NOTE  Bacteriology

Protective Effect of *Pasteurella multocida* Cell-Free Antigen and Toxoid against Challenge with Toxigenic Strains of *Pasteurella multocida* in Mice

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(Received 12 February 2002/Accepted 19 February 2003)

ABSTRACT. The cell-free antigen (CFA) obtained from the culture supernatant of *Pasteurella multocida* (*P. multocida*) and the toxin (PMT) purified from CFA were inactivated and mixed with oil adjuvant to prepare a trial vaccine. Both of the mice immunized with CFA and PMT toxoid vaccine were noticeably protected against intratracheal challenge with toxigenic strains of *P. multocida*. Nevertheless, the protective indices of the mice immunized with CFA vaccine indicate that it is more protective and clears away the bacteria more promptly than in the mice immunized with PMT vaccine. The results suggested that CFA would possibly be good as an effective antigen to toxigenic strains of *P. multocida* infection.

KEY WORDS: cell-free antigen, *Pasteurella multocida*, toxin

Respiratory disease causes one of major economic losses to the swine industry, resulting in mortality, growth retardation and carcasses damaged by pneumonic lesions. Although several pathogens are associated with respiratory diseases, *Pasteurella multocida* (*P. multocida*), *Actinobacillus pleuropneumoniae* and *Mycoplasma hyopneumoniae* are the most important causal agents.

Various antimicrobial agents have been used for the prophylaxis and cure of these diseases. There have been an increasing number of pathogens resistant to antimicrobials on various farms recently [2, 16, 19], but no vaccine for pneumonia from *P. multocida* infection has yet been developed in Japan. To develop an effective vaccine for pneumonia from *P. multocida* infection, we examined the protective activities of the cell-free antigen (CFA) obtained from the culture supernatant of toxigenic *P. multocida* B-45 strain (type A; isolated from pneumonic lesions in a pig) and the toxin (PMT) purified from the culture supernatant with toxigenic strains of *P. multocida* B-45 and B-67 (type D; isolated from a nasal swab obtained from swine with symptoms of atrophic rhinitis) in mice.

Strain B-45 was cultured in brain heart infusion broth (Difco Lab., U.S.A.) at 37°C for 72 hr. The culture was automatically maintained at pH 7.0 by the addition of 2 M NaOH. The cultured fluid was centrifuged at 10,000 × g for 1 hr to remove the bacterial cells. The supernatant was concentrated to approximately 1/30 of the original volume by ultrafiltration, and the concentrated supernatant was used as CFA [13, 17]. PMT was collected by 50% (NH4)2SO4 precipitation from CFA, followed by Macro-prep Ceramic Hydroxyapatite (Bio-Rad Lab., U.S.A.) chromatography. PMT was examined for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [11] and silver staining (Silver Stain Plus Kit; Bio-Rad Lab., U.S.A.). The dermonecrotic activity of CFA and PMT were assayed by intradermal reaction [4] in female Hartley guinea pigs (Japan SLC Inc., Hamamatsu, Japan), and then CFA and PMT were inactivated with 0.3% formalin. Minimal dermonecrotic units (MU) of CFA and PMT in guinea pigs were determined according to criteria for the minimal size of a positive skin lesion (diameter: 10 mm) and the length of the period between intradermal injection and reading of the result (48 hr). Twenty thousand MU/mL of CFA and PMT antigens were respectively mixed with oil adjuvant [a mixture of one part of anhydromannitol octadecenoate-ether (AMOE; Sepic Co., Ltd., France) and nine parts of liquid paraffin] at the volume ratio of 3 (antigen): 7 (oil adjuvant), and homogenized to prepare a water/oil vaccine.

Two hundred and fifty-five female BALB/c mice (Japan SLC Inc., Hamamatsu, Japan) were assigned to three groups, two groups immunized with either CFA or PMT vaccine and a control group. Eighty-five mice (4 weeks old) in each of two immunized groups were injected twice intramuscularly with 0.1 mL of vaccine at 3-week intervals. Two weeks after the final immunization, 10 mice in one of the immunized groups and the control group were challenged intratracheally with toxigenic strains by making an incision in the throat under general anesthesia as follows: the immunized mice were challenged with strain B-45 at 5.5 × 103 to 5.5 × 106 CFU/0.025 mL, with strain B-67 at 3.1 × 103 to 3.1 × 106 CFU/0.025 mL, the control mice were challenged with strain B-45 at 5.5 × 103 to 5.5 × 106 CFU/0.025 mL, with strain B-67 at 3.1 × 103 to 3.1 × 106 CFU/0.025 mL. The LD50 of the challenge strains in each group was calculated by the Behrens-Kärber method 7 days after the challenge, and expressed as indices. The protective index was indicated by the difference in LD50 between the immunized and control groups.

At the time of challenge, blood samples were collected from the rest of the mice to measure antibody titers. Indirect hemagglutination (IHA) antibody titer was measured by IHA test with glutaraldehyde-fixed erythrocytes and heat extract antigen of strain B-45 [18], and anti-PMT antibody titer was measured by enzyme linked immunosorbent assay.
(ELISA) as follows: 96-well microtiter plates were coated with PMT in carbonate buffer, pH 9.6. After incubation overnight at 4°C the plates were washed and blocked for 1 hr at 30°C with tween-PBS (T-PBS) containing 2% bovine serum albumin (WAKO Lab., Japan). After another wash, serum samples diluted 1:2 to 1:256 in T-PBS were added and incubated for 1 hr at 30°C. The plates were washed and to them was added HRP-conjugated antibody to mouse IgG (Zymed Lab., Inc., U.S.A.) diluted in T-PBS and incubated for 30 min at 30°C. After a final wash, enzyme substrate (10 mg 1.2-orthophenyldiamine dihydrochloride, 25 ml 0.1 M citrate buffer, pH 5.0 and 10 µl H2O2) was added and incubated for 30 min at 30°C. After the incubation colour development was stopped with 1 M H2SO4. The spectrophotometric absorption was read at 490 nm with 650 nm as the reference. The anti-PMT antibody titer was expressed as the reciprocal of the highest dilution of serum with an absorbance of more than 0.5. These titers were expressed as the geometric mean for 5 mice.

To investigate the multiplication-inhibition of *P. multocida* in lungs of mice, 120 female BALB/c mice were assigned to three groups, two groups immunized with either CFA or PMT vaccine and a control group. Immunization was performed as mentioned above. Forty mice in each of two immunized groups and the control group were divided into 2 subgroups, 20 mice in each, and challenged intratracheally with strain B-45 (5.5 × 10^2 CFU/0.025 ml) or strain B-67 (3.1 × 10^5 CFU/0.025 ml). Then the number of viable bacteria in the lungs was determined at 0, 1, 2 and 3 days after challenge, using 5 mice each time, and expressed as the geometric mean.

As shown in Fig. 1, CFA contained over 20 polypeptide species (reduced monomer), covering an estimated molecular weight range from 25 kDa to 250 kDa. On the other hand, PMT had a single band of about 150 kDa [5, 7, 8, 12]. Antibody titers in immunized mice and the protective effects of CFA and PMT vaccine against intratracheal challenge with *P. multocida* are shown in Table 1. The IHA and anti-PMT antibody titers in the mice immunized with CFA vaccine were 3.5 and 36.8, and those in the mice immunized with PMT vaccine were 1.0 and 48.5. Both the mice immunized with CFA and PMT vaccine were noticeably protected against intratracheal challenge. Protective indices in the mice immunized with CFA vaccine were 3.7 to strain B-45, 2.1 to strain B-67, and those in the mice immunized with PMT vaccine were 3.2 to strain B-45 and 1.9 to strain B-67.

The quantity of bacterial recovery after intratracheal challenge of the immunized mice was very low as compared with the control mice. The difference in the index between

| Table 1. Protective effect of CFA and PMT against intratracheal challenge with *P. multocida* |
|---|---|---|---|---|
| Immunogen | Antibody titer (GM) | Challenge strain | LD₅₀ | Protective indices |
| | IHA | Anti-PMT | B-45 | B-67 |
| CFA | 3.5 | 36.8* | 5.04 | 3.7 |
| | | | 7.29 | 2.1 |
| PMT | 1.0 | 48.5 | 4.54 | 3.2 |
| | | | 7.09 | 1.9 |
| - | 1.0 | 1.0 | 1.34 | - |
| | | | 5.19 | - |

Serum antibody titers of mice at the time of challenge were measured by indirect hemagglutination (IHA) and enzyme linked immunosorbent assay to Anti-PMT antibody. The antibody titers are the geometric means for 5 mice. LD₅₀ of challenge strain was calculated by the Behrens-Kärber method and expressed as indices. The protective indices for each group were indicated the difference in LD₅₀ between the immunized group and the control.

*: no significant difference between CFA and PMT found by Student’s t-test (P=0.4608).
the control mice and mice immunized with CFA vaccine was 2.9 and the mice immunized with PMT vaccine was 2.1 in challenge with strain B-45 at 3 days after the challenge (Fig. 2). On the other hand, the difference in the index between the control mice and mice immunized with CFA and PMT vaccine was about 3 in challenge with strain B-67.

Although many strains of *P. multocida* isolated from pneumonia of swine, most *P. multocida* are nontoxigenic strains, which rarely induce pneumonia experimentally. But Kamp and Kimman reported that some pigs inoculated intranasally with toxigenic *P. multocida* strains had chronic pneumonia [9]. In our study, nontoxigenic strains of B-18 and B-24 (type A; isolated from pneumonic lesion of swine) showed no pathogenicity in mice on intratracheal challenge (LD$_{50}$: $\geq 10^{18}$ CFU/0.025 ml), whereas toxigenic strains showed signs of strong pathogenicity. Therefore we inferred that the virulence of *P. multocida* correlated with the ability to produce a PMT, and the PMT of toxigenic *P. multocida* may be a colonization factor in lungs, and toxigenic *P. multocida* strain can be a primary cause of pneumonia.

It is well known that PMT is a causative agent of atrophic rhinitis in swine and anti-PMT antibody protects against atrophic rhinitis [1, 3, 6, 10, 14, 15]. In the present studies, the mice immunized with CFA and also those immunized with PMT showed strong resistance to the intratracheal challenge with toxigenic strains, but the mice immunized with CFA derived from a nontoxigenic strain showed a slight resistance (data not shown). Therefore it seemed that anti-PMT antibody plays an important role in protection against *P. multocida* infection. Bording and Foged reported that pure toxoid vaccine caused a significantly higher anti-PMT antibody than crude toxoid vaccine, and when equal quantities of formaldehyde detoxified *P. multocida* toxin were present in the pure and crude toxoid vaccines the pure toxoid vaccines were the more effective [1]. In our study, the anti-PMT antibody titer in the mice immunized with PMT was slightly higher than that in the mice immunized with CFA, but there is no statistical difference between the anti-PMT antibody titers in mice immunized with CFA and those immunized with PMT ($P=0.4608$). In the challenge test of strain B-45, the mice immunized with CFA are better protected and clear away the bacteria more promptly than the mice immunized with PMT. These findings indicate that not only PMT but also capsular antigen participate in protection against toxigenic strains of *P. multocida* infection, although PMT certainly is an effective antigen. Moreover, it is suggested that the protective effect is extended as a result of the multiplier effect associated with PMT, lipopolysaccharide and antigens to some capsular polysaccharides. From the results of this study, we inferred that CFA would possibly be good as an effective antigen to toxigenic strains of *P. multocida* infection.

REFERENCES

1844–1849.