Immunogenicity of Actinobacillus pleuropneumoniae Outer Membrane Proteins and Enhancement of Phagocytosis by Antibodies to the Proteins

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To determine the opsonic effect of antibodies to Actinobacillus (Haemophilus) pleuropneumoniae outer membrane proteins on phagocytosis by porcine polymorphonuclear leukocytes (PMN), we separated the integral outer membrane proteins (IOMPs) by Triton X-114 extraction. Four major IOMPs with molecular masses of 76, 50, 39, and 29 kDa were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. These IOMPs were found to be essentially free of endotoxin in the Limulus amebocyte lysate assay. The 76-kDa protein exhibited a more intensely stained electrophoresis band when isolated from iron-restricted cultures, and a new band at 105 kDa was present in the whole-membrane fraction but not in the integral fraction, indicating that the 105-kDa iron-repressible protein is a peripheral membrane protein. The 76-, 50-, and 39-kDa proteins were shown to be surface exposed, since antibodies to these IOMPs could be absorbed out of convalescent-phase sera by whole cells. Percentages of phagocytosis by porcine PMN of A. pleuropneumoniae opsonized with convalescent-phase sera, convalescent-phase sera absorbed with IOMPs, or convalescent-phase sera absorbed with whole cells were 46.75, 21.81, and 7.96%, respectively. These results demonstrate that antibodies to IOMPs of A. pleuropneumoniae serve as important opsonins in phagocytosis by porcine PMN.

Actinobacillus (Haemophilus) pleuropneumoniae is the causative agent of a severe, often fatal, contagious disease known as swine pleuropneumonia. The symptoms range from sudden death with hemorrhagic pulmonary necrosis in the peracute stage to poor weight gain, pulmonary abscesses, fibrinous pneumonia, pleuritis, and occasionally diarrhea in the chronic stage (27, 36). Transmission is postulated to be aerosol mediated from chronic carriers to 2- to 6-month-old pigs just entering finishing houses (34, 37). The prevalence of A. pleuropneumoniae has increased steadily since 1976 (35). Currently available vaccination protocols can prevent death from the acute stage but have failed to provide satisfactory control of the other aspects of the disease process, including the spread of infection from chronic carriers (12, 14, 16, 33). It should be noted that current bacterin vaccines only elicit serotype-specific protection, whereas immunity from natural infections is cross-protective. Such failures in protection afforded by empirically derived vaccines indicate a need for a more complete understanding of the pathogenesis of A. pleuropneumoniae (36).

Several virulence factors, including lipopolysaccharide (LPS) (6, 13, 21, 45), capsular polysaccharide (CP) (17, 18, 41), heat-labile hemolysin (7, 11, 21), and outer membrane proteins (OMPs) (8, 9, 25, 30, 31), have been studied. However, additional studies are needed to evaluate specific interactions of these virulence factors and the host defense system.

Phagocytosis constitutes the major mechanism in the defense of the lungs against bacterial colonization (38). Certain components of A. pleuropneumoniae appear to be involved in interfering with the phagocytic and bactericidal mechanisms of porcine phagocytes. Hemolysin (46) has been reported to be cytotoxic for porcine macrophages and neutrophils. The capsule has also been shown to be antiphagocytic as well as to protect the bacteria against the host's bactericidal activities (18). However, antibodies to the capsule alone could not account for all of the opsonic activity seen with whole-cell antiserum. In addition, the protection elicited by CP antibodies was highly serotype specific. The OMPs of A. pleuropneumoniae have been partially characterized and shown to be antigenic in swine (8, 9, 30). Unlike antibodies to CP, antibodies to at least four of the OMPs were cross-protective for the majority of the nine serotypes tested (31). Antibodies to the OMPs developed concomitantly with protective immunity.

To determine the effect that antibodies to OMPs exert in the phagocytic process, we used a Triton X-114 membrane protein extraction procedure. With this procedure, a highly purified preparation of OMPs (proteins which span both the inner and the outer membranes) essentially free of LPS could be obtained; OMPs were detected primarily if not exclusively in the detergent phase, while soluble proteins and peripheral membrane proteins partitioned into the aqueous phase. It could be shown that a major portion of the opsonic activity in convalescent-phase sera from pigs infected with A. pleuropneumoniae was due to antibodies to OMPs.

MATERIALS AND METHODS

Bacteria. A. pleuropneumoniae Shope 1 (serotype 1) was cultivated in trypticase soy broth supplemented with 0.6% yeast extract and 0.01% NAD. For the IOMP preparations, 10-ml portions of an overnight culture were inoculated into 4-liter flasks each containing 1 liter of medium and grown to the mid-log phase with shaking (100 rpm) at 37°C. The iron chelator 2,2-dipyridyl was added at a 300 μM concentration for iron-limited growth conditions.

Preparation of IOMPs. Crude OMP fractions were prepared by a modification of the procedure described by Bricker et al. (5). In brief, the cells were harvested, washed,
and suspended in 1% Triton X-114 containing 10 U of DNase and 20 U of RNase per ml. This suspension was incubated at 37°C with shaking (100 rpm) overnight. Cellular debris was removed by centrifugation (8,000 × g) at 4°C, and the supernatant was stored at −20°C until used. The frozen samples were thawed at room temperature, and the IOMP fraction was separated (3). The crude protein samples were mixed with 2% Triton X-114 in a 20 mM Tris-buffered saline solution (20 mM Tris, 300 mM NaCl [pH 7.8]). After 10 min at 0°C, the samples were centrifuged (8,000 × g) to remove cellular debris. The supernatant was layered over a 6% sucrose cushion, incubated at 30°C for 5 min, and centrifuged (500 × g for 10 min). The aqueous phase was removed from the cushion, and the procedure was repeated with the same sucrose cushion to ensure that all IOMPs had been separated into the detergent phase. The detergent phase was collected and dialyzed against 10 mM Tris-buffered saline solution. Two samples (50 µl) were separated for protein determination and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The total protein concentration was determined by the colorimetric method described by Bradford (4).

**LPS assay.** The concentration of LPS in the IOMP preparation was determined by the colorimetric Limulus amoebocyte lysate assay with a kit provided by Whittaker Bioproducts, Walkersville, Md.

**SDS-PAGE.** SDS-PAGE was performed by the procedure described by Laemmli (23). Protein samples were heated in SDS-PAGE sample buffer for 5 min at 100°C, and 5-µg samples were loaded onto a 4.5% stacking gel overlaying a 15% separating gel. Modified Laemmli running buffers were used on all gels. Gels were run in a Mini Protean II electrophoresis cell (Bio-Rad, Rockville Centre, N.Y.) in accordance with the manufacturer’s suggestions. After electrophoresis, gels were stained with 2.5% Coomassie blue for 60 min and destained with a 10% methanol-10% acetic acid solution.

**Immunoblots.** The proteins separated by SDS-PAGE were transferred to nitrocellulose paper by a modification of the transfer process described by Towbin et al. (42). Transferred immunoblots were blocked by incubation in 5% bovine serum albumin and reacted with normal serum, convalescent-phase serum, convalescent-phase serum absorbed with whole cells, or convalescent-phase serum absorbed with IOMPs at 37°C for 20 min. Opsinized bacteria were washed three times to remove unbound antibodies and suspended in VBS plus 2% gelatin.

The phagocytosis mixture consisted of opsonized labeled bacteria and phagocytes (1:1 ratio) (approximately 10⁷ cells per ml) incubated at 37°C for 20 min with shaking. Bacteria which had not been phagocytized were removed by washing the cells three times in VBS plus gelatin and centrifuging the cells at 800 × g for 5 min. The pelleted cells were suspended in liquid scintillation fluid and counted. Percent phagocytosis was calculated by comparing the number of intracellular radiolabeled bacteria to the total number of radiolabeled bacteria with the following formula: percent uptake = (mean cpm associated with phagocytes incubated with bacteria at 37°C/mean cpm of total available bacteria) × 100.

**Statistics.** The analysis of variance (ANOVA) procedure was used to calculate the one-way analysis of variance for the opsonization effect of IOMPs in convalescent-phase sera on the phagocytosis of labeled A. pleuropneumoniae organisms. Tukey’s test was performed to evaluate the significant differences between the individual effects of sera. All calculations were made with the PC-SAS software program (SAS Institute Inc., Cary, N.C.).

**RESULTS**

**Purification of IOMPs.** SDS-PAGE analysis of the cells treated with Triton X-114 revealed the presence of four major bands, of 76, 50, 39, and 29 kDa, on a 15% separating gel (Fig. 1). The 76-kDa band was more intense in cultures grown under iron-limited conditions. This observation has been reported by other investigators (8), and since the host presents an iron-depleted environment (47), all tests were performed with cells grown in iron-depleted media. Deneer and Potter (8) have also reported the presence of a 105-kDa
protein that is iron repressible. In the Triton X-114 extraction, the 105-kDa band was in the whole-cell membrane fraction but not in the detergent phase, suggesting that it is a peripheral protein (Fig. 1, lane W). The IOMPs were found to be essentially free of endotoxin, containing less than 50 pg/mg of IOMP, as measured by the Limulus amebocyte lysate assay.

**Immunoblots.** To ascertain the immunogenicity of the four major protein bands, we subjected the bands to immunoblot analysis with convalescent-phase and normal sera. The convalescent-phase serum contained antibodies to all four proteins, whereas the normal serum lacked antibodies to all four proteins (Fig. 2). This result is similar to those obtained with an OMP-enriched fraction prepared by solubilization with N-lauroyl sarcosinate (31). To verify that these proteins are indeed surface exposed, we immunoblotted them with convalescent-phase sera that had been absorbed with whole bacteria (Fig. 3). The only antibodies remaining were those binding to the 29-kDa protein, and even these were reduced in concentration, as determined by the decrease in the intensity of the band (Fig. 3, CPS-Abs cells). The fact that antibodies to the three larger proteins were eliminated after absorption to whole cells is evidence that they contain surface-exposed epitopes (24, 40). It is possible that the 29-kDa protein is only exposed after cell disruption by Triton X-114 and denaturation during SDS-PAGE processing. It has been suggested (30) that this band may cross-react with other gram-negative bacteria. Immunoblots with convalescent-phase sera absorbed with IOMPs demonstrated that the antibodies to these proteins were effectively removed by absorption.

**Phagocytosis assay.** To verify that all differences noted were due to the opsonic effect of different serum fractions and were not related to variations in the PMN collected, we performed a block-type analysis of variance. The ANOVA procedure yielded an F value of 0.75 for the experimental procedure, indicating that no significant difference (P > 0.01) was attributable to different PMN populations. The different serum fractions had an F value of 28.56, which is significant (P < 0.01). Thus, the differences in phagocytosis were due to the opsonic effect of the different antibodies on the bacteria.

Opsonization of labeled *A. pleuropneumoniae* organisms with normal sera and convalescent-phase sera absorbed with whole bacterial cells resulted in phagocytosis rates of 3.91 and 7.96%, respectively. These values are not significantly different but do show that some non-antibody-mediated phagocytosis does occur (Table 1). Incubation at 4°C with the sera tested reduced the uptake to 1.45%. This basal level was used to distinguish surface adherence from active phagocytosis and showed that three washings effectively removed 98% of the nonphagocytized bacteria. Neither sera absorbed with whole cells nor normal sera produced uptake significantly greater than that obtained at 4°C. Absorption of convalescent-phase sera with IOMPs reduced phagocytosis to 21.81%, as compared with 46.75% phagocytosis for the unabsorbed convalescent-phase sera. These results (Table 1) show that opsonization can be divided into three specific groups consisting of (i) convalescent-phase sera, (ii) conva-

![FIG. 1. SDS-PAGE of *A. pleuropneumoniae* grown in iron-depleted medium. Lanes: A, low-molecular-weight protein standards; W, whole-cell membrane protein extract; X-114, IOMPs prepared by Triton X-114 extraction. The apparent molecular weights of the isolated proteins are given on the right.](image)

![FIG. 2. Immunoblots of the Triton X-114-extracted IOMPs obtained from cells grown under iron-depleted conditions and reacted with serum collected from pigs prior to exposure to *A. pleuropneumoniae* (NPS) or with convalescent-phase serum (CPS), both at a 1:1,000 dilution. The numbers (1, 2, 3, and 4) on the right correspond to those in Fig. 1.](image)

![FIG. 3. Immunoblots of the Triton X-114-extracted IOMPs obtained from cells grown under iron-depleted conditions and reacted with convalescent pig serum (CPS), convalescent pig serum absorbed with whole bacteria grown to the mid-log phase (CPS-Abs cells), or convalescent pig serum absorbed with Triton X-114-extracted IOMPs (CPS-Abs OMP). All sera were reacted at a dilution of 1:1,000. The numbers (1, 2, 3, and 4) on the right correspond to those in Fig. 1.](image)
Lescent-phase sera absorbed with IOMPs, and (iii) convalescent-phase sera absorbed with whole cells, normal sera, or passive adhesion. Antibodies to IOMPs thus are important in the phagocytic process but are not the only antibodies that are opsonic. To quantitate the contribution of IOMP antibodies to opsonization, we can extrapolate from Table 1. If 47% represents the maximum opsonic effect of convalescent-phase sera and 22% represents the effect of all surface-exposed epitopes except for IOMPs, then antibodies to IOMPs must account for 25% or approximately 53% of the total effect of opsonization on phagocytized bacteria.

**DISCUSSION**

OMPs are a dynamic feature of bacterial cells. They have been shown to undergo specific changes that are mediated by environmental fluctuations and to have a significant role in the pathogenesis of several bacterial species. A few examples are the 50-kDa antiphagocytic protein of Pasteurella multica-s (43), the seven in vivo-specific antigens in Vibrio cholerae (20), and iron-repressible proteins expressed in numerous species (10, 26, 28).

Capsules are effective virulence factors for several bacterial species (1, 2, 22, 29, 41). The host’s main line of defense against bacterial invasion of the respiratory tract is phagocytosis by “professional phagocytes.” It is known that the capsule is one important component that bacteria use in preventing phagocytes from carrying out their protective function. Antibodies to capsules have been used to provide passive protection against infection with encapsulated bacteria (15, 18, 32). In this study, an attempt was made to determine whether antibodies to IOMPs could serve as effective opsonins.

We were interested in examining the role that IOMPs play in A. pleuropneumoniae-induced swine pleuropneumonia and, specifically, in determining whether antibodies to these proteins promote phagocytosis. Triton X-114, a nonionic detergent of the Triton series, has the unique capacity to separate into two visible phases when incubated at temperatures in excess of 20°C; one phase is a detergent containing the amphiphilic proteins. The detergent binds to the proteins by replacing the bound LPS molecules and thus is a more accurate and milder technique than are the other purification techniques currently in use (39).

*A. pleuropneumoniae* grown in iron-depleted conditions produced four major Triton X-114-soluble proteins in SDS-PAGE. The 76-kDa band was an iron-repressible OMP that was still produced at measurable basal levels in the presence of iron. The only iron-repressible OMP that was shown to be completely repressed (a 105-kDa protein) did not separate out in the Triton X-114 detergent phase but was present in the whole-cell sample, suggesting that it is a peripheral protein (Fig. 1). Antibodies reacting to all four IOMPs were present in sera obtained from convalescent pigs. However, only the 76-, 50-, and 39-kDa proteins were shown to be surface epitopes. SDS-PAGE causes unfolding of proteins and thus could produce new epitopes that are cross-reactive among gram-negative bacteria (40). This activity has been postulated to occur in *A. pleuropneumoniae* serotype 5 with the 29- and 43.5-kDa proteins (30). Absorption of convalescent-phase sera with whole bacterial cells did not eliminate the immunoblot reaction to the 29-kDa protein band. Since the immunoblot reaction could not be completely eliminated, one might postulate that two proteins migrate together at 29 kDa, one being a minor cross-reactive protein that is only exposed after SDS-PAGE denaturation, and the other being a surfaced-exposed epitope. This hypothesis would explain why the band in Fig. 3 was not completely deleted after absorption with whole cells. However, further investigation would be necessary to prove such a scenario.

Convalescent-phase sera effectively opsonized *A. pleuropneumoniae* for phagocytosis by porcine PMN. Absorption of convalescent-phase sera with IOMPs resulted in a significant decrease in the phagocytosis of bacteria, but normal sera and convalescent-phase sera absorbed with whole cells did not show a significant opsonizing effect. In studies with *A. pleuropneumoniae* serotype 5, 37.3% of the bacteria were phagocytized after opsonization with porcine antiserum (18). After opsonization of bacteria with antiserum absorbed with purified CP, the phagocytic ability was reduced to 19% (18), indicating that CP antibodies accounted for 18% or approximately 48% of the total opsonins in the antiserum. Thus, CPs did not account for all of the opsonocytaphagic effect noted. For the total of 46.75% of the bacteria phagocytized in our study after opsonization with convalescent-phase sera, antibodies to IOMPs accounted for 25% or approximately 53% of the total activity (Table 1).

<table>
<thead>
<tr>
<th>Oposized sera</th>
<th>% Phagocytosis of IOMPs</th>
<th>Tukey's analysis of groupings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal porcine</td>
<td>3.91</td>
<td>A</td>
</tr>
<tr>
<td>Convalescent phase</td>
<td>46.75</td>
<td>B</td>
</tr>
<tr>
<td>Absorbed convalescent phase</td>
<td>7.96</td>
<td>C</td>
</tr>
<tr>
<td>Absorbed convalescent phase</td>
<td>21.81</td>
<td>A</td>
</tr>
<tr>
<td>None (4°C)</td>
<td>1.45</td>
<td>A</td>
</tr>
</tbody>
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* TABLE 1. Tukey's analysis of groupings indicating a high significant difference

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* Mean percent uptake by phagocytes for five experiments.
* Rows with the same letter indicate that there was no significant difference in the means (P = 0.05).
* Obtained from pigs prior to exposure to *A. pleuropneumoniae*.
* Obtained from convalescent pigs.
* Absorbed with whole bacteria.
* Absorbed with Triton X-114-extracted IOMPs.
* Phagocytosis experiments incubated at 4°C.

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REFERENCES


