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Mac-1-mediated Uptake and Killing of *Bordetella bronchiseptica* by Porcine Alveolar Macrophages

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Abstract

The role of Mac-1 as a receptor for *Bordetella bronchiseptica* infection of alveolar macrophages (AMΦ) was examined using 6 strains (2 ATCC and 4 pathogenic field isolates) to assess *B. bronchiseptica* binding, uptake and replication in primary porcine AMΦ. All B. bronchiseptica strains were rapidly killed by porcine serum in a dose- and time-dependent manner. However, heat-inactivated porcine serum (HIS) did not demonstrate any bacterial-killing activity, suggesting that complement may have a direct killing activity. All field isolates were more resistant to direct complement-mediated *B. bronchiseptica* killing. The uptake of *B. bronchiseptica* into AMΦ was inhibited approximately 50% by antiMac-1 monoclonal antibodies in the medium. However, *B. bronchiseptica* phagocytosed in the presence of serum or HIS was not altered by anti-Mac-1 antibodies although more bacteria were internalized by addition of serum or HIS. These data suggest that Mac-1 is a target for direct uptake of *B. bronchiseptica* via opsonin-independent binding. The phagocytosed *B. bronchiseptica*, either via direct or serum-mediated binding, were efficiently killed by AMΦ within 10 hr post-tinfection. This demonstrates that Mac-1-mediated *B. bronchiseptica* uptake is a bacterial killing pathway not leading to productive infections in AMΦ.

Key words: Mac-1, *Bordetella bronchiseptica*, alveolar macrophage, pig

Introduction

Bordetella bronchiseptica is an important respiratory tract pathogen of several mammals, including swine, dogs and laboratory animals [8]. Although *B. bronchiseptica* is not considered as a human pathogen, several cases of *B. bronchiseptica*-associated human infections have been reported in immunocompromised patients [29]. In swine, *B. bronchiseptica* is primarily a respiratory pathogen causing atrophic rhinitis, a disease which is responsible for considerable economic losses in the swine industry. *B. bronchiseptica* was considered to be an extracellular pathogen which localizes and multiplies on and among cilia of the respiratory epithelial cells [20]. However, several studies have demonstrated the capability of *B. bronchiseptica* for invasion and intracellular survival in upper respiratory tract epithelial cells and dendritic cells [9, 14, 26]. A study also demonstrated that *B. bronchiseptica* was rapidly ingested by porcine PMN in the absence of complement and antibody, and that internalization was mediated by multiple adhesion mechanisms, including CD18- and carbohydrate-dependent pathways [21]. The utilization of CD18-integrin, Mac-1, has also been identified in the attachment and internalization of the human macrophage intracellular pathogen *B. pertussis* [22, 25].

Mac-1 is a noncovalent heterodimer composed of an α-chain (CD11b) and a β-chain (CD18), and is primarily expressed on granulocytes, monocytes, macrophages and natural killer cells [2]. Mac-1 serves as a multifunctional receptor for a wide range of ligands, including ICAM-1, C3bi, LPS, β-glucan, Factor X and several bacterial adhesins [23]. Several studies demonstrated that macrophage Mac-1 expression was utilized as a receptor for attachment and internalization by a group of pathogenic respiratory microorganisms [13]. *B. pertussis* [22, 25], *Legionella pneumophila* [19], *Mycobacterium tuberculosis* [10], *Histoplasma capsulatum* [5], group *B streptococci* [1] and *Rhodococcus equi* [11] can utilize Mac-1 to gain entry into macrophages. Utilization of Mac-1 for internalization allows pathogens to bypass critical killing pathways, such as the production of H_2O_2 and toxic oxygen free radicals [28, 31]. Mac-1 also serves as a complement receptor type 3 (CR3) which promotes macrophage-mediated phagocytosis of

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complement (C3bi) opsonized targets [4]. Therefore, uptake of microorganisms via Mac-1 occurs either by the surface coating of the pathogen with C3bi or direct binding to a surface-localized ligand encoded by the microorganism. Mac-1-mediated pathogen internalization into macrophages to avoid intracellular killing mechanisms suggests that Mac-1 may serve as an entry point to a pathway for productive bacterial infections of alveolar macrophages (AMΦ).

AMΦ are the first line of host defense in the lung and their interaction with respiratory pathogens may determine the fate of pathogen either for bacterial killing or for evading host killing mechanisms. The mechanism(s) of binding and the subsequent fate of bacteria, either extracellular adhesion or internalization, are unknown in the interaction of *B. bronchiseptica* with porcine AMΦ. Previously, the expression of Mac-1 (CD11b/CD18) by porcine AMΦ has been studied in our laboratory [16, 17]. In the present study, we tested the hypothesis that- Mac-1-mediated *B. bronchiseptica* binding to AMΦ results in uptake into AMΦ leading to intracellular replication indicative of a productive infection. Two ATCC strains and four field isolates of *B. bronchiseptica* were used to examine the binding, uptake and replication in AMΦ in the absence or presence of anti-Mac-1 antibodies.

Materials and Methods

Reagents. Mouse IgG1 isotype control (MOPC-21) and anti-CD18 (MHM23) monoclonal antibody (mAb) were obtained from Sigma Chemical Co. (St. Louis, MO) and DAKO Corp. (Carpinteria, CA), respectively. Anti-CD11b mAb (TMG6-5) was kindly provided by I. Ando (Institute of Genetics, Szeged, Hungary). Gentamicin and other general chemicals were purchased from Sigma Chemical Co.

Preparation of *B. bronchiseptica*. Two ATCC strains including the type strain were obtained from ATCC (American Culture Type Collection, Rockville, MD) and four field isolates of *B. bronchiseptica* were kindly provided by J.E. Collins (Veterinary Diagnostic Laboratory, University of Minnesota) (Table 1). *B. bronchiseptica* strains were prepared for the bacterial invasion assays as follows. *B. bronchiseptica* was inoculated onto blood agar plate and incubated for 24 hr at 37℃. The bacteria were washed with 10 ml phosphate-buffered saline (PBS) by scraping cells from the surface of plates and then separated by centrifugation (1,000 x g, 4℃ for 10 min), washed with PBS, recentrifugated and resuspended in PBS. To prepare frozen bacterial stocks, an equal volume of 15% sterile glycerol supplemented with 5% DMSO was added and small aliquots of the suspension were dispensed into small sterile vials. Vials were stored at -80℃ and the number of CFU/ml was determined by plating 10-fold dilutions on blood agar plates.

Preparation of porcine AMΦ. Porcine AMΦ was collected from 8- to 10-week old pigs as previously described (3). Briefly, after flushing the lungs with 500 to 1,000 ml of PBS, cells were collected by centrifugation (500 x g at 4℃ for 10 min) and resuspended in PBS for counting.

Swine sera. Swine sera were prepared from the blood of clinically healthy 12-week-old pigs that were immunized with *B. bronchiseptica* at 6-week-old. Briefly, the blood was allowed to clot for 1 hr at room temperature and centrifuged at 2,000*g* for 20 min at 4℃. The sera were stored at -80℃ in 1.0 ml aliquots. Heat-inactivated serum (HIS) was prepared by incubation at 56℃ for 30 min.

Bacterial invasion assay. Bacterial invasion assays were performed to measure the bacterial binding, uptake and intracellular replication as previously described (6) with some modification.

a. Binding: One ml of macrophages (5x 105 cells) in RPMI 1640 was added to each well of a 24-well tissue culture plate (Corning, NY) and incubated for 1.5 hr at 37℃ in 5% CO2 After washing twice to remove adherent cells, macrophages were incubated in the presence of blocking mAb (i.e., anti-CD18, anti-CD11b with 0.1 ~ 0.5 μg of mAb/106 cells) in 200 μl PBS at 4℃ for 30 min. Macrophages were washed twice with 1 ml of PBS prior to inoculation of the wells. Bacteria (1.25 x 107 CFU) were added in 1 ml of RPMI 1640 in the presence or absence of the indicated % of serum or HIS with gentle shaking to distribute the inoculum throughout the tissue culture medium. The number of bound bacteria to AMf (CFU/well) was determined by the plate count, after incubation of the 24-well plate at

Table 1. *B. bronchiseptica* strains used in this study

Strain	Classification	Strain number	Source
Bb1	Field isolate	95-12430	VDL1)
Bb2	Field isolate	95-12977	VDL
Bb3	Field isolate	95-13492	VDL
Bb4	Field isolate	95-13538	VDL
Bb5	Type strain, dog isolate	19395	ATCC2)
Bb6	Reference strain, pig isolate	31437	ATCC

1) VDL, Veterinary Diagnostic Laboratory, University of Minnesota, St. Paul, MN.
2) ATCC, American Type Culture Collection, Rockville, MD.

37℃ for 30 min in a 5% CO2 incubator following washing three times with PBS (prewarmed to 37 ℃).

b. Uptake: To determine the number of internalized viable intracellular bacteria, the incubation time of macrophages with bacteria was extended from 30 min to 1 hr. Macrophage-associated extracellular bacteria were eliminated by adding 1.5 ml of RPMI 1640 supplemented with gentamicin (50 μg/ml) following the 2 hr incubation at 37℃ in 5% CO2 incubator. The gentamicin was removed by washing with PBS and the macrophages were selectively lysed by adding 1 ml of 0.1% Triton X-100 in distilled water. The total number of viable bacteria (CFU/well) was determined by plating on blood agar plates.

c. Intracellular replication: The extent of intracellular replication of bacteria was determined in gentamicin medium. Recovered bacteria from the various time points, up to 26 hr post-infection, were compared with the recovery at the initial time point.

The results of an invasion assay were presented as the invasion index: invasion index= (No. of uptaken bacteria ÷ No. of bound bacteria) x 100 %. All bacterial binding, uptake and replication assays were performed at least in duplicates for statistical analysis.

Results

Optimization of *B. bronchiseptica* binding and uptake by AMΦ.

Six different *B. bronchiseptica* strains including 2 ATCC strains and 4 field isolates were used in this study (Table 1). The growth patterns of each strain were similar except strain Bb6 (Fig. 1). Strain Bb6 demonstrated a 12 hr lag period. The same length of time requirement for Bb6 growth was also observed on blood agar plate culture (data not shown). In order to optimize the ratio of bacteria to macrophages showing a linear range of response in bacterial invasion assays, the binding and uptake pattern of *B. bronchiseptica* at various ratios of bacteria to AMΦ was determined. Linear responses of bacteria binding and uptake were demonstrated in less than 50:1 (bacteria/AMf) ratio (data not shown), and the ratio 25:1 (bacteria/AMf) was therefore selected in further bacterial binding and uptake assay. The early saturation of *B. bronchiseptica* uptake by porcine AMΦ at lower bacteria ratios (data not shown) suggests that binding of B. bronchiseptica to AMf is mediated by multiple bacteria-AMΦ contacts, whereas intracellular uptake of *B. bronchiseptica* is mediated by specific receptor(s)-mediated events between *B. bronchiseptica* and AMΦ.

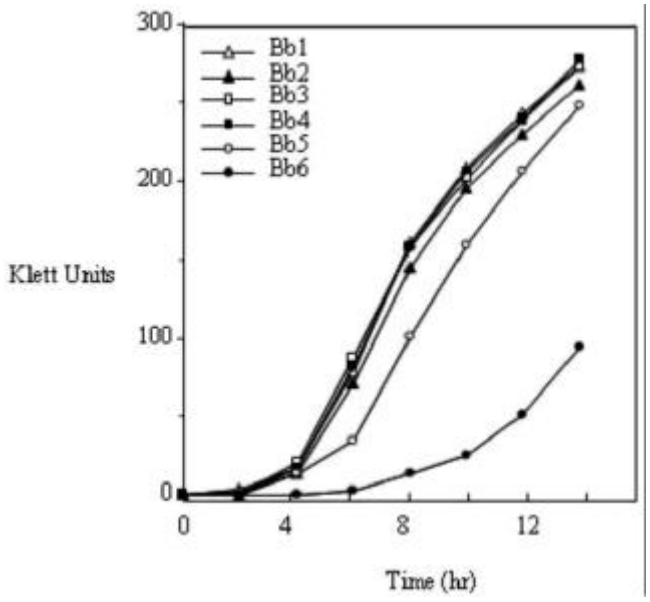

Fig. 1. *B. bronchiseptica* growth gurves. *B. bronchiseptica* (1 x 108 CFU) was inoculated from frozen bacterial stocks into 4 ml of LB medium to a final concentration of 2.5 x 107 CFU/ml. Bacteria were grown at 37℃ with 250 rpm shaking for 14 hr. The bacterial growth was monitored by measuring Klett Units using Klett colorimeter every 2 hr. Results shown are the mean of duplicate measurements.

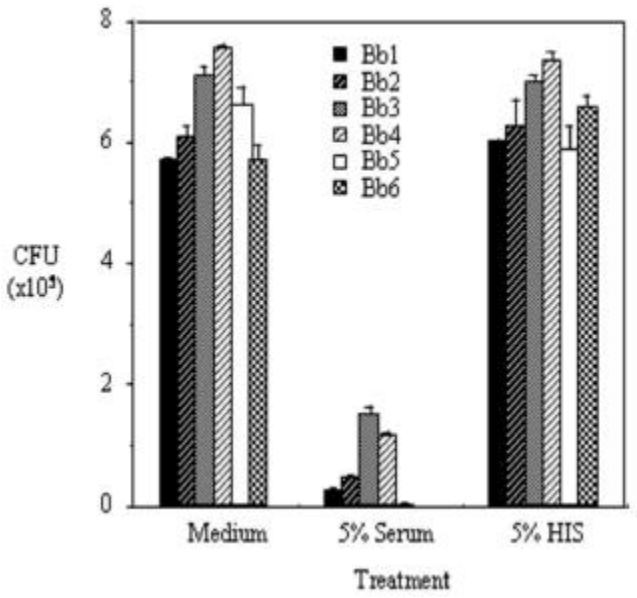

Fig. 2. Serum-mediated *B. bronchiseptica* bactericidal activity. Each strain of *B. bronchiseptica* (5 x 105 CFU) was incubated in 200μl RPMI 1640 medium at 37℃ for 1 hr in the absence or presence of 5% serum or 5% HIS. After adding 800 μl of cell lysis buffer (0.1% Triton X-100 in dH2O), the tube was rotated for 5 min to facilitate AMΦ lysis. The number of viable bacteria (CFU) was determined by plate count. Results shown are the mean of duplicate blood agar plate cultures.

Porcine serum has direct *B. bronchiseptica* killing activity.

In order to test the direct effect of serum from Bordetella-vaccinated pigs on *B. bronchiseptica* viability, *B. bronchiseptica* was incubated for 1 hr at 37℃ in the absence or presence of 5% serum or 5% HIS. Medium or HIS did not reveal any bactericidal activity for any of the strains examined. However, overall 90% of *B. bronchiseptica* were killed within 1 hr at 5% serum (Fig. 2). These results suggest that serum complement may have *B. bronchiseptica* bactericidal activity through the activation of complement cascade since HIS did not show killing activity. The serum-mediated direct *B. bronchiseptica* killing was dose- and time-dependent (data not shown). Pathogenic field isolates of *B. bronchiseptica* were more resistant to serum-mediated killing compared to ATCC strains (Fig. 2).

The effect of serum for *B. bronchiseptica* binding and uptake.

To test the role of serum for *B. bronchiseptica* binding and uptake, AMΦ were incubated with *B. bronchiseptica* in the various concentrations of serum, and the number of bound and phagocytosed bacteria was determined. Although serum itself has *B. bronchiseptica* killing activity (Fig. 2), low concentrations (1%) of serum resulted in a 2-fold increased binding to AMΦ (Fig. 3A). Surprisingly, serum-enhanced binding led to a dramatic 26-fold increase in the uptake of *B. bronchiseptica* (Fig. 3B). Even at relatively high concentrations (10%) of serum, the uptake of *B. bronchiseptica* was elevated 5-fold compared to medium (Fig. 2 and 3).

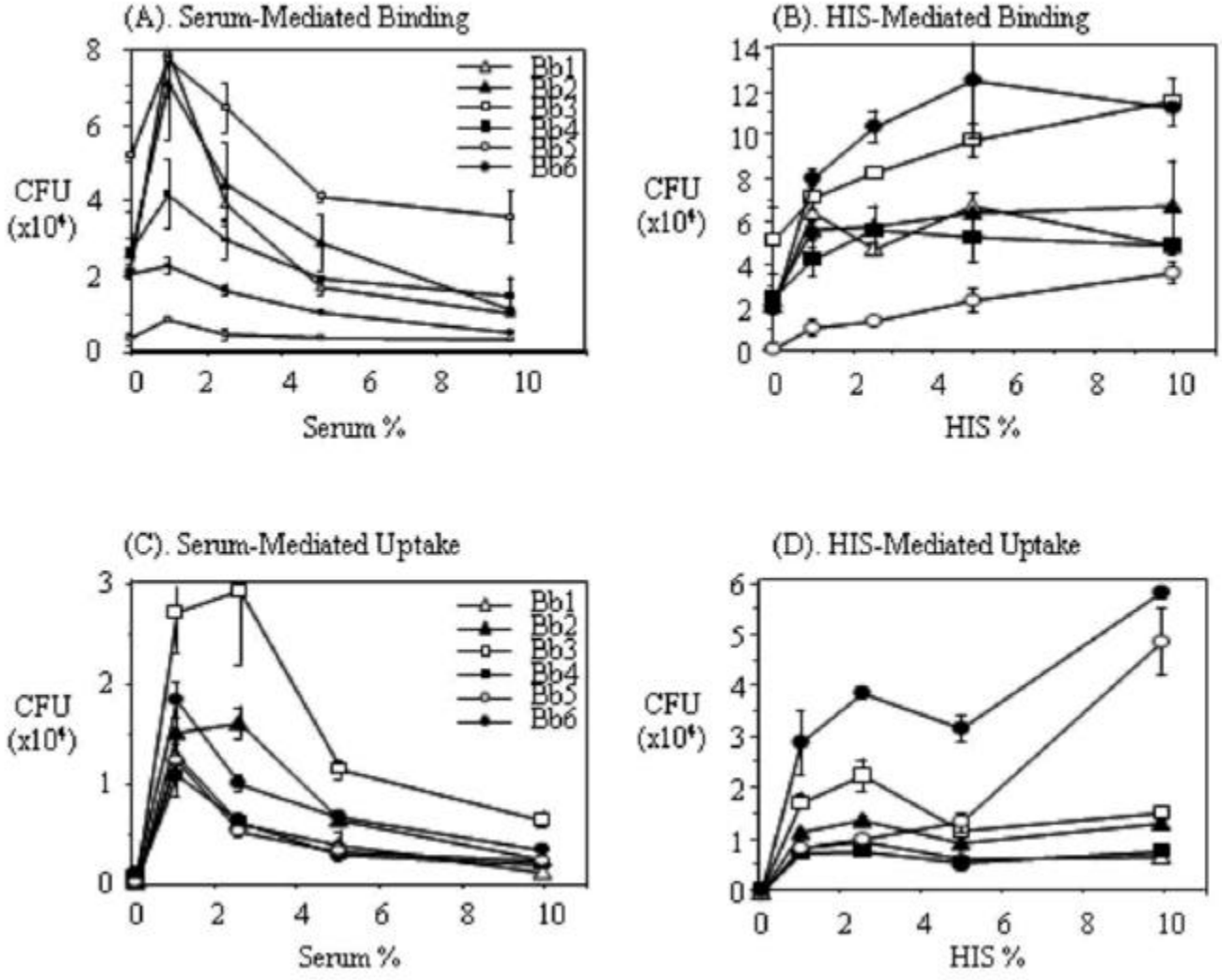

Fig. 3. Serum-mediated *B. bronchiseptica* binding and uptake by AMΦ. Binding (A, B) and uptake (C, D) assays were performed as described in Materials and Methods using various concentrations of serum (A, C) or HIS (B, D) with 6 different *B. bronchiseptica* strains. Results shown are the mean of duplicate blood agar plate cultures.

Mac-1 is a target for direct uptake of *B. bronchiseptica*.

Since the human pathogen, *B. pertussis*, uses Mac-1 receptor to infect macrophages, the role of Mac-1 in *B. bronchiseptica* binding and uptake was examined using anti-CD11b and anti-CD18 antibodies in the bacterial invasion assay. In order to determine Mac-1-mediated direct *B. bronchiseptica* invasion, the binding and uptake of *B. bronchiseptica* was measured in the absence of serum. The binding of *B. bronchiseptica* was not inhibited by anti-Mac-1 antibodies. However, *B. bronchiseptica* uptake was inhibited approximately 50% by anti-Mac-1 antibodies, primarily by anti-CD11b antibody (Table 2). The invasion of B. bronchiseptica was inhibited overall 11%, 37% and 56% by anti-CD18, anti-CD11b and a combination of anti-CD18 and anti-CD11b antibodies (Table 2), respectively, demonstrating that CD11b has more significant effect for *B bronchiseptica* uptake rather than CD18. However, in the presence of serum, *B. bronchiseptica* uptake was not inhibited by anti-Mac-1 antibodies (data not shown). These data indicate that Mac-1 can mediate the direct uptake of *B. bronchiseptica* via opsonin-independent binding. This also suggests that direct binding utilizes CD11b and CD18 epitopes different than those used for direct binding of *B. bronchiseptica*.

Internalization of *B. bronchiseptica* is bactericidal.

In order to determine whether internalized *B. bronchiseptica* can replicate within AMΦ leading a productive infection, we measured the increase of viable intracellular bacteria up to 26 hr postinfection in the absence of serum or HIS. However, the uptaken bacteria were efficiently killed in AMΦ by 10 hr postinfection at a 1:1 ratio of bacteria to AMΦ (Fig. 4). Also, to further confirm that *B. bronchiseptica* did not replicate in AMΦ, we monitored the number of extracellular and intracellular *B. bronchiseptica* during coculture with AMΦ at various time points. While the number of extracellular bacteria dramatically increased, the number of intracellular bacteria did not show any significant increase (data not shown). These results indicate that Mac-1-mediated *B. bronchiseptica* uptake into AMΦ is a bacterial killing pathway not leading to a productive infection of *B. bronchiseptica* in AMΦ.

Discussion

The early interaction between microbial pathogens and immune cells determines the localization of the microorganisms on the surface of the host cell or internalization into an intracellular niche [12]. The initial microbial recognition is mediated by multiple host receptors on leukocytes, such as Fcγ receptors, complement receptors, lipopolysaccharide receptors, mannose receptors, integrins, and toll-like receptors [18]. Previously, it was shown that leukocyte β2-integrin Mac-1 can be utilized as a pathogen receptor for productive bacterial infections of macrophages [13].

In order to test Mac-1-mediated direct *B. bronchiseptica* infection pathway, the role of Mac-1 was examined in the absence of serum for *B. bronchiseptica* binding, uptake and replication in AMΦ. The direct binding of *B. bronchiseptica* to AMΦ was not inhibited by anti-Mac-1 antibodies. However, the uptake of *B. bronchiseptica* was inhibited overall 11%, 37% and 56% by anti-CD18, anti-CD11b and a combination of anti-CD18 and anti-CD11b antibody (Table 2), respectively. This suggests that CD11b epitopes structurally and/or conformationally contribute more to *B bronchiseptica* uptake rather than CD18. Also the synergism of anti-CD18 and anti-CD11b antibody suggests that CD18 cooperates with CD11b for *B. bronchiseptica* uptake and maybe other CD18-integrins such as LFA-1 or p150,95 are involved in *B. bronchiseptica* uptake.

The reason for the increase of Bb2 uptake by anti-Mac-1 blocking is unknown. A possible explanation is that the Bb2 strain lacks the Mac-1 binding ligand due to strain variability or uses different Mac-1 epitope triggering different immune response. An alternative explanation is that the Bb2 strain uses different phagocytosis pathway which is activated through intracellular signalling events by binding of Mac-1 and its ligand. The role of Mac-1 for pathogen binding is believed to vary with different pathogenic microorganisms depending on their surface structures which may recognize different receptor or different epitope of same receptor because different Mac-1 ligand binding to Mac-1 induces different immune responses through interactions with neighboring different receptors [32]. It is unclear, however, whether Mac-1-mediated *B. bronchiseptica*-AMΦ interactions are direct or a consequence of local opsonization of bacteria by complement components (C3bi) even in the absence of serum, as shown in *Leishmania* [30] and zymosan [7]. Compared to porcine isolates, the type strain Bb5 of canine origin, showed lower binding and uptake. This may explain results of a previous study in which an isolate of pig origin produced atrophic rhinitis, while the isolate of dog origin was incapable of causing atrophic rhinitis in swine [24].

Because other studies had demonstrated the capability of *B. bronchiseptica* to invade and survive intracellularly in host cells [9, 26], the replication of *B. bronchiseptica* in porcine AMΦ was examined. The internalized *B. bronchiseptica* was efficiently killed within 10 hr postinfection by AMΦ (Fig. 4). These data support the concept that AMΦ are not utilized for productive *B. bronchiseptica* infection via Mac-1-mediated binding and subsequent uptake in porcine AMΦ. The absence of intracellular replication of *B. bronchiseptica* in AMΦ presented in this paper and strong *B. bronchiseptica* infectivity to epithelial cells as previously observed [26] may explain why *Bordetella-mediated* infections induce primarily upper respiratory diseases such as atrophic rhinitis in pig and whooping cough caused by *B. pertussis* in humans.

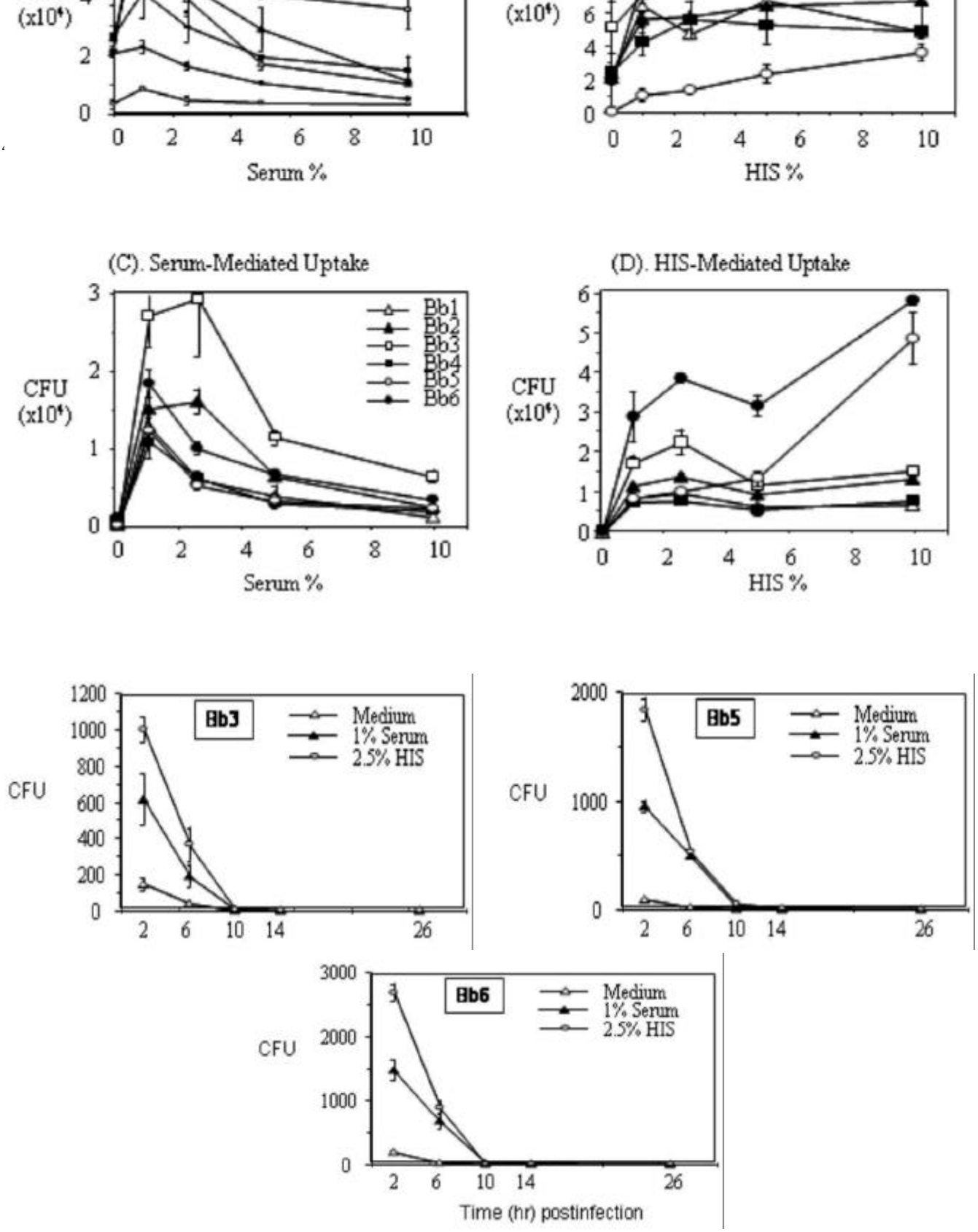

Fig. 4. Viability of uptaken intracellular *B. bronchiseptica*. *B. bronchiseptica* (5 x 105 CFU) and alveolar macrophages (5 x 105 cells), at a 1:1 ratio, were incubated in RPMI 1640 medium for 1 hr. After 2, 6, 10, 14, or 26 hr of incubation in gentamicin solution, macrophages were lysed and the number of viable intracellular bacteria (CFU) was determined using blood agar plate as described in Materials and Methods.

Table 2. Anti-Mac-1 blocking of AMΦ binding and uptake of *B. bronchiseptica*

strain	Ab treatment	Binding (CFU)	Uptake (CFU)	Invasion index (%)	△Invasion index (% of control Ab)
	MOPC-21	7300±900	355±145	4.86	0%
	MHM23	13250±1050	460±100	3.47	-28.6%
	TMG6-5	16000±2600	695±385	4.34	-10.7%
	MHM23+TMG6-5	13900±1600	210±90	1.51	-68.9%
	MOPC-21	15550±650	235±65	1.51	0%
	MHM23	17500±2000	420±110	2.40	+58.9%
	TMG6-5	14000±1200	410±160	2.93	+94.0%
	MHM23+TMG6-5	17950±2450	445±65	2.48	+64.2%
	MOPC-21	29950±1950	695±55	2.32	0%
	MHM23	31700±1100	715±45	2.26	-2.59%
	TMG6-5	49900±1200	560±290	1.12	-51.7%
	MHM23+TMG6-5	36100±4000	560±250	1.55	-33.2%
	MOPC-21	12700±2200	360±70	2.83	0%
	MHM23	15700±3600	290±70	1.85	-34.6%
	TMG6-5	11150±3950	185±115	1.66	-41.3%
	MHM23+TMG6-5	19050±4650	190±70	1.00	-64.7%
	MOPC-21	3380±340	140±60	4.14	0%
	MHM23	5600±150	130±20	2.32	-44.0%
	TMG6-5	8235±615	155±15	1.88	-54.6%
	MHM23+TMG6-5	5515±75	90±10	1.63	-60.6%
	MOPC-21	10200±1800	2170±360	21.27	0%
	MHM23	10750±1750	2165±155	20.14	-5.31%
	TMG6-5	11900±1200	1515±35	12.73	-40.2%
	MHM23+TMG6-5	10600±1300	785±95	7.41	-65.2%

Bacterial invasion assays were performed to measure binding and uptake of 6 different *B. bronchiseptica* strains in RPMI 1640 medium as described in Materials and Methods. Invasion index (II, %) was calculated by the equation: II (%)= [number of uptaken bacteria ÷ number of bound bacteria] x 100 %. Mouse IgG1 isotype control Antibody (MOPC-21), anti-CD18 mAb (MHM23) and/or anti-CD11b mAb (TMG6-5) were used as blocking antibodies in *B. bronchiseptica* invasion assays.

In order to test the role of serum in Mac-1-mediated *B. bronchiseptica* infection to AMΦ, bacterial invasion assays were performed in the presence of porcine serum. The uptake of *B. bronchiseptica* was dramatically enhanced by serum. However, the increased binding and uptake of *B. bronchiseptica* by addition of serum was not inhibited by anti-Mac-1 antibodies, indicating that the dramatic increase of *B. bronchiseptica* uptake by serum is mediated via other macrophage cell surface receptor(s), most probably via Fcγ R because porcine serum used in experiments were prepared from *B. bronchiseptica*-vaccinated pigs. Serum-mediated *B. bronchiseptica* uptake appears to be complex involving multiple bacterial- and host-derived molecules, as shown similarly in *M. tuberculosis* in which multiple receptors are involved for *M. tuberculosis* invasion to AMΦ such as CR1, Mac-1, p150,95, mannose receptor [27]. Serum-mediated internalized bacteria were also efficiently killed in AMΦ by 10 hr postinfection (Fig. 4). This data indicate that *B. bronchiseptica* uptake by AMΦ is a bacterial killing pathway not leading to productive infections in AMΦ, regardless of how they are taken up via either Mac-1-mediated direct binding or serum opsonin-mediated binding. On the other hand, at least three different Mac-1 epitopes have been identified and each epitope binding with specific ligands induces different immune responses [23]. Therefore, it is possible that serum-mediated *B. bronchiseptica* binding and uptake is also mediated through different epitope of Mac-1 binding. In this case, anti-Mac-1 antibodies bind to receptor but do not block the binding of *B. bronchiseptica* recognizing other Mac-1 epitope.

Among the opsonic, chemotactic and lytic functions of the complement cascade, the primary role of complement against *B. bronchiseptica* seems to be lytic functions inducing direct B. bronchsieptica killing (Fig. 2). However, it is unknown whether complement-mediated direct *B. bronchiseptica* killing is triggered by complement activation via

either classical complement cascade or alternative complement cascade. The immunized serum may have high titer of anti-*B. bronchiseptica* antibodies which may activate complement-mediated direct bactericidal activity via classical complement cascade and also enhance phagocytosis through Fcg γ-mediated pathways. The increased resistance of field isolates against complement-mediated *B. bronchiseptica* killing suggests that pathogenic *B. bronchiseptica* has been developed to evade complement-mediated host defense mechanisms as demonstrated in other pathogenic bacterial infections by expression of virulence factor that inhibits complement fixation [15].

In this study, we demonstrate that Mac-1 is a target for direct uptake of *B. bronchiseptica* via opsonin-independent binding. However, Mac-1-mediated uptake by AMΦ does not lead to productive infections of *B. bronchiseptica* in AMΦ. The better understanding of *B. bronchiseptica*-AMΦ interactions will facilitate to develop new therapies against pathogenic respiratory bacterial infections.

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