows clinical isolates; WN391 (Fisher type 1), WN202 (Fisher type 2), C6077 (Fisher type 3) and WN42 (Fisher type 6).

Each attenuated strain was cultured and processed until the last purification step so that the quality of each preparation could be easily monitored. In the purification process, treatment with acetone was used to inactivate the bacteria and to remove the outer membrane lipid components, loosening the outer membrane structure at the same time. Extraction was carried out with stirring at 50~200 rpm at 4°C. At this step, special precautions were taken to avoid cell lysis so that cytoplasmic proteins including enzymes would not leak, thus causing side effects as well as lowering the efficacy of the vaccine. We continuously monitored cytoplasmic protein contamination by measuring LDH activity. As shown in Table 1B, LDH activity of OM protein preparations was approximately 40-fold lower than that of CFCPA10142 total cell lysate except that of OM protein preparation from CFCPA20215, which was 16-fold lower than that of CFCPA10142 cell lysate. This indicates that cytoplasmic protein leakage was minimal. Ultrafiltration was performed in two steps to remove proteins smaller than 10 kDa, which are less immunogenic, and molecules larger than 100 kDa, which are mainly LPS. Subsequent ultracentrifugation at low pH (pH 6.5) further reduced LPS contamination by enhancing LPS aggregation. We determined LPS content at every step. LPS contamination in the crude extract from small scale preparation was 0.1% (w/w) of the protein but ultrafiltration removed 99.5% of the contaminating LPS and ultracentrifugation processing further decreased it 10 fold to 0.005% (Table 1A). LPS in the final preparation on a large scale was as low as 24 ng/mg protein, which is less than 0.003% for all the strains (Table 1B). After sterilization, the final yield of OM proteins was 25.9% for a small scale preparation but was raised up to 52% on a large scale (Table 1A & B). This result indicates that approximately 2 mg of the vaccine protein can be obtained

Table 1. Preparation of outer membrane proteins from *P. aeruginosa*. (A) Small scale cultivation[a]

Purification step	Volume (ml)	Protein concentration (mg/ml)	Total protein (mg)	Yield (%)	LPS content[b] (ng/mg protein)
Crude extract	1,200	0.21	252	100	100 (0.100)[c]
Ultrafiltration	1,370	0.06	82.2	32.6	50 (0.050)
Ultracentrifugation	67	1.03	69.0	27.4	5 (0.005)
Sterilization	54	1.21	65.3	25.9	–

[a]*P. aeruginosa* CFCPA30720 was cultured in 1 liter of TSB and approximately 70 g of wet cell was obtained. [b]LPS content was determined by the LAL test as described in Materials and Methods. One ng of LPS is equal to 6.5 endotoxin unit. [c]The number in the parenthesis is the percentage of LPS to protein (w/w).

Table 1. Continued. (B) Large scale fermentation[a]

Strains (CFCPA)	Wet cell weight (kg)	Protein (g)		Yield (%)	LDH[b] (U/mg protein)	LPS content (ng/mg protein)
		crude extract	final			
10142	2.2	8.9	3.90	43.8	0.003	26.2 (0.0026)[c]
20215	2.8	11.2	5.85	52.2	0.011	28.3 (0.0028)
30720	1.5	7.9	3.68	46.5	0.001	24.0 (0.0024)
60534	3.0	16.0	5.77	36.0	0.004	31.5 (0.0031)

[a]Each strain was grown in 100 liters of TSB and processed as described in Materials and Methods. [b]LDH activity for total cell lysate of CFAPA10142 was 0.16 U/mg protein. [c]The number in the parenthesis is the percentage of LPS to protein (w/w).

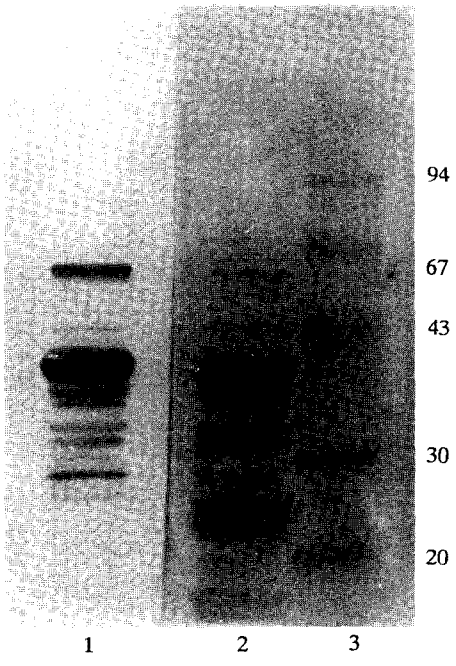

Fig. 2. SDS-polyacrylamide gel electrophoresis and western immunoblot analysis of the *Pseudomonas* vaccine prepared from four attenuated strains.

The *Pseudomonas* vaccine was resolved on a 12% SDS-polyacrylamide gel and the gel was stained with Coomassie Blue (lane 2). Protein on the gel was transferred to a nitrocellulose membrane and probed with the serum from a rabbit immunized with the *Pseudomonas* vaccine (lane 1). Lane 3 contains protein size markers. The molecular weight sizes are shown on the right of the gel.

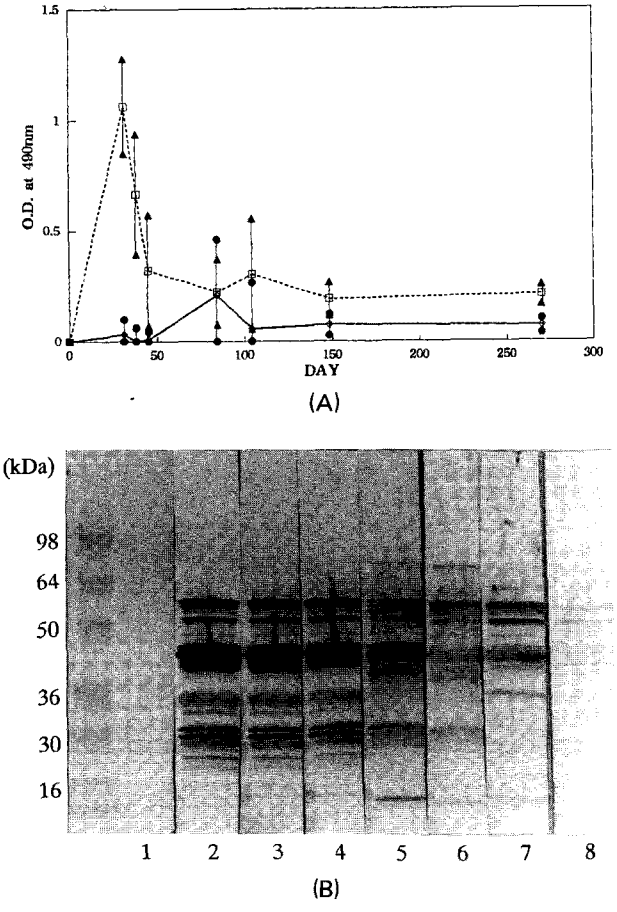

Fig. 3. A: Antibody response of the rabbits immunized with the *Pseudomonas* vaccine. B: Reactivity of the antibody from the rabbits immunized with the *Pseudomonas* vaccine to the vaccine proteins.

A: Rabbits were given by a subcutaneous injection with 100 μg of the vaccine 3 times at 7-day intervals, and the serum was collected on the day as noted on the x-axis. Each group contains 5 rabbits. Bars indicate standard errors. A solid line indicates the control group; a broken line the vaccinated group. B: The *Pseudomonas* vaccine was resolved on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose filter, followed by probing with antisera from rabbits immunized with 200 μg of the *Pseudomonas* vaccine. Protein size markers are on the left; lanes 1, 2, 3, 4, 5, 6, 7 and 8 are probed with the antisera collected at day 0, 31, 38, 45, 84, 104, 148 and 270, respectively, after the first immuization.

from 1 g of wet cell. An SDS-polyacrylamide gel analysis of the vaccine revealed the presence of several protein bands mainly with MW between 23-60 kDa (Fig. 2, lane 2).

Immunogenicity and Protective Efficacy of the *Pseudomonas* Vaccine

After quality analysis, four OM protein preparations were mixed in equal amounts and used to immunize rabbits. The antibody titer of the serum from the immunized rabbits reached a peak on day 31 and decreased till day 84, and thereafter remained at a low level but was significantly

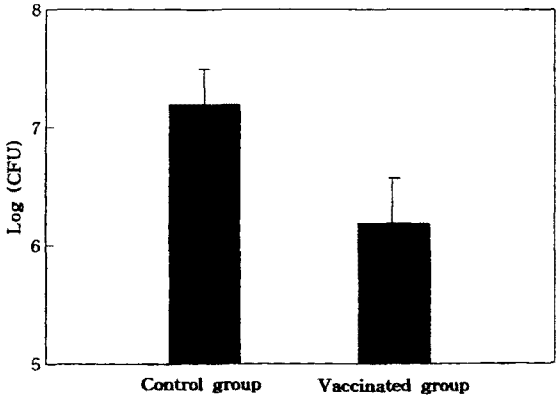

Fig. 4. Recovery of live *P. aeruginosa* from the infected skin of burned mice.

Mice were injected with saline or the *Pseudomonas* vaccine 3 times at 2-day intervals. Five days later mice were subject to burning, followed by a challege with 9×10^7 cfu of *Pseudomonas*. The number of live bacteria in the infection site was counted as described in Materials and Methods. Bar indicates the mean±standard error of the mean for a group containing ten mice ($p < 0.001$).

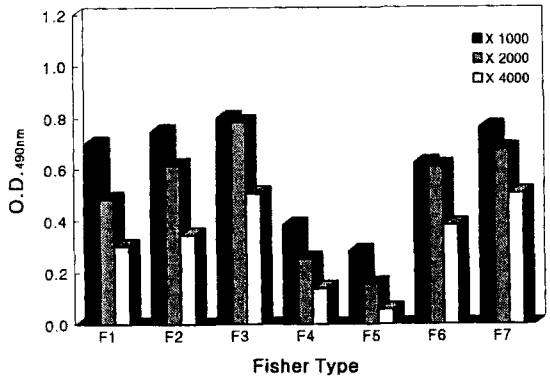

Fig. 5. Reactivity of antibody elicited by the *Pseudomonas* vaccine to various Fisher types.

Reactivity of antibody to *Pseudomonas* strains was measured by ELISA as described in Materials and Methods.

higher than that of the unimmunized control group up to day 270 (Fig. 3A). Western blot analysis was carried out to verify the reactivity of the antibody induced in rabbits (Fig. 3B). The *Pseudomonas* vaccine protein was separated on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose membrane for immunoblot analysis. Our vaccine contained proteins with a MW range of 10-100 kDa, but antibodies elicited were reactive mainly to proteins larger than 25 kDa (Fig. 2, lane 1). This result was again confirmed by the result of western blot analysis carried out with antisera collected at various times after the first immunization (Fig. 3B). Binding activity of the antibody to the vaccine protein was good till day 45 and thereafter decreased to a detectable level till day 270, which is in good agreement with the pattern of the antibody titer of the immunized group shown in Fig. 3A. It was also notable that antibodies reactive to protein bands with a higher molecular weight persisted for a longer period than those reactive to smaller proteins.

The protective efficacy of the vaccine preparation has been shown in previous studies (11, 16). Since bacterial clearance from infection sites has been used to measure protective efficacy (4), we used the efficiency of bacterial clearance in a burned mouse infection model, which is more representative of human infection, to investigate effectiveness of the vaccine. As shown in Fig. 4, the number of live bacteria recovered from the infection site was significantly reduced in the immunized group when compared with that of the unimmunized group ($p < 0.001$).

Our vaccine was made of OM proteins from 4 different Fisher types which were classified based on O-antigen. Although OM proteins are known to be conserved among *Pseudomonas* strains, we investigated the reactivity of the antibody elicited by the vaccine to 7 Fisher types (Fig. 5). The antibody was highly reactive to the Fisher types used for vaccine preparation (Fisher types 1, 2, 3 and 6) and also to Fisher type 7 ($OD_{490nm} > 0.6$ at 10^3-fold dilution). It also showed significantly high reactivity to Fisher types 4 and 5 ($OD_{490nm} > 0.2$ at 10^3-fold dilution).

DISCUSSION

We performed the present study to develop an effective method for preparation of LPS-free OM proteins from *P. aeruginosa*. The OM proteins isolated from 7 strains of different serotypes were investigated for their cross-reactivities (16), and four strains were selected for a wide protection range of *Pseudomonas* infection. The most abundant strains found in Korean and foreign hospitals belong to the Fisher types 1, 2, 3, and 6, which covered 65% of *Pseudomonas* bacteremic isolates in Seoul National University Hospital (2). Other minor Fisher types (35%) were shown to be cross-reactive to antisera raised by the OM proteins of major serotypes (16), and this was also confirmed by *in vitro* tests (Fig. 5). Thus, we expect that the protection range of our polyvalent vaccine would be higher than 95% for *Pseudomonas* infections.

The method for OM protein preparation described here provided a very high yield of the vaccine (35~52%) with minimal contamination of LPS and cytoplasmic proteins using a simple purification process. With this method we were able to reduce LPS content dramatically to 0.003% (w/w), which was 20 fold lower than that of the *Pseudomonas* vaccine described by Stanislavsky *et al.* (22). As LPS content higher than 300 ng may cause toxicity such as erythema and pyrogenic symptoms, the high level of LPS contamination in the previous vaccine

was not acceptable for human vaccination as proven in the human study where 26% of all volunteers experienced febrile reaction and 40% experienced a local reaction (21). In addition, LPS was highly immunogenic and the protective efficacy index of an monoclonal antibody to LPS was 40~200 fold higher than that of an monoclonal antibody to proteins (20). Thus, it was not clear whether the efficacy of the vaccine used by Stanislavsky was due to the OM proteins or to contaminating LPS. The vaccine we developed contained substantially no LPS, assuring that the efficacy was mainly from OM proteins. This was also confirmed by western blot analysis where the antisera from the immunized rabbits recognized OM proteins (Fig. 2 & 3B). These data also suggest that immunogenicity increased with the size of proteins and that proteins smaller than 25 kDa were not very immunogenic. Our vaccine preparation contained all the OM proteins of various sizes, which would induce good antibody response for a long period. Studies on the immunological effect of mixed OM proteins in active and passive animal challenge tests demonstrated that this vaccine had an excellent protective efficacy (11, 16) and a series of tests on antigenicity (1), acute (10), subacute (3) toxicity and pharmacological activity (17) of this vaccine candidate confirmed the safety of the vaccine.

In conclusion, the advantages of our vaccine are that 1) since it contains multiple OM proteins with various epitopes, it would give an prophylactic efficacy higher than those of vaccines made of a single purified or recombinant protein, 2) The polyvalency of the vaccine provides a wide range of protection, 3) safety of the vaccine is higher than killed whole cell or live attenuated bacterial vaccines, 4) safety is once again assured by the effective elimination of LPS to a minimum level, and 5) in addition, the simple and efficient production procedures provide high productivity at a low cost, thus making this vaccine very attractive to manufacturers.

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