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Demonstration of an Outer Membrane Protein with Antiphagocytic Activity from *Pasteurella multocida* of Avian Origin

WAVA M. TRUSCOTT AND DWIGHT C. HIRSH*

Department of Veterinary Microbiology and Immunology, School of Veterinary Medicine, University of California, Davis, California 95616

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A strain of *Pasteurella multocida* of avian origin was found to inhibit phagocytosis of *Candida albicans* by mononuclear phagocytes in vitro. Whole-cell lysates of *P. multocida* showed this effect, as did a 50-kilodalton (kDa) protein eluted from sodium dodecyl sulfate-polyacrylamide gels obtained by electrophoresis of whole-cell lysates. Heat, digestion with trypsin, and antibody specific for this 50-kDa protein neutralized the antiphagocytic effects of *P. multocida*, of the whole-cell lysates, and of the 50-kDa protein itself. Evidence that this protein was in the outer membrane of the bacterial cell included the findings that (i) treatment of encapsulated or unencapsulated *P. multocida* with trypsin reduced the antiphagocytic effect; (ii) whole-cell lysates prepared from trypsinized, unencapsulated *P. multocida* had reduced antiphagocytic activity; and (iii) antibody to outer membrane proteins neutralized the antiphagocytic effect. Turkeys given antibodies specific for the 50-kDa outer membrane protein were protected against lethal challenge with *P. multocida*.

Fowl cholera, a frequently fatal septicemic disease of fowl, is one of the leading infectious causes of loss to the poultry industry (26). Prevalence in the turkey industry, for example, has been estimated to be about 10%, with mortality in some flocks reaching 60% (34). Despite the fact that the etiology of fowl cholera has been recognized since the late 1700s, little progress has been made in its prevention (26). Inability to control this condition is primarily due to a lack of understanding of its pathogenesis.

Pasteurella multocida appears to multiply extracellularly in the turkey and, by inference, in other birds as well (4, 7, 8, 16, 35). This is, in part, facilitated by a capsule that is responsible for making the microorganism resistant to the action of the complement system (36). The presence of a capsule, however, does not seem to affect whether the microorganism associates with phagocytic cells; association is minimal regardless of the presence or absence of capsule (35). These observations suggest that *P. multocida* produces a substance(s) that interferes with the function of phagocytic cells. Since toxins with similar activity have been demonstrated in *Pasteurella haemolytica* isolated from cattle and in *P. multocida* isolated from rabbits, there is high likelihood that a toxin plays a role in the pathogenesis of fowl cholera (1, 2, 5, 9, 18, 20, 22, 25, 27, 28, 32, 33). To test this hypothesis, extracts and culture filtrates of *P. multocida* were examined for substances that interfere with the function of phagocytic cells.

MATERIALS AND METHODS

Microorganisms. *P. multocida* P1059-1 was used. P1059-1 is a serotype 3, capsular type A (composed of hyaluronic acid), nalidixic acid-resistant mutant of *P. multocida* P1059 that was isolated from the tissues of a turkey that had died of fowl cholera. Strain P1059-1 has a 50% lethal dose for 14-week-old Nicholas broad-breasted white hen turkeys of 1.3×10^3 organisms. *Candida albicans* VMTH-1 was used in studies involving uptake by phagocytic cells.

Preparation of mononuclear phagocytes. Blood was drawn from the brachial vein of Nicholas broad-breasted white turkeys into heparinized syringes. For each experiment, blood was drawn and pooled from at least three turkeys. The blood was centrifuged at 400 × *g* for 12 min at room temperature. The pellet was suspended in phosphate-buffered saline (PBS) (pH 7.2) and layered onto a solution of Ficoll-diatrizoate (Histopaque 1077; Sigma Chemical Co., St. Louis, Mo.), and the suspension was centrifuged for 30 min at 400 × *g* and room temperature (13). The mononuclear cells were removed, washed with M199 (GIBCO Laboratories, Grand Island, N.Y.), and suspended in growth medium that contained 79% M199, 10% tryptose phosphate broth, 5% newborn calf serum, 5% chicken serum, and 1% streptomycin-penicillin (10 mg/ml and 10,000 U/ml, respectively; GIBCO Laboratories). The cells were distributed into 75-cm^2 culture flasks (Corning Glassworks, Corning, N.Y.) and incubated at 37°C in the presence of 5% CO_2. After 24 h of incubation, nonadherent cells were removed and the flasks were reincubated for 24 h in fresh growth medium, after which they were rinsed with sterile PBS and removed from the culture flask after the addition of a solution containing 0.5% trypsin, 0.2% EDTA, 7.5% sodium bicarbonate, and 1% penicillin-streptomycin in 0.85% NaCl. Cells were suspended in growth medium to a concentration of 3.75×10^5 cells per ml. Samples of 4 ml of the suspension were added to 35-mm-diameter wells into which precleaned sterile 22-mm^2 cover slips had been placed. Cells were incubated at 37°C in the presence of 5% CO_2 for 24 h before being assayed.

Preparation of *P. multocida* used in whole-cell studies. *P. multocida* was grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) at 37°C in air with shaking (100 rpm) for 24 h. Cultures were centrifuged at 12,000 × *g* for 20 min at 4°C. The pellet was washed twice and then suspended in PBS to an optical density of 1.00 (540 nm), representing approximately 3.0×10^9 microorganisms per ml.

Preparation of antiphagocytic principle from culture supernatants. *P. multocida* was grown in 1 liter of BHI broth at 37°C in air with shaking (100 rpm) for 24 h. Cultures were centrifuged at 12,000 × *g* for 20 min at 4°C, and EDTA (final concentration 4 mM), pepstatin A (0.1 μM), phenylmethyl-

* Corresponding author.

sulfonyl fluoride (1 mM), and dithioerythritol (12 mM) were added to the supernatant, which was then passed through a 0.45-μm-pore-size filter. Ammonium sulfate was added to achieve 45% saturation. The precipitate was removed by centrifugation at 10,000 × *g* for 10 min at 4°C and suspended in 2 ml of PBS. The preparation was dialyzed against PBS overnight at 4°C and stored at −70°C until used.

Preparation of the antiphagocytic principle from bacterial cells treated with polymyxin B. *P. multocida* was grown in 1 liter of BHI broth overnight at 37°C in air. The culture was centrifuged at 12,000 × *g* for 20 min at 4°C, and the pellet was washed twice with PBS and then adjusted to an optical density of 1.0 (540 nm) with 10 mM Tris-buffered saline (0.9%, pH 7.4) containing 4 mg of polymyxin B sulfate per ml. The suspension was incubated at 37°C for 30 min, the debris and remaining cells were removed by centrifugation (12,000 × *g*, 15 min, 4°C), and EDTA (final concentration, 4 mM), pepstatin A (0.1 μM), and phenylmethylsulfonyl fluoride (1 mM) were added. Ammonium sulfate was added to achieve 45% saturation. After centrifugation (10,000 × *g*, 10 min, 4°C), the precipitate was suspended in 2 ml of PBS and dialyzed against PBS overnight at 4°C. The preparation was stored at −70°C until used.

Preparation of the antiphagocytic principle from bacterial cells. *P. multocida* was grown overnight in 1 liter of BHI at 37°C. The cultures were centrifuged at 12,000 × *g* for 20 min at 4°C. The bacterial pellet was washed twice with PBS and then suspended to 4 ml with PBS containing 6 mM $MgCl_2$, 4 U of DNase, 4 mM EDTA, and 0.1 μM pepstatin A. The bacterial cells were processed twice through a French pressure cell at 7,059 kg/cm^2. Phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM, followed by centrifugation at 10,000 × *g* at 4°C for 15 min. The supernatant was passed through a 0.45-μm-pore-size filter. Ammonium sulfate was added to the filtrate to achieve 45% saturation. After centrifugation at 10,000 × *g* for 15 min at 4°C, the precipitate was suspended in 2 ml of PBS containing 4 mM EDTA (pH 7.2), dialyzed, and stored as described above.

Preparation of the antiphagocytic principle from hyaluronidase-treated cells was as described above except that *P. multocida* was grown overnight in BHI containing 9 × 10^2 U of hyaluronidase per ml. The antiphagocytic principle was prepared from trypsinized *P. multocida* as described above except that bacterial cells were treated with trypsin (1 mg/ml) for 1 h at 30°C just before or immediately after lysis in the French pressure cell. Trypsin inhibitor (2 mg/ml) was added to stop the reaction.

SDS-PAGE. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed as described (19). Briefly, protein samples were boiled in the presence of 2% SDS and 20% 2-mercaptoethanol (omitted if purifying the antiphagocytic principle) in 0.04 M Tris (pH 6.8) and loaded into wells made in a 3% stacking gel overlying a 12.5% separating gel. Samples were electrophoresed at 25 mA for approximately 5 h, using a buffer consisting of 25 mM Tris, 0.192 M glycine, and 0.1% SDS (pH 8.7). After electrophoresis, proteins were stained with 0.25% Coomassie brilliant blue.

Bis-acrylylcystamine (Bio-Rad, Richmond, Calif.) was used to form gels from which proteins were to be eluted for antibody production. In such instances, a 7.5% separating gel using bis-acrylylcystamine and a 3% stacking gel prepared with *N,N'*-methylenebisacrylamide was used. Before use, the separating gels were run overnight (50 V, 17 mA) in Tris-glycine buffer.

PAGE (nondenaturing conditions). Proteins for nondenaturing PAGE were prepared as detailed for SDS-PAGE except that neither SDS nor 2-mercaptoethanol was added, nor was the sample heated. Samples were loaded onto a 3% stacking gel over a 10% separating gel. The running buffer was the same as that described for SDS-PAGE except that SDS was omitted. Samples were electrophoresed as described above for SDS-PAGE.

Preparation of immunoblots. After separation by PAGE, proteins were electrotransferred to nitrocellulose paper (37). Antiserum prepared in turkeys to the antiphagocytic principle was used at a 1/250 dilution. The secondary antiserum was affinity-purified goat anti-turkey immunoglobulin G labeled with horseradish peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) (38).

Ultrafiltration. Samples (2 ml) of preparations containing the antiphagocytic principle were each diluted with 23 ml of PBS. The solution was separated into five equal portions, and each was filtered (Amicon model 12 filtration unit; Amicon Corp., Danvers, Mass.) through separate filters with molecular weight exclusions of 10,000 (PM10), 30,000 (PM30), 50,000 (XM50), and 100,000 (PM100), with the fifth portion used as a control. Retentates were diluted to 10 ml with PBS and refiltered three more times. The final suspension, the filtrates, and controls were precipitated with ammonium sulfate (45%) saturation.

Purification of the antiphagocytic principle. Preparations containing antiphagocytic principle were boiled in the presence of 2% SDS in 0.04 M Tris (pH 6.8) and were subjected to PAGE. Sections of gels (approximately one-fourth of the length) were removed, and the contents were eluted and then assayed for antiphagocytic activity. Sections which included the antiphagocytic activity were cut from other gels and serially cut perpendicular to the direction of electrophoresis. The contents of each gel slice (1 to 3 mm) were eluted and assayed for antiphagocytic activity. Electroelution was for 4 h (40 mA) with PAGE buffer diluted half-strength in the wells (15). The running buffer was changed once. Eluted samples were dialyzed against 25% isopropanol in 0.04 M Tris (pH 8) at room temperature for 4 h and then dialyzed against PBS at 4°C overnight. The precipitate of SDS was removed by centrifugation in a Microfuge (1 min, 4°C), and the supernatant was assayed for antiphagocytic activity. Protein concentration was determined using the Bradford method (Bio-Rad Microassay, Bio-Rad Laboratories).

Antiphagocytic assay. Medium was removed from monolayers containing adhering mononuclear cells and replaced with medium without antibiotics before the assay was run. This maneuver was repeated two times. *C. albicans* was incubated overnight in 10 ml of BHI broth at 37°C in a shaking water bath (150 rpm). The culture was centrifuged at 12,000 × *g* for 15 min at 4°C, and the pellet was suspended in PBS. This was repeated two times. The final suspension was adjusted to an optical density of 0.5 at 540 nm and then heated for 30 min at 60°C. The suspension was diluted 1/3 with M199, and turkey plasma was added to a final concentration of 5%. The suspension was incubated for 10 min at 37°C and used immediately. Suspensions of opsonized, heat-shocked *C. albicans* were added to the monolayers of mononuclear cells to a final concentration of approximately 2.4 × 10^7 yeasts per well (yeast-to-phagocytic cell ratio of 20:1). *P. multocida* (30 bacteria to 1 phagocytic cell) or preparations with antiphagocytic activity (whole-cell lysate, 1% final concentration; culture supernatant, 1%; culture supernatant of polymyxin B-treated whole cells, 1%; purified toxin, 2 μg/ml) were added at various times or 30 min before addition of *C. albicans* to the monolayers of mononuclear

cells. All assays were performed in duplicate. These mixtures were incubated at 37°C for 30 min, and the cover slips were then removed, gently rinsed with PBS, rapidly dried, and stained (Diff-Quik; Dade Diagnostics, Inc., Aguade, Puerto Rico). A minimum of 100 phagocytic cells were examined per cover slip. A *t* test was used to measure significance.

Preparation of antibodies to outer membrane proteins. Outer membranes were prepared by the method utilizing *N*-lauroylsarcosine (Sigma Chemical Co.) (11, 31). A 100-ml sample of bacterial culture was centrifuged (25,000 × *g*, 15 min, 4°C), and the pellet was suspended in 4 ml of 0.05 M Tris (pH 8.0) containing 1 mM EDTA. The suspension of cells was passed twice through a French pressure cell at 7,059 kg/cm^2. Cell debris was removed by centrifugation (6,500 × *g*, 15 min, 4°C). The outer membranes were pelleted by centrifugation (40,000 × *g*, 60 min, 4°C) and treated twice with 5 ml of 1.5% *N*-lauroylsarcosine. Outer membranes were centrifuged (40,000 × *g*, 60 min, 4°C) once again, suspended in 0.05 M Tris (pH 8.0) containing 1 mM EDTA, centrifuged again, and suspended in 0.15 ml of water. An equal volume of a solution of 2% SDS–20% 2-mercaptoethanol in 0.25 M Tris (pH 6.8) was added to solubilize the membranes. Water was added to a final volume of 3.75 ml. The suspension was mixed with 1.25 ml of adjuvant (LES + STM, a lipid emulsion containing a proteinaceous fraction from an Re mutant of *Salmonella typhimurium*; RIBI Immunochemical Research, Inc., Hamilton, Mont.) and vortexed. A 1-ml volume of the suspension was injected intramuscularly and repeated in 2 weeks. Turkeys were bled 10 days after the last injection.

Preparation of antibodies to the antiphagocytic principle. Outer membrane proteins were prepared by the Sarkosyl method as described above and electrophoresed in a 7.5% bis-acrylylcystamine gel. The gel was then stained with Coomassie brilliant blue. A slice of gel containing the band identified to have antiphagocytic activity was removed (see above, "Purification of antiphagocytic principle") and dissolved in 0.8 ml of 1 M dithioerythritol per g of gel. The dissolved protein-gel combination was then dialyzed against PBS at 4°C overnight and mixed (1:5) with adjuvant (see above). Samples of 1 ml were injected intramuscularly into 14-week-old Nicholas broad-breasted white turkeys, two times, 3 weeks apart. Turkeys were bled 10 days after the last injection.

Neutralization assay. The neutralization assay was performed as described above except that 0.04 ml of serum from turkeys immunized with purified antiphagocytic principle was added 30 min (at room temperature) before the addition of *C. albicans*.

Passive protection trial. Ten 12-week-old Nicholas broad-breasted white hen turkeys were injected intravenously with 1 ml of anti-antiphagocytic principle (diluted 1:1 with normal turkey serum), and 10 were injected with 1 ml of normal turkey serum by the same route. Each group of 10 birds was housed together on the floor, each group in a separate pen. Contact between birds in different groups was not possible. One hour later, the oropharynges of five birds from each group were inoculated with a cotton-tipped swab that had been dipped into an overnight broth culture of *P. multocida* P1059. We estimate that each turkey was inoculated with approximately 10^8 organisms. Birds were observed for 7 days.

TABLE 1. Effect of *P. multocida* on phagocytosis of *C. albicans* by avian mononuclear phagocytes[a]

Time *P. multocida* added relative to *C. albicans*	No. of phagocytes scored	% Phagocytes with ≥2 yeast cells	No. of *C. albicans* per phagocyte (± SE)
Concurrent	193	89.1	7.0 ± 0.3[b]
15 min before	173	78.6	4.7 ± 0.2[c]
30 min before	202	56.9	3.3 ± 0.2[d]
60 min before	220	16.3	2.6 ± 0.2[e]
No *P. multocida* added	155	99.4	12.0 ± 0.5

[a] Data presented represent one of two experiments.
[b] Significantly different from culture with no *P. multocida* added: $t_{346} = -9.95$; $P < 0.001$.
[c] Significantly different from culture with no *P. multocida* added: $t_{326} = -14.76$; $P < 0.001$.
[d] Significantly different from culture with no *P. multocida* added: $t_{355} = 20.27$; $P < 0.001$.
[e] Significantly different from culture with no *P. multocida* added: $t_{373} = 22.09$; $P < 0.001$.

RESULTS

Phagocytosis of *C. albicans* by avian mononuclear phagocytic cells was inhibited by *P. multocida* as evidenced by a decrease in the number of phagocytosing cells as well as the number of yeast cells per phagocyte (Table 1). The antiphagocytic principle was determined to be a part of the bacterial cell and was not found in culture supernatants of *P. multocida* (which in fact stimulated uptake) regardless of whether the microorganism was treated with polymyxin B, indicating that it was not sequestered in the periplasmic space (Table 2, experiment 1).

To determine the chemical nature of the antiphagocytic principle, whole-cell lysates were treated with trypsin and heated to 80°C for 30 min. Both treatments significantly reduced the antiphagocytic effect (Table 2, experiment 2), suggesting that the active principle was protein.

The antiphagocytic principle was found not to be a part of the hyaluronic acid capsule. Instead, it appeared located beneath the capsule, but exterior to or part of the cell wall since removal of the capsule actually increased the antiphagocytic effect (Table 2, experiment 3). Treatment of unencapsulated bacterial cells with trypsin virtually eliminated the antiphagocytic effect, supporting the notion that the protein was exterior to or part of the cell wall. Too, after treatment of unencapsulated cells with trypsin, these cells were now found inside phagocytes (approximately 0 to 5 bacteria per cell before trypsinization, >50 per cell after).

Antibodies to outer membrane proteins obtained from *P. multocida* neutralized the antiphagocytic effect, demonstrating that the antiphagocytic principle was part of the outer membrane of the cell (Table 3).

The antiphagocytic principle was isolated from whole cell lysates by ultrafiltration as well as by SDS-PAGE. The protein(s) was found to be between 50,000 and 100,000 in molecular weight as judged by ultrafiltration. No antiphagocytic activity was found in any part of the gel, except for that part containing a protein of about 50 kilodaltons (Table 4 and Fig. 1). Treatment of this protein with either trypsin or heat (80°C) eliminated the antiphagocytic activity (results not shown). Antibodies made against this protein neutralized the antiphagocytic effect (Table 4 and Fig. 2).

All turkeys died that had been inoculated in the oropharynx with strain P1059 and injected with normal turkey serum (mean time to death, 1.4 days). Four of five turkeys inoculated in the oropharynx with strain P1059, and also

TABLE 2. Effect of various constituents of *P. multocida* on phagocytosis of *C. albicans*

Sample	No. of phagocytes scored	% Phagocytes with ≥2 yeast cells	No. of *C. albicans* per phagocyte (± SE)
Expt 1[a]			
Whole-cell lysate	186	62.9	3.9 ± 0.2[b]
Culture supernatant	162	95.7	12.0 ± 0.4[c]
Culture supernatant after treatment of whole cells with polymyxin B	151	94.7	8.5 ± 0.3[d]
Culture medium	128	90.1	8.0 ± 0.4
Expt 2[e]			
Whole-cell lysate	133	68.4	4.7 ± 0.3[f]
Whole-cell lysate treated with trypsin	172	86.6	9.3 ± 0.4[g]
Whole-cell lysate heated to 80°C	152	83.6	9.1 ± 0.5[h]
PBS	274	86.5	9.2 ± 0.4
Expt 3[i]			
Encapsulated *P. multocida*	255	89	7.0 ± 0.4[j]
Unencapsulated *P. multocida*	206	73	4.6 ± 0.3[k]
Unencapsulated *P. multocida* treated with trypsin	193	95	9.3 ± 0.3[l]
PBS	197	95	9.9 ± 0.4

[a] Data presented represent one of five experiments, except for the cultures with polymyxin B added (three experiments).
[b] Significantly different from culture with culture medium added: $t_{312} = 9.19$; $P < 0.001$.
[c] Significantly different from culture with culture medium added: $t_{288} = -6.55$; $P < 0.001$.
[d] Not significantly different from culture with culture medium added: $t_{277} = -0.86$; $P < 0.4$.
[e] Data presented represent one of six experiments.
[f] Significantly different from culture with PBS added: $t_{405} = 7.57$; $P < 0.001$.
[g] Not significantly different from culture from PBS added: $t_{444} = -5.21$; $P < 0.96$.
[h] Not significantly different from culture with PBS added: $t_{424} = 0.27$; $P < 0.8$.
[i] Data presented represent one of two experiments.
[j] Significantly different from culture with PBS added: $t_{450} = 3.47$; $P < 0.001$.
[k] Significantly different from culture with PBS added: $t_{401} = 8.64$; $P < 0.001$. Also significantly different from culture with encapsulated bacteria added: $t_{459} = 7.49$, $P < 0.001$.
[l] Not significantly different from culture with PBS added: $t_{388} = 0.43$; $P < 0.7$.

injected with antibodies to the antiphagocytic principle, died (mean time to death, 2 days). All five turkeys that had been injected with normal serum and left in contact with turkeys inoculated with P1059 died (mean time to death, 3.2 days). Of the five birds injected with antibodies to the antiphagocytic principle and left in contact with turkeys inoculated with P1059, one died (on day 3).

DISCUSSION

We have shown that a strain of *P. multocida* of avian origin produces a cell-associated protein that interferes with phagocytosis of *C. albicans* by avian mononuclear phagocytes. This protein appears to be in the outer membrane of the cell wall.

Evidence that the antiphagocytic moiety is protein includes its lability to heat and digestibility by trypsin. It is difficult to prove that the partially purified product was not contaminated with lipopolysaccharide and that the toxicity was due in part to endotoxin. Heat and enzymatic lability, however, make it unlikely that lipopolysaccharide played a significant role in preventing phagocytosis.

Our data strongly suggest that the protein is part of the outer membrane of the cell wall. Three lines of evidence support this claim. First, removal of the capsule increased antiphagocytic activity when the whole microorganism was used to inhibit phagocytosis. This finding may be coupled with the fact that if the unencapsulated microorganisms were treated with trypsin, antiphagocytic activity was abolished. Finally, antibodies made against outer membrane proteins neutralized the activity of the isolated protein.

Antibodies specific for the antiphagocytic principle passively protected turkeys. However, this protection was only demonstrated when the birds were challenged by contact with birds inoculated with virulent *P. multocida*. This manner of challenge is similar to what might occur during an outbreak of fowl cholera. The oropharyngeal challenge, on the other hand, was too severe and simply overwhelmed the

TABLE 3. Effects of antiserum to outer membrane proteins on number of *C. albicans* taken up by avian mononuclear cells treated with the antiphagocytic principle[a]

Sample	Assayed in the presence of:			
	Normal serum		Anti-outer membrane proteins	
	% Phagocytes with ≥2 yeast cells[b]	No. of *C. albicans* per phagocyte (± SE)	% Phagocytes with ≥2 yeast cells[b]	No. of *C. albicans* per phagocyte (± SE)
P. multocida	79.6 (103)	5.5 ± 0.4[c]	94 (183)	9.6 ± 0.5[c]
P. multocida lysate	60.9 (115)	4.2 ± 0.4[d]	91.4 (209)	9.4 ± 0.4[d]
PBS	94.4 (143)	9.4 ± 0.6	87.3 (126)	9.1 ± 0.5

[a] Data represent one of two experiments.
[b] Parentheses indicate number of phagocytes scored.
[c] Significantly different: $t_{284} = 5.72$; $P < 0.001$.
[d] Significantly different: $t_{322} = 8.8$; $P < 0.001$.

TABLE 4. Effects of antiserum to the antiphagocytic protein on number of *C. albicans* taken up by avian mononuclear cells treated with the antiphagocytic principle[a]

Sample	Assayed in the presence of:			
	Normal serum		Anti-antiphagocytic protein	
	% Phagocytes with ≥2 yeast cells[b]	No. of *C. albicans* per phagocyte (± SE)	% Phagocytes with ≥2 yeast cells[b]	No. of *C. albicans* per phagocyte (± SE)
P. multocida	94.2 (171)	6.5 ± 0.2[c]	98.9 (185)	9.7 ± 0.3[c]
P. multocida lysate	89.1 (183)	6.0 ± 0.3[d]	93.1 (185)	8.2 ± 0.3[d]
Antiphagocytic protein	87.5 (208)	5.8 ± 0.2[e]	96.5 (171)	9.6 ± 0.3[e]
PBS	93.7 (174)	9.0 ± 0.3	96.6 (174)	8.8 ± 0.3

[a] Data represent one of two experiments.
[b] Parentheses indicate number of phagocytes scored.
[c] Significantly different: $t_{355} = 8.35$; $P < 0.001$.
[d] Significantly different: $t_{366} = 6.32$; $P < 0.001$.
[e] Significantly different: $t_{354} = 6.86$; $P < 0.001$.

turkeys. That passively administered antibody protected the birds is evidence that the antiphagocytic principle plays a role in fowl cholera.

P. multocida strains isolated from a variety of sources have been shown to have cytotoxic activity. The best characterized is the dermonecrotic toxin produced by some strains belonging to serogroup D (9, 29). This toxin, so named because it causes necrosis upon injection into the skin, is thought to play a significant role in the initiation of atrophic rhinitis in swine. The toxin increases osteoclastic activity while decreasing osteoblastic activity of osteocytes in the nasal passages of young swine (12). This toxin has a molecular weight of between 143,000 and 160,000 (as measured by SDS-PAGE) (6, 12, 23, 24). In sum, the dermonecrotic toxin is cell associated, heat labile, and digested by trypsin.

A similar toxin has been isolated from group D *P. multocida* affecting rabbits. This toxin, about 112 to 158 kilodaltons, was shown to be lethal for mice and cell associated (27).

P. multocida strains of a variety of serotypes and sources, including swine, have been shown to produce other proteins that are toxic, as measured by their effect on embryonic bovine lung cells (25, 29). Whether this activity is due to one or several proteins is unknown. Though the sizes of the toxic principles are unknown, they differ from the dermonecrotic toxin by being liberated into culture supernatants.

P. multocida from bovine sources also produces a saline-extractable substance that appears to be associated with the hyaluronic acid capsule of serotype A strains (30). This substance (about 300 kilodaltons) was shown to decrease uptake of staphylococci by bovine polymorphonuclear leukocytes. Removal of the capsule resulted in an increased uptake of *P. multocida* by bovine leukocytes. These effects are difficult to reconcile with the results of experiments of others which show that *P. multocida* type A cells do not affect the chemiluminescence response of bovine leukocytes (5).

The antiphagocytic protein(s) described herein differs from those previously described for *P. multocida* in a number of important ways. First, the protein we describe is cell associated (like the dermonecrotic toxin), but different from those toxins that are active on embryonic bovine lung cells (which are not cell associated). Second, the antiphagocytic protein we describe has a molecular weight of about 50,000. All the other toxins from *P. multocida* so far described have molecular weights in excess of 100,000. It is

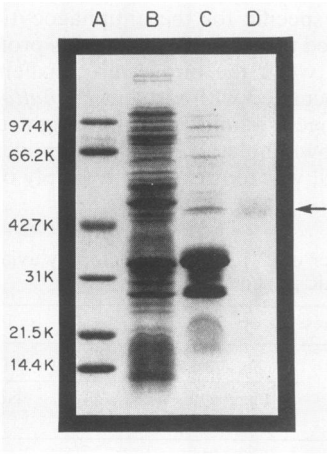

FIG. 1. SDS-PAGE of proteins obtained from *P. multocida*. Lanes: A, molecular weight standards (K, 10^3); B, whole-cell proteins; C, outer membrane proteins: D, eluted antiphagocytic principle. Numbers at the left represent molecular weights of the molecular weight standards; arrow indicates the position of the antiphagocytic principle. The gel was stained with Coomassie brilliant blue.

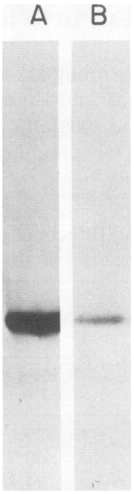

FIG. 2. Immunoblot of proteins separated by PAGE under nondenaturing conditions. The primary antibody was made against the antiphagocytic principle. Lanes: A, whole-cell proteins; B, antiphagocytic principle.

possible, of course, that the protein we are studying is part of a larger moiety. We feel that this is unlikely for two reasons: first, antiserum to the eluted antiphagocytic principle does not bind to anything else on immunoblots, and second, subunits of other *P. multocida* toxins are not active (23).

It is premature to generalize concerning the role of the antiphagocytic protein in the pathogenesis of fowl cholera, since our experiments were conducted using only one strain of *P. multocida*, strain P1059. This strain possesses the somatic antigen 3 and is of the A capsular type (composed of hyaluronic acid). The model that follows is one constructed from data obtained from experiments with strain P1059. It should be pointed out, however, that a majority of the strains of *P. multocida* isolated from turkeys possess capsule type A, and most of these possess somatic antigen 3, though not solely (14, 27; unpublished observations).

We propose that the role of the toxin in vivo is to prevent uptake of *P. multocida* by phagocytic cells (supported by the observation that trypsinized, unencapsulated *P. multocida* are readily phagocytosed). *P. multocida* apparently multiplies in the extracellular compartment of the turkey (35). To do so, it must escape destruction not only by the humoral components of the blood, such as the complement proteins, but by the cellular as well (3, 10, 17). We have previously shown that resistance to killing by components of the complement system resides in the capsule (36). Though the capsule may possess some antiphagocytic properties, data presented herein suggest that a toxin also interferes with uptake (1, 21). As predicted, serum-sensitive unencapsulated *Pasteurella* strains are poorly phagocytosed (35), presumably due to the expression of antiphagocytic activity of the protein in the outer membrane. These properties, taken in sum, allow *P. multocida* to multiply to 10^7 to 10^8/ml of blood.

It is important that antibodies to the antiphagocytic principle neutralize its effect on the phagocytic cell as well as protecting turkeys from lethal challenge. This makes feasible the use of epitopes on the toxin as part of an immunizing product in the form of an inactivated toxoid or as a subunit vaccine. Antibodies directed against the antiphagocytic moiety would not only bind and neutralize, but, as importantly, trigger the complement system, generating other opsonins as well as membrane attack complexes. This would either lead to killing of the microorganism outright, or promote its phagocytosis and subsequent killing.

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