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Capsular Hyaluronic Acid-mediated Adhesion of Pasteurella multocida to Turkey Air Sac Macrophages

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SUMMARY. Serogroup A strains of *Pasteurella multocida,* the major cause of fowl cholera, are resistant to phagocytosis in nonimmunized birds. Adherence studies with a capsulated strain of *P. multocida* (serotype A:3) and turkey air sac macrophages in culture showed that the bacteria were capable of adhering in large numbers to the macrophages but were not internalized. A noncapsulated variant of the bacteria (serotype -:3) showed little or no adherence and was not internalized. These data indicated that the adhesive properties were caused by the presence of a capsule on the bacteria. The role of capsular hyaluronic acid in adherence to macrophages was investigated. Depolymerization of the bacterial capsule with hyaluronidase increased phagocytosis by macrophage cultures, and addition of hyaluronic acid to the macrophages inhibited bacterial adherence. Additionally, exposure of macrophages to chondroitin sulfate B, an anionic polysaccharide similar to hyaluronic acid, did not affect the adhesive properties and resistance to phagocytosis of capsulated organisms. Treatment of macrophages with sodium metaperiodate or trypsin suppressed bacterial binding. Collectively, these data indicate that *P. multocida* adhesion to air sac macrophages, but not internalization, is mediated by capsular hyaluronic acid and suggest that recognition of this bacterial polysaccharide is a result of a specific glycoprotein receptor.

RESUMEN. Adhesión capsular mediada por el ácido hialurónico de la Pasteurella multocida a los macrófagos de los sacos aéreos de pavos.

Las cepas de *Pasteurella multocida* pertenecientes al serogrupo A, la mayor causa de cólera en aves, son resistentes a la fagocitosis en aves no inmunizadas. Estudios de adherencia con una cepa capsulada de *P. multocida* (serotipo A:3) y con cultivos de macrófagos de los sacos aéreos de pavos, demostraron que la bacteria fue capaz de adherirse a grandes cantidades de los macrófagos pero sin penetrarlos. Una variante de la bacteria sin cápsula (serotipo -:3) mostró poca o ninguna adherencia y no fue penetrada. Estos resultados indicaron que las propiedades adhesivas de la bacteria son debido a la presencia de cápsula. Se investigó el papel del ácido hialurónico capsular en la adherencia a los macrófagos. La despolimerización de la cápsula bacteriana por la enzima hialuronidasa aumentó la fagocitosis por los cultivos de macrófagos, y la adición de ácido hialurónico a los macrófagos inhibió la adherencia a las bacterias. Además, la exposición de los macrófagos al sulfato de condroitina B, un polisacárido aniónico similar al ácido hialurónico, no afectó las propiedades adhesivas y la resistencia a la fagocitosis por organismos capsulados. El tratamiento de los macrófagos con metaperiodato de sodio o tripsina suprimió la fusión bacteriana. Estos resultados indican que la adhesión de la *P. multocida* a los macrófagos de los sacos aéreos, pero no la penetración, es mediada por el ácido hialurónico capsular y sugiere que el reconocimiento de este polisacárido bacteriano es el resultado de un receptor específico de glicoproteína.

Key words: adhesion, hyaluronic acid, capsule, *Pasteurella multocida*

Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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Abbreviations: ASM = air sac macrophages; FITC = fluorescein isothiocyanate; MEM = minimum essential eagle medium; mMEM = MEM + penicillin + streptomycin + fungizone + heat-inactivated fetal bovine serum; mPBS = PBS + fetal bovine serum; mRPMI = RPMI-1640 without sodium bicarbonate and phenol red; PBS = phosphate-buffered saline; PI = propidium iodide

Pasteurella multocida causes fowl cholera in both domestic and wild birds. The high morbidity and mortality associated with fowl cholera result in significant economic losses to the poultry industry, especially the turkey industry. Transmission of the disease usually occurs following contamination of drinking water with mucous secretions from infected birds. Experimentally, a small inocula of virulent organisms (30–100) is capable of causing nearly 100% mortality within 48 hr. *Pasteurella multocida* usually enters tissues of birds through mucous membranes of the pharynx and upper air passages (8). Septicemia, pneumonia, and airsacculitis are common clinical features of turkeys that are affected by the disease.

Serogroup A strains of *P. multocida* are the major cause of fowl cholera in poultry. An ability to evade host phagocytic cells and resistance to complement-mediated lysis by normal turkey plasma are considered important virulence factors of pathogenic strains (5). Survival of *P. multocida* outside the host and resistance to phagocytosis in the host are associated with the presence of a capsule. With serogroup A strains of *P. multocida,* the capsule contains hyaluronic acid, an anionic polysaccharide that also occurs in host tissues (10). Hyaluronic acid is found in the capsules of other bacteria, such as group A streptococci, where it is recognized as a virulence factor (16). In turkeys, the capsule seems to inhibit opsonization or create physical interferences of receptor–ligand binding between phagocytes and opsonized bacteria (11). Whether those effects in turkeys are exclusively a result of the presence of hyaluronic acid is not yet clear. Although antiphagocytic properties are generally attributed to the capsule, a 50-kD outer membrane protein capable of inhibiting phagocytosis has been described (13).

Phagocytosis is an event that follows adhesion of host cells to opsonized or nonopsonized bacteria. Although adhesion of *P. multocida* to respiratory epithelia has received some interest, studies on adhesion to professional phagocytic cells are sparse. The adhesive properties of *P. multocida* to cultured HeLa cells and mammalian phagocytes have been examined (2,3). Use of the HeLa cell model revealed that capsular hyaluronic acid could mediate the bacterial adhesion (2). Nevertheless, studies on *P. multocida* adhesion to avian phagocytes have not been described.

This study was designed to investigate whether 1) capsulated *P. multocida* would adhere to turkey air sac macrophages, 2) adherence occurred by means of hyaluronic acid contained in its capsule, and 3) a receptor specific for hyaluronic acid occurred on the surface of macrophages.

MATERIAL AND METHODS

Animals. Normal Beltsville small white turkeys, 8–12 wk old, from the National Animal Disease Center were used.

Bacteria. Capsulated (serotype A:3) and noncapsulated (serotype -:3) *P. multocida* strain P-1059 were used. The noncapsulated variant was derived by serial passage on dextrose starch agar and selection of colonies that appeared blue in oblique-transmitted light. The bacteria were grown overnight at 37 C on dextrose starch agar (Baltimore Biological Laboratories, Cockeysville, Md.). Bacterial cells were resuspended in RPMI-1640 without sodium bicarbonate and phenol red (mRPMI; Sigma Chemical Co., St. Louis, Mo.). The suspensions were adjusted to a density equivalent to that of a number 2 MacFarland nephelometer standard ($\sim 2 \times 10^9$ bacteria/ml) using a spectrophotometer (model 35; Perkin-Elmer, Oak Brook, Ill.).

Phagocytic cell recruitment and collection. A sterile suspension of Sephadex G-100 superfine (Pharmacia Fine Chemicals AB, Uppsala, Sweden), preswollen in 0.85% sterile saline, was the eliciting agent used to recruit phagocytic cells. For recruitment, left and right posterior thoracic air sacs were inoculated with the eliciting agent. The suspension was injected about 2 cm deep with syringe and needle at a site located dorsally in the last intercostal space. The injected volume was 1 ml of suspension/100 g of body weight. After 72 hr, turkeys were euthanatized (Sleepaway; Fort Dodge Laboratories, Inc., Fort Dodge, Iowa) and opened to expose the anterior and posterior thoracic air sacs. Care was taken to avoid cutting major blood vessels. Recruited cells were col-

lected by flushing the air sacs with cold Hanks' balanced salt solution (Gibco Laboratories Inc., Grand Island, N.Y.) that contained 0.5 U heparin/ml; collection was done with a siliconized Pasteur pipet. The cells were centrifuged at 600 × *g* for 10 min at 22 C, and the pellet was resuspended in minimum essential eagle medium (MEM; Sigma) supplemented with 100 U/ml penicillin, 50 μg/ml streptomycin, 2 μg/ml fungizone, and 10% heat-inactivated fetal bovine serum (mMEM).

Removal of the heterophils. The cell suspension was layered on a density gradient medium (Accuprep [sp. G. 1.077]; Accurate Chemical & Scientific Corporation, Westbury, N.Y.) in 13 × 100-mm tubes according to the instructions provided by the manufacturer. Tubes were centrifuged at 600 × *g* for 15 min at 22 C. The cell layer above the gradient medium was collected and diluted 1:3 with 0.85% sterile saline supplemented with 0.13% ethylenedinitrilotetraacetate acid (Eastman Fine Chemicals, Rochester, N.Y.) to reduce the density of the solution. The diluted layer was centrifuged at 500 × *g* for 10 min at 22 C. The cell pellets containing the air sac macrophages (ASM) were resuspended in mMEM, and cell counts were made using a cell counter (Nova Cell Track; Alicia Diagnostics, Oveido, Fla.). Four-well chamber slides (Lab Tek®; Nunc Inc., Naperville, Ill.) or 25-cm^2 tissue culture flasks (Corning Inc., Corning, N.Y.) were seeded with 2–4 × 10^5 macrophages/cm^2. After 2 hr of incubation at 37 C under 5% CO_2, the medium was changed, and macrophage cultures were further incubated overnight before use in adhesion assays.

Adherence assays. Overnight ASM cultures were rinsed three times with MEM plus 10% heat-inactivated fetal bovine serum, and adjusted suspensions of *P. multocida* were added to give a final concentration of $10^{8.2}$ bacteria/10^6 macrophages. After addition of bacteria, incubation was 2 hr at 37 C in an atmosphere containing 5% CO_2. For enumeration of bacteria adhering to ASM, the slides were rinsed three times with MEM, stained by Diff Quick (Baxter Healthcare Corporation, McGaw Park, Ill.), and examined with a light microscope. Adhesion was evaluated by observing 50–100 randomly chosen macrophages. Each experiment was repeated with three different birds.

A modification of the double fluorescence technique of Detilleux *et al.* (1) was used to discriminate between adherent and internalized bacteria as follows: 1) chamber slides of ASM exposed to bacteria were washed (three times) in phosphate-buffered saline (PBS) supplemented with 3% fetal bovine serum (mPBS); 2) residual ASM-associated bacteria were labeled for 1 hr at 4 C in the dark with a 1:3000 dilution of rabbit anti-Pasteurella serum; 3) the slides were washed three times with mPBS, macrophages were fixed for 5 min in methanol at 4 C, dehydrated for 5 min in acetone at 4 C, and then air dried; 4) a 1:50 dilution of fluorescein isothiocyanate (FITC)-conjugated, goat anti-rabbit antiserum (Kirkegarrde and Perry Laboratories, Rockville, Md.) was applied for 1 hr at 4 C in the dark; 5) the slides were washed with mPBS (three times) and treated for 3 min with propidium iodide (PI; Sigma) solution (25 μg PI/ml PBS); and 6) slides were washed with mPBS, rinsed with distilled water, and immediately mounted (Vecta Mounting Medium; Vector Laboratories, Inc., Burlingame, Calif.) with coverslips. Fluorescence was observed with a BX50 microscope (Olympus Optical Co., Tokyo, Japan) equipped with a reflected light fluorescent attachment and a filter cube (U-M51005) for propidium iodide and FITC. Because fluorescent bacteria could not be precisely enumerated, the adherence or the internalization was determined subjectively with 0 indicating no adherence or internalization and 1+ indicating slight (1–25 bacteria/macrophage), 2+ indicating moderate (26–75 bacteria/macrophage), and 3+ indicating heavy (≥76 bacteria/macrophage) adherence or internalization.

Antiserum. Anti-Pasteurella serum was made in rabbits with capsulated strain P-1059 as described by Rimler and Brogden (9). The antiserum was absorbed with turkey liver powder to remove any cross-reactive antibodies for turkey cells and tissues.

Decapsulation and adherence inhibition. To demonstrate the role of hyaluronic acid in binding, capsulated *P. multocida* were treated with 250 U of hyaluronidase (Sigma)/1 × 10^9 bacteria in mRPMI for 2 hr at 37 C before addition to macrophage cultures.

Adherence inhibition studies were performed by treating macrophage cultures in MEM for 2 hr at 37 C and 5% CO_2 with one of the following substances: hyaluronic acid (5 mg/ml; Sigma), trypsin (250 U/ml; Millipore Corporation, Freehold, N.J.), chondroitin sulfate B (5 mg/ml; Calbiochem, La Jolla, Calif.), or 4.278 mg/ml sodium metaperiodate. After each treatment, *P. multocida* were added to the treated macrophage cultures in MEM plus 10% fetal bovine serum, and incubation was done for an additional 2 hr. The final concentration was 2 × 10^8 bacteria/1 × 10^6 macrophages.

Electron microscopy. To confirm adherence and internalization observed by the fluorescence technique, the macrophages were transferred to sterile siliconized centrifuge tubes with aid of a rubber policeman. The cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4 C. After fixation, the tubes were lightly centrifuged (for 5 min at 1000 rpm) to pellet the cells. The glutaraldehyde was discarded, and the cells were stored in 0.1 M sodium cacodylate buffer at 4 C. Macrophages were postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer at 4 C, dehydrated in ethanol, infiltrated and embedded in epoxy resin, sec-

tioned at 70–90 nm, and examined with a Philips 410 electron microscope.

Statistical analysis. Student's *t*-test was used to determine statistical probabilities.

RESULTS

Adhesion assays. Enumeration of bacteria associated with ASM was determined by light microscopy, and distinction between adherent and internalized bacteria was determined by fluorescence microscopy. An example of capsulated *P. multocida* adhered to ASM is shown in Fig. 1. Note that the bacteria are closely associated with macrophages and there are no bacteria free in the intercellular spaces. With the double fluorescence method, primary antibodies do not penetrate the plasma membrane of unfixed cells (1). Therefore adherent bacteria, but not internalized bacteria, react to produce bright-green fluorescence with labeled secondary antibodies (Fig. 2). Because methanol-acetone fixation results in membrane permeability, both macrophage DNA (Figs. 2, 3) and internalized bacteria fluoresce bright red (Fig. 3). Similar to light microscopic observations with the Diff Quik stain procedure, fluorescent microscopy showed bacteria were intimately associated with macrophages and there were no bacteria free in the intercellular spaces. The findings observed with fluorescence microscopy were confirmed by electron microscopy, and an example of a macrophage with internalized bacteria is shown in Fig. 4.

Influence of hyaluronic acid on bacterial adhesion. Capsulated bacteria were adherent but not internalized by ASM (Table 1). Comparisons of adhesion between capsulated P-1059 and its noncapsulated variant indicated that the former had significant higher incidence of adherence ($P < 0.05$). These data suggested that the presence of a capsule promoted adherence. Depolymerization of the bacterial capsule with hyaluronidase resulted in a high degree of bacterial association with ASM ($P < 0.05$). In contrast to nontreated bacteria, hyaluronidase-treated bacteria were internalized.

Preincubation of the ASM with chondroitin sulfate B, an anionic polysaccharide similar to hyaluronic acid, did not significantly affect adherence of capsulated bacteria (Table 2). Although exposure of ASM to a concentration of 5 mg/ml of hyaluronic acid diminished adherence of capsulated bacteria, adherence of hyaluronidase-treated *P. multocida* was not affected (data not shown).

Oxidation of the macrophage surface with sodium metaperiodate markedly reduced the bacterial adhesion. Moreover, proteolytic treatment of the macrophages with trypsin suppressed bacterial binding (Table 2).

DISCUSSION

Death of turkeys from acute fowl cholera usually results following a septicemia, and microscopic examination of their blood shows little or no phagocytosis by heterophils or monocytes. The failure of circulating phagocytes to ingest *P. multocida* has been associated with the presence of a capsule on the bacterium. This antiphagocytic property of the capsule is considered to be a major virulence factor because noncapsulated bacteria are greatly reduced in virulence (10,14). Pneumonia and airsacculitis are common findings in acute as well as chronic fowl cholera, and recent studies have suggested that damaged air sac epithelium may be a portal of entry for *P. multocida* into the systemic circulation (4). Therefore we were interested in determining the response of respiratory tract macrophages to capsulated *P. multocida.*

Respiratory tract macrophages in many species are considered a first line of defense. However, numbers of resident macrophages in the respiratory tract of normal chickens and turkeys are very low compared with those in mammals of similar body weight (12). Moreover, experimental exposure to *P. multocida* by tracheal inoculation in chickens and air sac inoculation in turkeys resulted in a response predominantly composed of heterophils (4,6). Although respiratory tract infection with *P. multocida* attracts macrophages, nonopsonic phagocytosis by these cells has not been demonstrated. Adhesion to phagocytic cells and subsequent internalization are significant features in the pathogenesis, as well as the prevention, of many bacterial diseases. In the present study with recruited air sac macrophages, capsulated *P. multocida* were adherent but not internalized. These findings are in contrast to those seen in preliminary studies in which bacteria neither adhered nor were internalized by peripheral blood monocytes (Pruimboom, unpubl.). Capsule depolymerization with hyaluronidase increased adhe-

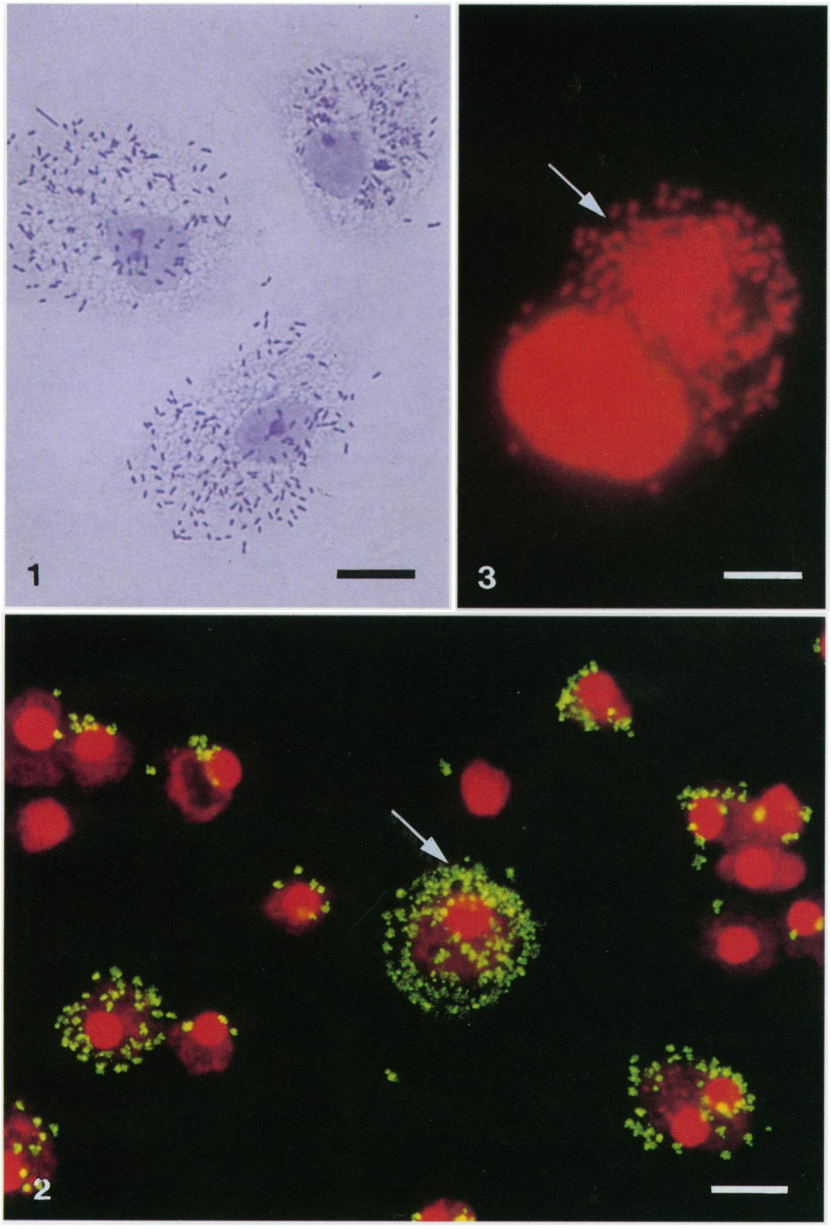

Fig. 1. Example of Diff Quick stain of capsulated *P. multocida* adhering to recruited air sac macrophages. Bar: 10 mm = 3.57 μm.

Fig. 2. Double fluorescence technique showing adherent capsulated *P. multocida*. Adherent bacteria (3+; see text) appear green (arrow) and macrophage nuclei appear red. The cytoplasm of the phagocyte is lightly stained in red. Bar: 10 mm = 8.93 μm.

Fig. 3. Double fluorescence technique showing internalization of *P. multocida* (3+; see text) after depolymerization of the capsule. Internalized bacteria (arrow) appear bright red. Bar: 10 mm = 2.98 μm.

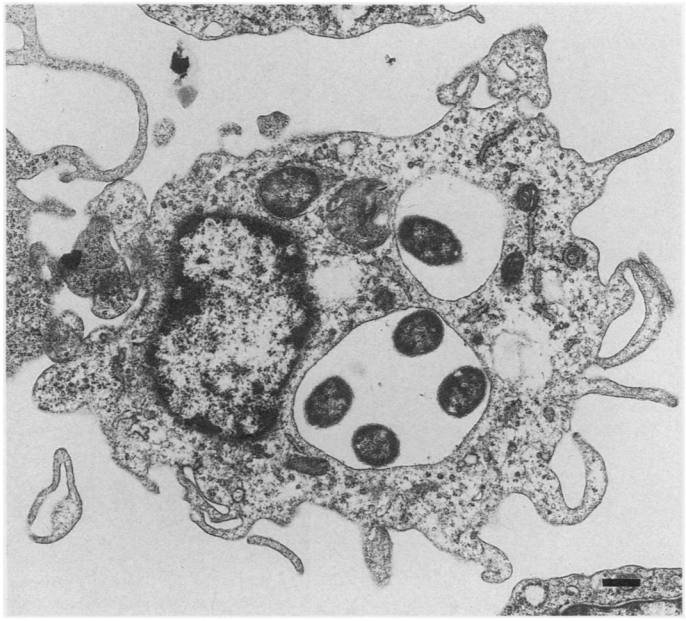

Fig. 4. Electron micrograph of turkey air sac macrophage with internalized bacteria. Bar: 4.9 mm = 0.5 μm.

sion and subsequent internalization of the bacteria by recruited macrophages. This observation together with the findings of the inhibition studies demonstrated the presence of a specific hyaluronic acid receptor. Apparently, this hyaluronic acid receptor is absent from normal peripheral blood monocytes.

This report showed that hyaluronic acid is the capsular component that mediates adhesion to recruited air sac macrophages. Hyaluronic acid seems to prevent internalization as described for group A streptococcus (16). It is clear from our studies, as well as others (5,14), that enzymatic depolymerization of the capsule enhances phagocytosis of *P. multocida.* This finding implies that a receptor other than that for hyaluronic acid is necessary for adherence and nonopsonic internalization of capsule-depolymerized bacteria.

Treatment with trypsin and metaperiodate abolished or diminished hyaluronic acid-mediated adherence to the recruited macrophages. These findings suggest that the hyaluronic acid receptor is a glycoprotein. Further investigations will be required to confirm this observation.

The role of the macrophage hyaluronic acid receptor in fowl cholera is not clear. Following experimental intravenous infection, capsulated *P. multocida* are rapidly cleared from the blood and are found primarily in the liver and spleen (7,14,15). Because little or no nonopsonic

Table 1. Adherence and internalization of *P. multocida* by air sac macrophages.

	Adhesion of bacteria[A]	Internalized bacteria[A]	Number of bact./macr.[B]
Capsulated P-1059	2+[C]	1+	50.2 ± 6.2
Noncapsulated P-1059	1+	1+	2.8 ± 0.6
Hyaluronidase-treated P-1059	1+	3+	101.2 ± 16.3

[A]Determined by fluorescent microscopy,
[B]Determined by Diff Quick stain (mean of three experiments).
[C]1+ = slight, 2+ = moderate, 3+ = heavy (see text).

Table 2. Inhibition of adhesion of capsulated *P. multocida* to air sac macrophages.

Treatment	Adhesion inhibition[A]	Number of bact./macr.[A]	*P*-value
None	No	50.2 ± 6.2[B]	—
Hyaluronic acid (5 mg/ml)	Yes	3.8 ± 1.7	$P < 0.05$
Chondroitin sulfate B (5 mg/ml)	No	45.4 ± 1.5	Not Significant
Metaperiodate (4.278 mg/ml)	Yes	6[C]	ND[D]
Trypsin	Yes	1.1 ± 0.9	$P < 0.05$

[A]Determined by Diff Quick stain.
[B]Mean of three experiments (see text).
[C]Experiment done once.
[D]Not determined.

phagocytosis occurs and the bacteria tend to concentrate and reproduce in these organs, it seems probable that a hyaluronic acid receptor on the resident macrophages may be a colonization factor. Identification of a hyaluronic acid receptor on resident macrophages of the liver and spleen and determination as to whether it is involved in clearance of *P. multocida* from circulating blood await further study.

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