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The Intracellular Bacterium *Rhodococcus equi* Requires Mac-1 To Bind to Mammalian Cells

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***Rhodococcus equi* is a facultative intracellular bacterium of macrophages that causes disease in immunocompromised individuals, particularly those with AIDS. In this report, we demonstrate that *R. equi* binding to mammalian cells requires complement and is mediated primarily by the leukocyte complement receptor, Mac-1. Bacteria bind to macrophages poorly unless exogenous complement is added to the incubation medium. The addition of fresh nonimmune serum, which contains no detectable antibodies to *R. equi*, greatly enhances bacterial binding to macrophages, whereas heat inactivation of this serum or immunological depletion of C3 from the serum reduces binding to levels only slightly higher than those of binding under serum-free conditions. Human serum depleted of C2 or C4 is fully opsonic, indicating that complement activation and fixation occur by the alternative pathway. The serum-dependent binding of rhodococci to macrophages is mediated primarily by the macrophage complement receptor type 3, Mac-1 (CD11b/CD18). Bacteria do not bind to fibroblastoid or epithelial cells that lack this receptor. Most of the bacterial binding to macrophages is inhibited by a monoclonal antibody to Mac-1 but is unaffected by a monoclonal antibody to complement receptor type 1. Furthermore, opsonized, but not unopsonized, bacteria bind to purified Mac-1 immobilized on plastic. In addition, in the presence of opsonic complement, rhodococci bind efficiently to fibroblastoid cells transfected with cloned Mac-1 but relatively poorly to cells transfected with the complement receptor type 1. Hence, *R. equi* fixes complement by activating the alternative complement pathway, and this fixation is a requirement for bacterial adhesion and invasion. Furthermore, complement fixation defines rhodococcal host cell tropism, since *R. equi* binds specifically and exclusively to cells expressing Mac-1.**

An increase in infections caused by a variety of opportunistic pathogens has coincided with the rising incidence of human immunodeficiency virus infection. One such opportunistic organism is *Rhodococcus equi*, formerly called *Corynebacterium equi*. *R. equi*, initially described as a veterinary pathogen (63, 65) that caused a severe and sometimes fatal pneumonia in young horses, is now recognized as a pathogen of humans, in whom a similar pulmonary disease results (20, 45). At present, the organism is being isolated with increasing frequency from sputum and blood cultures of individuals with AIDS (12, 51, 52) and patients undergoing immunosuppressive therapy (23, 34). The disease most often presents as a cavitary pneumonia, which may be misdiagnosed or go undiagnosed (3, 34, 51). Misdiagnosis occurs because clinical microbiologists sometimes incorrectly classify *R. equi* as a nonpathogenic diphtheroid (9, 23, 34), and physicians are frequently unaware of its pathogenic potential (32). Histologically, the pulmonary lesions associated with *R. equi* are granulomatous in nature, with macrophages and multinucleate giant cells containing numerous intact bacteria (25). *R. equi* is a facultative intracellular pathogen of macrophages, since it can survive and multiply within infected macrophages in vitro (22, 64). Until now, the receptors on the macrophage mediating the binding and ingestion of rhodococci had not been identified.

A number of intracellular microorganisms of macrophages are able to interact with macrophage receptors for complement. These organisms include *Leishmania major* (39), *Legionella pneumophila* (44), *Cryptococcus neoformans* (33), *Listeria monocytogenes* (10), and two species of mycobacteria (49, 50). In each case, these organisms can fix complement by activating the alternative complement pathway (ACP) and then bind to macrophages via their complement receptors. In virtually all of these cases, however, the organisms have an alternative method of binding to mammalian cells that does not depend on complement, and often these organisms adhere to several other nonmacrophage cell types.

Macrophages express two (possibly three) receptors which bind to fragments of the third component of complement, C3. The complement receptor type 3 (CR3), also termed Mac-1, is a member of the leukocyte integrin family. Structurally, this glycoprotein is a heterodimer composed of two noncovalently associated subunits, CD11b and CD18 (29). Mac-1 binds several cell surface and soluble ligands, including the complement protein iC3b, a cleavage product of C3b (1). The complement receptor type 1 (CR1) is a monomer which is expressed on a variety of cell types, including macrophages and polymorphonuclear leukocytes. It preferentially binds to C3b and C4b (60). Because the CR1 functions more as a binding site than as an endocytic receptor in resting cells (19), its role in microbial clearance is indeterminate. It may play an ancillary role in cooperating with other receptors (13), perhaps with Mac-1, to facilitate complement-mediated phagocytosis. A third putative complement receptor, p150,95, is structurally related to Mac-1, having a unique α subunit paired with the common CD18 β subunit. It has been implicated in the binding of degradation

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fragments of C3b (35, 42) and indirectly in microbial recognition by macrophages (55).

In the work described in this paper, we have begun to characterize the interaction of *R. equi* with its host cell, the macrophage. We report that *R. equi*, a gram-positive encapsulated bacterium, must fix complement before it can invade mammalian cells. Complement fixation occurs by activation of the ACP and leads to opsonic C3 deposition on *R. equi*. Thus, host-derived C3 is the ligand recognized by the host cell receptor, primarily CR3. Therefore, in a nonimmune individual, *R. equi* attachment to mammalian cells is dependent on complement and is restricted to cells expressing Mac-1, which may explain why *R. equi* cellular invasion in vivo is limited to macrophages.

MATERIALS AND METHODS

Bacteria. Aliquots of *R. equi* ATCC 6939 (the type strain of *R. equi*) were stored at −70°C. Prior to use, they were thawed and grown on chocolate agar plates for 36 h at 37°C. Approximately three isolated colonies of bacteria were inoculated into 10 ml of Mueller-Hinton broth for radiolabeling with tritiated uracil as described previously (24).

Equine clinical isolates of *R. equi* (isolates 238 and 250) obtained from foals with pneumonia were provided by the Veterinary Microbiology Laboratory, New Bolton Center, University of Pennsylvania, Kennett Square, Pa. Both clinical isolates were handled in a manner similar to that for the type strain.

Monocytes and macrophages. Murine resident peritoneal macrophages were washed from the peritoneal cavity of adult female BALB/c mice (National Cancer Institute, Frederick, Md.) with cold cation-free Dulbecco's phosphate-buffered saline (PD). The cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Grand Island, N.Y.) supplemented with 7% fetal calf serum, 2 mM glutamine, and penicillin G and streptomycin (100 U/ml and 100 μg/ml, respectively) (D-7). Then 5×10^5 peritoneal exudate cells were placed on 13-mm-diameter glass coverslips in 24-well plates. The cells were allowed to adhere for 1 h at 37°C, washed with warm DMEM, and incubated overnight in D-7 as described previously (38). Following washing, approximately 10^5 cells remained per coverslip. Prior to the binding assay, macrophages were washed free of serum.

Mononuclear cells were isolated from human peripheral blood by using Lymphoprep (Nycomed Pharma, Oslo, Norway) as specified by the manufacturer (41). The cells were resuspended in RPMI 1640 (GIBCO) and adhered to 13-mm-diameter glass coverslips in 24-well plates for 30 min at 37°C. Following washing, the monocytes were used immediately. Bacteria were added in the presence or absence of autologous serum. To obtain monocyte-derived macrophages, monocytes were incubated in RPMI 1640 on gelatin-coated tissue culture flasks for 1 h at 37°C in 5% CO_2. Nonadherent cells were removed by washing. Adherent monocytes were released from the gelatin with 5 mM EDTA in PD. The cells were washed and then allowed to adhere to 13-mm-diameter glass coverslips for 72 h in RPMI 1640 supplemented with 20% autologous serum, 2 mM glutamine, and penicillin G and streptomycin (100 U/ml and 100 μg/ml, respectively) as described previously (21). Prior to use in the binding assay, cells were washed free of serum.

Fibroblastoid and epithelioid cells. HEp-2 human epidermoid carcinoma cells (36) were obtained from the American Type Culture Collection (CCL 23) and were maintained in D-7. For use in binding assays, the cells were trypsinized, washed once in PD, resuspended in D-7, and cultured at 37°C overnight in 24-well plates. Primary foreskin fibroblast cells were obtained from 2-day-old human males and were provided by Earl Hendersen, Temple University School of Medicine, Philadelphia, Pa. The cells were maintained in DMEM supplemented with 10% fetal calf serum, glutamine, penicillin G, and streptomycin, as described above. The cells were trypsinized and cultured at 37°C overnight in 24-well plates for use in binding assays.

Transfected cells. Chinese hamster ovary (CHO) cell lines stably expressing wild-type human Mac-1 (CHO-Mac-1) or a third immunoglobulin-like-domain-deleted form of human ICAM-1 (CHO-F185.1) were developed as described previously (7). All data were generated with a high-receptor-expressing subclone, CHO-Mac-1.3C5. Sheep erythrocytes (SRBC) opsonized with iC3b bound to the Mac-1 transfectant but not to the ICAM-1 control transfectant, thus confirming the functional activity of the transfected complement receptor (data not shown).

To generate CR1 transfectants, 10^7 L-929 cells were cotransfected with 2 μg of pRSVneo and either 20 μg of paABCD or Ap^{r}M8. Plasmid paABCD, which contains the complete cDNA of the F allotype of human CR1 cloned into the Ap^{r}M8 expression vector, and plasmid Ap^{r}M8 were provided by Lloyd Klickstein, The Center for Blood Research, Boston, Mass. (27, 30). Plasmid pRSVneo was obtained from Yung-Wu Chen, Temple University School of Medicine (18). Transfections were performed by electroporation using a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) set at 1.5 kV and 25 μF. To obtain linear DNA for transfections, paABCD and Ap^{r}M8 were digested with *Sfi*I and pRSVneo was digested with *Bam*HI. Transfection with pRSVneo allowed for the selection of stable transfectants in medium containing 800 μg of G418 sulfate (GIBCO) per ml. G418 sulfate-resistant cells were cloned by limiting dilution and then screened for cell surface CR1 expression by flow cytometry. Lneo.1E3 and LCR1.1D9 are clonal cell lines which were transfected with Ap^{r}M8 and pRSVneo and with paABCD and pRSVneo, respectively. The transfected cell lines were maintained in D-7.

Functional activity of the CR1 transfectant was confirmed by rosetting of C3b-coated SRBC. Rabbit anti-SRBC immunoglobulin M (IgM) (Diamedix Corp., Miami, Fla.) was used to opsonize SRBC. Following washing, IgM-coated SRBC were incubated with 20% human C8-deficient serum for 10 min at 37°C. Monolayers of transfected L-929 cells on 13-mm-diameter glass coverslips in 24-well plates were incubated with 2.5×10^6 complement-opsonized (EAC3b) or unopsonized (EA) IgM-coated SRBC for 45 min at 37°C in a buffer consisting of equal parts medium 199 and DMEM supplemented with 1% bovine serum albumin (BSA) and 12 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES). The monolayers were extensively washed, fixed with 1.25% glutaraldehyde, and Giemsa stained. The coverslips were examined by light microscopy.

MAbs. The following monoclonal antibodies (MAbs) were used as purified IgG: M1/70 (IgG2b, rat anti-murine CD11b) (53), OKM1 (IgG2a, murine anti-human CD11b) (61), 7G6 (IgG, rat anti-murine CR1) (28), and J3D3 (IgG1, mouse anti-human CR1; AMAC, Westbrook, Maine). M18/2 (IgG2a, rat anti-murine CD18) (47) was diluted from culture supernatant. TS1/22 (IgG1, murine anti-human CD11a) (48) and CBRM1/29.1 (IgG1, murine anti-human CD11b) (7) were diluted from ascitic fluid.

Complement reagents. Purified human C3 was purchased

from Quidel Corp., San Diego, Calif. Serum deficient in C2 or C3 was purchased from Sigma Chemical Co., St. Louis, Mo., or Quidel, respectively. C4-deficient serum was provided by Gary Noel, Cornell University Medical School, New York, N.Y. Immune-mouse serum was obtained by immunizing BALB/c mice subcutaneously twice at 14-day intervals with 5×10^8 CFU of heat-killed rhodococci. Heat-inactivated serum was obtained by heating normal nonimmune sera from BALB/c mice at 56°C for 1 h. Serum was functionally depleted of complement by treatment with either 80 μg of zymosan (Sigma) per ml, hydroxylamine-NaOH (40 and 360 mM, respectively), or 10 U of cobra venom factor (Sigma) per ml for 1 h at 37°C. Zymosan-treated serum was centrifuged to remove the zymosan particles, and all sera were used at a final concentration of 10% for bacterial opsonization. Functional complement depletion was confirmed by failure of the sera to allow IgM-opsonized SRBC to rosette with murine peritoneal macrophages as described previously (46). C2, C4, and C3 deficiencies were confirmed by a classical complement pathway hemolytic assay, performed as described previously (37). Antigenic C4 deficiency was confirmed by a radial immunodiffusion assay performed by Gary Noel, Cornell University Medical School, in which C4 was undetectable.

Integrin-coated substrates. Human leukocyte integrins were purified (8, 11, 54) and adsorbed to 96-well plates (41) as previously described. Murine Mac-1 was purified from cell lysates by a protocol that was modeled on the purification of human Mac-1 (8). Briefly, to obtain 250 μg of purified, functional heterodimer, 20 g of frozen P388D1 cells, a macrophage-like cell line obtained from the American Type Culture Collection (TIB 63), was solubilized in 300 ml of lysis buffer (100 mM Tris-HCl [pH 7.8], 150 mM NaCl, 2.0 mM $MgCl_2$, 1% Triton X-100, 0.025% NaN_3, 1 mM phenylmethylsulfonyl fluoride, 0.22 TIU [trypsin inhibitory unit] of aprotinin per ml, 5 mM iodoacetamide, 1 mM diisopropylfluorophosphate) for 1 h at 4°C while stirring gently. The resultant lysate was centrifuged at $10,000 \times g$ for 2 h at 4°C. The supernatant was decanted and then ultracentrifuged at $100,000 \times g$ (Ti45 rotor; Beckman Instruments, Inc., Palo Alto, Calif.) for 1 h at 4°C. The clarified lysate was precleared with human IgG coupled to Sepharose CL-4B (Pharmacia, Uppsala, Sweden), and 40 μl of a slurry of IgG-Sepharose was added per ml of lysate and rotated overnight at 4°C. The Sepharose was pelleted, and the precleared lysate was passed over an M1/70 (rat anti-mouse CD11b) immunoaffinity column (bed volume, 6 ml; 3.2 mg of M1/70 per ml) that was prepared by attaching protein G-purified M1/70 to cyanogen bromide-activated Sepharose CL-4B (Pharmacia). The column was preequilibrated with 10 bed volumes of 50 mM Tris-HCl (pH 7.8)–150 mM NaCl–2 mM $MgCl_2$–0.1% Triton X-100, and the precleared lysate was loaded at a rate of 15 to 20 ml/h. The column was sequentially washed at 20 to 30 ml/h with 10 bed volumes of 50 mM Tris-HCl (pH 7.8)–150 mM NaCl–0.1% Triton X-100 and then 10 bed volumes of 50 mM Tris-HCl (pH 7.8)–150 mM NaCl–2 mM $MgCl_2$–1% *n*-octyl-β-D-glucopyranoside. Mac-1 was eluted with 5 bed volumes of 50 mM triethylamine (pH 10.3)–400 mM NaCl–2 mM $MgCl_2$–1% *n*-octyl-β-D-glucopyranoside into tubes with neutralizing buffer (10% by volume, 1 M Tris-HCl [pH 7.4]). The peak fractions were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Fractions were aliquoted and stored at −70°C.

Rhodococcus-binding assay. Radiolabeled bacteria were washed and resuspended in phagocytosis buffer, which consists of 0.1% gelatin in equal parts of medium 199 and DMEM buffered with 12.5 mM HEPES. Bacteria were added to either purified integrins on 96-well plates or cells on 24-well plates for 45 min at 37°C as described previously (24). For assays performed with purified integrins, bacteria were preincubated in saline or 10% human or mouse serum for 15 min and washed prior to their addition to the wells. For macrophage-monocyte and transfected CHO cell assays, normal mouse serum or normal human serum was added directly to the wells at a final concentration of 3, 5, or 10%. For L-929 transfected-cell assays, human serum deficient in complement protein C8 was used because normal human serum was lytic to the L-929 cells. After incubation with the bacteria, the cells and plates were washed extensively to remove any unbound organisms. Integrin-bound bacteria were released by solubilization with 0.5% Triton X-100 and 0.1 M NaOH. After neutralization with 0.1 M HCl, lysates were analyzed in a Packard 1900-CA liquid scintillation counter. In assays with intact cells, monolayers were lysed with 0.5% Triton X-100, and the radioactivity associated with the cellular lysates was similarly analyzed.

Flow cytometry. Rhodococci (1.8×10^8 CFU) were incubated in 10% serum for 20 min at 37°C and then washed in Hanks balanced salt solution (GIBCO) containing 1% BSA. Bacteria were incubated on ice for 45 min with MAb to human iC3b (Quidel) at a final concentration of 70 μg/ml. Following washing with Hanks balanced salt solution containing BSA, the bacteria were stained with fluorescein isothiocyanate-conjugated goat anti-murine IgG (heavy- and light-chain specific; Jackson ImmunoResearch, West Grove, Pa.) for 45 min on ice. The bacteria were washed with Hanks balanced salt solution containing BSA, fixed in 1% paraformaldehyde, and analyzed on an Epics Elite Flow Cytometer (Coulter Diagnostics, Hialeah, Fla.). Sera used included normal human serum, C2-deficient human serum (C2D), C4-deficient human serum (C4D), C3-deficient human serum (C3D), C3-deficient human serum with 100 μg of purified human C3 added (C3D + C3), and heat-inactivated human serum (HI). Fluorescence levels observed were compared with values obtained in the absence of sera. Similar experiments were also performed by direct immunofluorescence and staining with fluorescein isothiocyanate-labeled goat anti-murine C3c (Jackson ImmunoResearch).

Transfected L-929 cells (0.5×10^6 to 1×10^6) were incubated with MAb J3D3 for 45 min at 4°C. The cells were washed with cold wash buffer (PD plus 1% BSA and 0.05% sodium azide) and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG for 45 min at 4°C. Following washing with cold buffer, the cells were fixed with 1% paraformaldehyde in PD and analyzed by flow cytometry as described above. Control cells were stained with fluorescein isothiocyanate-conjugated goat anti-mouse IgG alone.

Whole-bacterial-cell enzyme-linked immunosorbent assay (ELISA). We coated 96-well plates with 50 μl of 100-μg/ml poly-L-lysine (Sigma) and incubated them at room temperature for 20 min. The plates were washed with PD, and 50 μl of live, washed *R. equi* cells in PD (5×10^7 to 9×10^7 cells) was added to the wells. The plates were incubated at room temperature for 1 h and blocked overnight at 4°C with 200 μl of 3% BSA per well in PD. The BSA solution was removed, and the plates were rinsed with PD. Then 50 μl of twofold serial dilutions of sera was added to the wells, and the plates were incubated for 1 h at 37°C. The plates were rinsed four times with PD prior to the addition of peroxidase-labeled goat antibody to mouse Ig. Then 50 μl of goat anti-murine

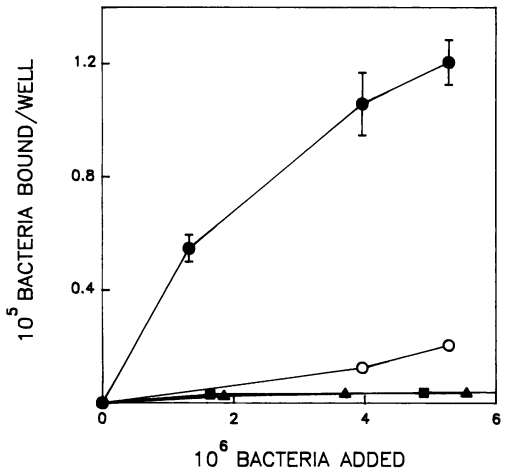

FIG. 1. Binding of increasing amounts of *R. equi* 6939 to human peripheral blood monocytes, human primary foreskin fibroblasts, and human HEp-2 cells. The figure represents a compilation of three separate experiments, one per cell type, performed on three separate occasions. Binding of bacteria to monocytes in the presence of 5% autologous serum (●) was compared with binding in the absence of serum (○). The binding of bacteria to both human foreskin fibroblasts (▲) and HEp-2 cells (■) was performed in the presence of 5% fresh normal human serum. Although not shown, binding to these cells under serum-free conditions was also evaluated, and results were indistinguishable from the values obtained under serum-dependent conditions. Error bars express the standard deviations from the means of three determinations, and where not shown they are smaller than the symbols.

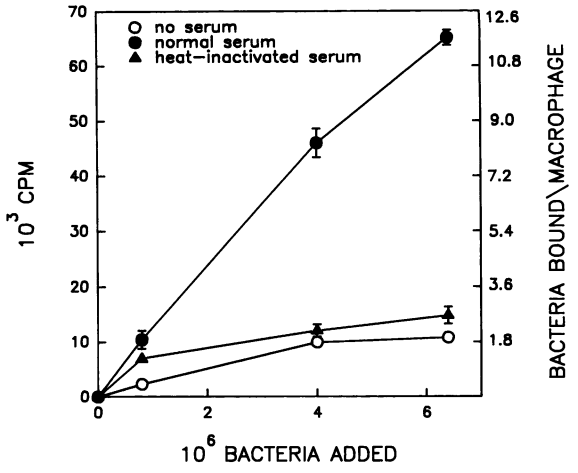

FIG. 2. Binding of increasing amounts of radiolabeled *R. equi* 6939 to murine peritoneal macrophages. Binding to macrophages in the presence of 5% normal mouse serum was compared with binding in the absence of serum and binding in the presence of 5% heat-inactivated normal mouse serum. In this representative experiment, bacteria incorporated an average of 0.054 cpm CFU^{-1} and there was an average of 10^5 cells per coverslip. Binding proceeded for 45 min at 37°C, and unbound bacteria were removed by washing. Error bars illustrate the standard deviations from the means of three determinations. The absence of error bars indicates that they are smaller than the symbols.

IgG heavy- and light-chain-specific antibody (Jackson ImmunoResearch) was added in PD with 1% BSA and 1% normal goat serum. After 1 h at 37°C, the plates were washed four times with PD. The plates were developed with 100 μl of 0.2 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Sigma) and 0.02% hydrogen peroxide in 0.1 M citrate buffer per well for 20 min at room temperature. The A_{405} was determined on a Microplate Reader (Bio-Tek Instruments, Winooski, Vt.). All values were corrected for background binding to poly-L-lysine–BSA-coated plates in the absence of rhodococci. Experimental absorbance which exceeded background values by 0.1 was considered positive.

RESULTS

Binding of *R. equi* to mammalian cells. Radiolabeled *R. equi* ATCC 6939 cells were added to a variety of mammalian cell types in the presence of normal nonimmune serum, and their adherence to each mammalian cell type was quantitated. The bacteria did not bind to primary human foreskin fibroblasts or to the human epithelioid cell line HEp-2 at any of the bacterial doses used (Fig. 1). Likewise, they failed to bind to the murine fibroblastoid cell line L-929 (24), COS 7 cells (data not shown), or CHO cells (see Fig. 9A). Bacteria were also added to freshly isolated human monocytes in either the presence or absence of normal serum. In the absence of serum, bacteria bound poorly to monocytes, with differences from background binding becoming significant only at the highest bacterial input dose (Fig. 1). The presence of 3% fresh nonimmune serum, however, resulted in a dramatic increase in bacterial binding to monocytes, which was dose dependent (Fig. 1).

Increasing concentrations of *R. equi* were also added to adherent murine peritoneal macrophages (Fig. 2). Similar to monocytes, murine macrophages bound bacteria poorly unless exogenous normal serum was added to the incubation mixture. The serum-dependent enhancement in bacterial binding to macrophages was a consistent result. On average, in more than 20 separate experiments performed over a wide dosage range, the addition of serum resulted in a greater than 10-fold increase in bacterial binding. This enhanced binding in the presence of serum was readily apparent at the light-microscopic level (Fig. 3).

Opsonin-dependent bacterial adherence. Since bacterial binding required serum opsonization, the roles of antibody and complement in mediating the binding of *R. equi* to macrophages were examined. MAbs to murine Mac-1 were used to inhibit the serum-dependent binding of *R. equi* to murine peritoneal macrophages. M1/70 (anti-murine CD11b) (53) decreased bacterial binding by 70% compared with binding to cells in the absence of antibody (Fig. 4). An irrelevant antibody, OKM1 (anti-human CD11b) (61), and a nonblocking β-chain-specific control MAb, M18/2 (47), had little or no effect on binding. The concentration of M1/70 used, 20 μg/ml, partially inhibited the binding of IgG-coated SRBC to macrophages (data not shown), as previously reported (4).

The role of antibody in mediating the opsonin-dependent binding of *R. equi* to macrophages was then examined. Heat inactivation of normal mouse serum at 56°C for 1 h, a method of inactivating complement but not antibody, dramatically decreased the binding of rhodococci to murine peritoneal macrophages (Fig. 2). In addition, specific antirhodococcal antibodies were not found in opsonic nonimmune mouse serum under conditions in which these antibodies could be readily detected in rhodococcus-immune serum. Serum obtained from mice immunized with killed rhodococci exhibited titers in excess of 10,000, whereas normal nonimmune serum exhibited no detectable titer (Fig. 5). Therefore,

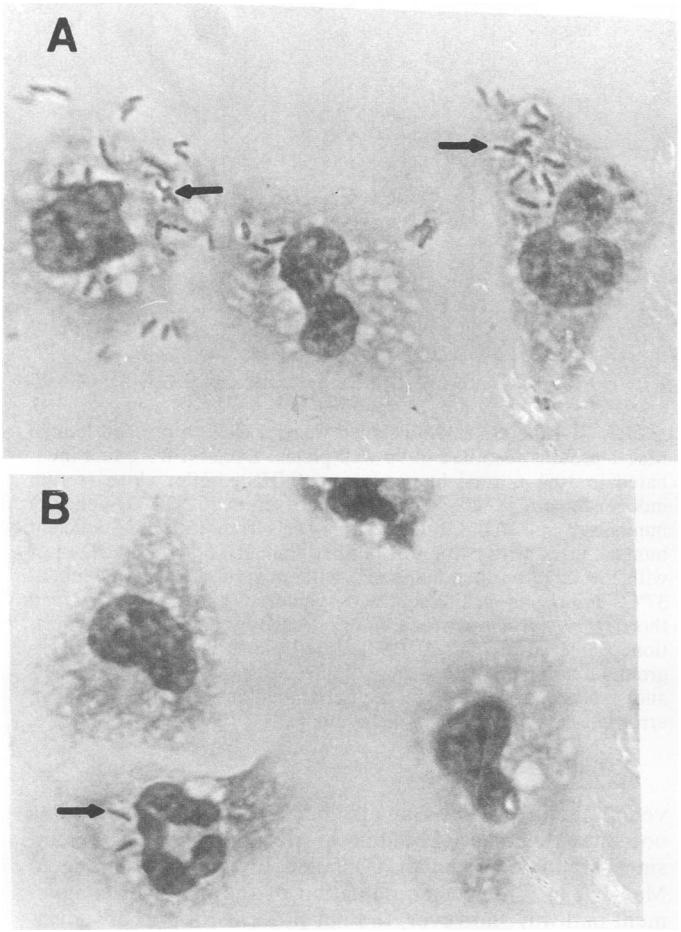

FIG. 3. (A) Light microscopy of a Giemsa-stained murine peritoneal macrophage monolayer showing *R. equi* in the cytoplasm of macrophages. Bacteria were incubated with macrophages for 45 min at 37°C in the presence of 5% normal mouse serum. Following washing, monolayers were fixed in 2.5% glutaraldehyde and stained with Giemsa (Accra Labs, Bridgeport, N.J.) for 2 min. Magnification, ×1,000; oil immersion. *R. equi* cells are indicated by arrows. (B) Parallel macrophage monolayer which was incubated with bacteria under serum-free conditions. Magnification, ×1,000; oil immersion.

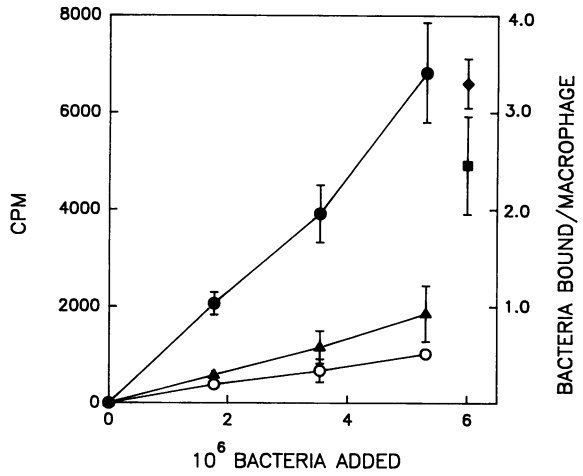

FIG. 4. The binding of increasing amounts of radiolabeled *R. equi* 6939 to murine peritoneal macrophages and the effects of antibody to Mac-1. Binding to macrophages in the presence of 5% normal mouse serum (●) was compared with binding in the absence of serum (○) and binding in the presence of MAb to Mac-1 (M1/70) (▲). Under conditions with antibody, the antibody was preincubated with the cell monolayer for 15 min before the addition of bacteria and remained in the wells throughout the experiment. Purified M1/70 and OKM1 (■) were used at a final concentration of 20 μg/ml, and M18/2 (◆) culture supernatant was used at a final dilution of 1:10. Normal mouse serum (5%) was present under all conditions when antibodies were used. There was an average of 10^5 cells per coverslip. Error bars express the standard deviations from the means of three determinations. Where not shown, error bars are smaller than the symbols. This experiment is representative of three separate experiments.

antibody opsonization was not likely to be responsible for the observed serum-enhanced binding of rhodococci to macrophages.

Complement fixation by *R. equi*. Complement fixation by rhodococci was evaluated by indirect immunofluorescence and flow cytometry. Bacteria incubated for 20 min in the presence of 10% normal human serum were 97.8% positive for C3 fixation, with a mean log fluorescence intensity (MFI) of 64.0, whereas bacteria that were not exposed to serum were essentially negative (1% positive, MFI of 0.2) (Fig. 6A). Similar complement fixation and fluorescence results were obtained when bacteria were incubated in 10% normal mouse serum and stained by direct immunofluorescence with antibody to murine C3 (data not shown). Incubation of bacteria in heat-inactivated serum resulted in a fluorescence profile that was comparable to serum-independent values (7% positive; MFI of 0.4). Fluorescence was also abolished by immunologically depleting human serum of C3 (0.8% positive; MFI of 0.3) (Fig. 6B). Significantly, the addition of purified human C3 to this C3-deficient serum restored C3 fixation and hence fluorescence (95.7% positive; MFI of 51.0).

To address the specific pathway of complement activation

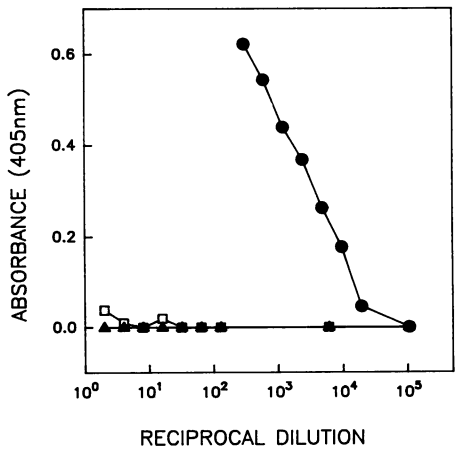

FIG. 5. Absorbance values from a whole-rhodococcus-cell ELISA performed on serial dilutions of mouse serum. Normal mouse serum (□) and mouse serum absorbed against *R. equi* 6939 (▲) were compared with rhodococcus-immune mouse serum (●). All values were corrected for background binding to plates without bacteria. Some variability in titer was seen at high concentrations of normal mouse serum, but in five separate assays nonimmune-serum titers never exceeded 1:32. Additionally, normal mouse serum (300 μl) absorbed against rhodococcus (3×10^9 CFU for 45 min at 4°C) exhibited a titer identical to that of unabsorbed serum ($n = 3$).

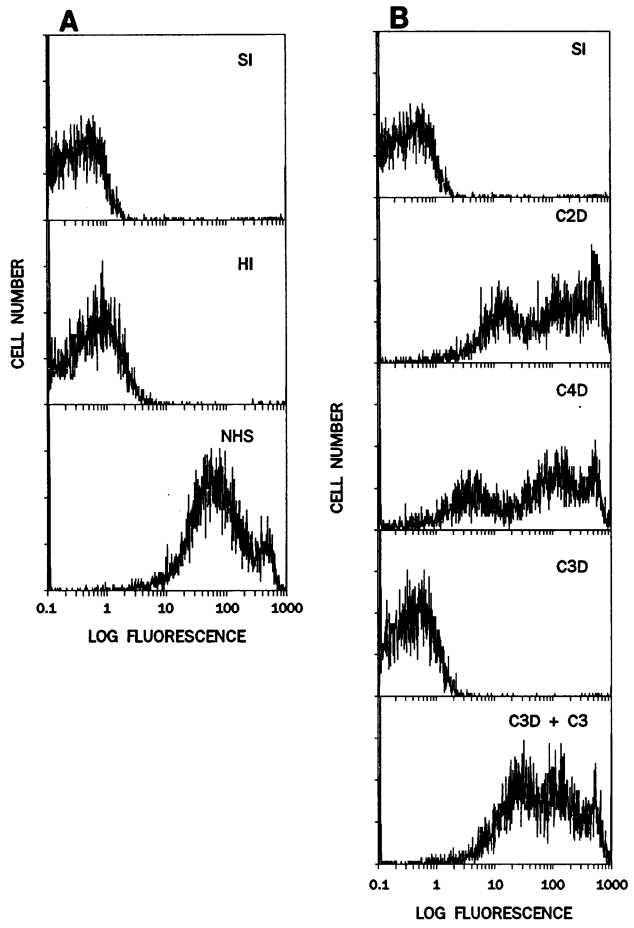

FIG. 6. Flow cytometry profiles of *R. equi* 6939. (A) Bacteria were stained by indirect immunofluorescence following incubation in 10% normal human serum (NHS), 10% heat-inactivated normal human serum (HI), or buffer (serum independent) (SI). Indirect immunofluorescence is described in detail in Materials and Methods. (B) Indirect immunofluorescence was performed in a manner identical to that in panel A, except that the primary incubation was performed in either buffer alone (serum independent) (SI), C2-deficient human serum (C2D), C4-deficient human serum (C4D), C3-deficient human serum (C3D), or C3D with 100 μg of purified human serum added (C3D + C3).

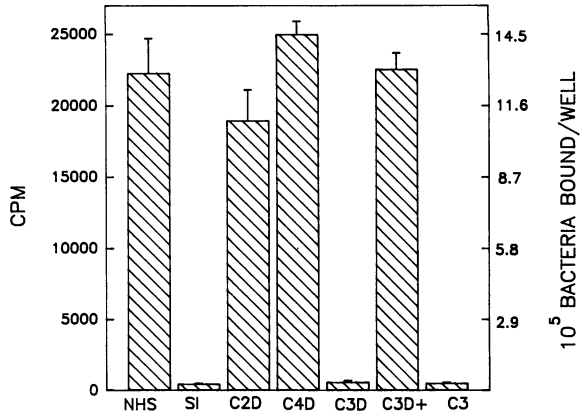

FIG. 7. Binding of radiolabeled *R. equi* 6939 to purified human Mac-1 protein adsorbed to 96-well plates. Bacteria were first incubated in 10% normal human serum (NHS), buffer alone (serum independent) (SI), C2-deficient human serum (C2D), C4-deficient human serum (C4D), C3-deficient human serum (C3D), C3-deficient human serum with 100 μg of purified human C3 added (C3D+) or with 100 μg of purified human C3 without serum (C3) for 20 min at 37°C. Following incubation with bacteria, plates were washed thoroughly, and the bacteria were released by detergent solubilization. Bacterial binding to BSA-coated plates resulted in a background binding of 391 ± 4 cpm. Values are expressed as the means and standard deviations of three determinations. Where not visible, error bars are smaller than the symbols.

that was used, we incubated the bacteria in sera that lacked components of the classical pathway. Bacteria incubated in serum deficient in complement factor C2 or C4 were positive for C3 fixation (91 and 73% positive, respectively, with MFIs of 46.4 and 13.1, respectively) (Fig. 6B). Thus, the classical complement pathway and therefore antibody were not required for complement fixation by *R. equi*.

Binding of rhodococci to integrin-coated plates. Having demonstrated that *R. equi* fixes complement, we examined whether complement fixation alone was functionally opsonic. Opsonization of *R. equi* by complement was addressed by evaluating the interaction of these bacteria with purified human Mac-1 adhered to plastic plates. Bacteria which were opsonized in normal mouse serum bound avidly to Mac-1, forming a nearly confluent lawn of bacteria across the well (data not shown). Unopsonized bacteria failed to bind to Mac-1-coated plates even at high bacterial inputs. Similarly, bacteria incubated in sera depleted of functional complement activity by treatment with hydroxylamine NaOH, cobra venom factor, or zymosan also failed to bind to Mac-1 (data not shown). Bacterial binding to Mac-1 was C3 dependent, since serum depleted of C3 failed to mediate binding to Mac-1 (Fig. 7). Specific depletion of the classical complement pathway, however, did not prevent *R. equi* opsonization. Bacteria opsonized in human serum deficient in either C2 or C4 bound to purified Mac-1 as well as bacteria opsonized in normal human serum did (Fig. 7).

To address the possibility of species-specific differences between human and murine macrophages with respect to microbial recognition, murine Mac-1 was isolated from the macrophage-like P388D1 cell line (Fig. 8A) and bacterial binding to murine Mac-1 was measured. Results were comparable to that of the human homolog in that bacteria opsonized in normal mouse serum bound to this receptor, whereas unopsonized organisms did not (Fig. 8B). This binding was specific, since it was blocked by a MAb to CD11b, M1/70, but was unaffected by the nonblocking MAb M18/2. In addition, as previously reported (62), binding to Mac-1 was cation dependent and was inhibited by 10 mM EDTA (Fig. 8B).

Rhodococcus binding to cells transfected with cloned complement receptors. To test whether Mac-1 expression on a cell surface was sufficient to mediate rhodococcus binding, CHO cells were stably transfected with either human CD11b/CD18 (Mac-1) or a truncated form of human CD54 (ICAM-1). Radiolabeled *R. equi* 6939 cells were added to CHO transfectants in the presence or absence of normal human serum. The rhodococci failed to bind to either transfected cell type in the absence of serum. However, the addition of increasing amounts of normal human serum resulted in a parallel increase in bacterial binding to the Mac-1-transfected cells but not to the ICAM-1 transfectants (Fig. 9A). The specificity of Mac-1 in this effect was confirmed by using MAb directed against human Mac-1. Two MAbs to Mac-1, CBRM1/29.1 and M1/70, inhibited serum-

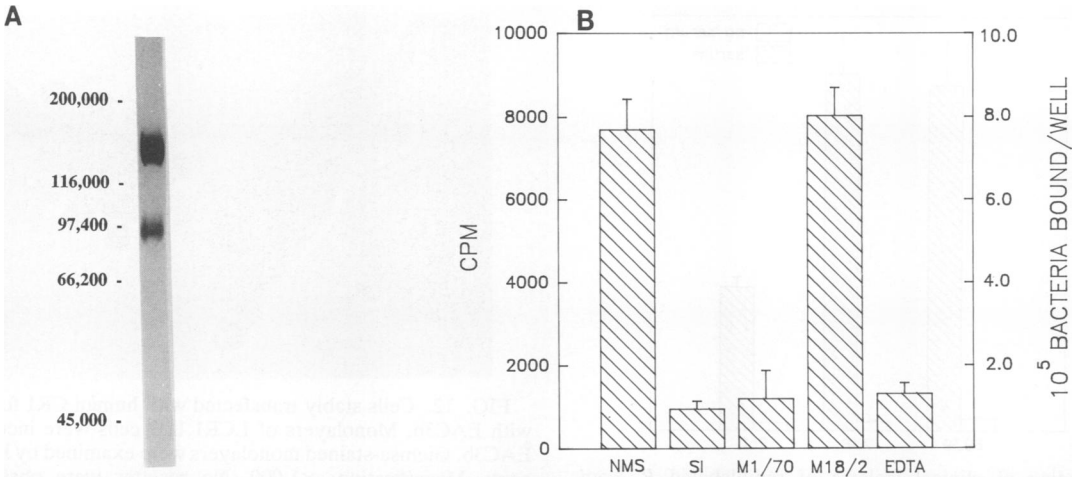

FIG. 8. (A) SDS-PAGE of purified murine Mac-1 showing the α chain (CD11b; M_r, 160,000) and the β chain (CD18; M_r, 95,000). Purified murine Mac-1 (15 μl of a 2-ml fraction from an M1/70 MAb affinity column) was subjected to reduction, SDS-PAGE, and silver staining. Molecular weight standards are marked on the left. (B) Binding of radiolabeled *R. equi* 6939 to purified murine Mac-1 adsorbed to plastic. Bacteria were preincubated in the presence of 10% normal mouse serum (NMS) or in buffer alone (serum independent) (SI) for 20 min at 37°C. Under conditions with MAbs, the MAbs were preincubated with the receptor 15 min before the addition of opsonized bacteria. MAbs remained in the wells throughout the binding period. Background binding of serum-opsonized bacteria to BSA control wells was 1,402 ± 651 cpm and was comparable to the binding of unopsonized bacteria to purified Mac-1. Determinations were performed in triplicate, and values are expressed as the means and standard deviations. This experiment is representative of three.

dependent binding to the Mac-1-transfected cells by approximately 90%, whereas an irrelevant antibody, TS1/22 (murine anti-human CD11a), had no effect on binding (data not shown). Bacterial binding to the Mac-1 transfectant was visualized by light microscopy. Many cells had in excess of 10 bacteria bound per cell when the assays were performed in the presence of serum (Fig. 9B). In the absence of serum, however, binding was negligible, with fewer than one bacterium detectable per high-powered field. In addition, the binding of clinical isolates of *R. equi* to human Mac-1 expressed on the CHO cell transfectants was compared with that of the type strain. The equine clinical isolates (isolates 238 and 250), obtained from foals with pneumonia, bound to the Mac-1-transfected cells in a serum-dependent manner and failed to bind to the ICAM-1 transfectant control, results consistent with that of *R. equi* ATCC 6939 (Fig. 10).

The role of CR1 in binding of *R. equi* to macrophages was examined by evaluating the effect of anti-CR1 MAb on bacterial adherence. The addition of 7G6, a blocking MAb to murine CR1 (28), had no effect on serum-dependent binding

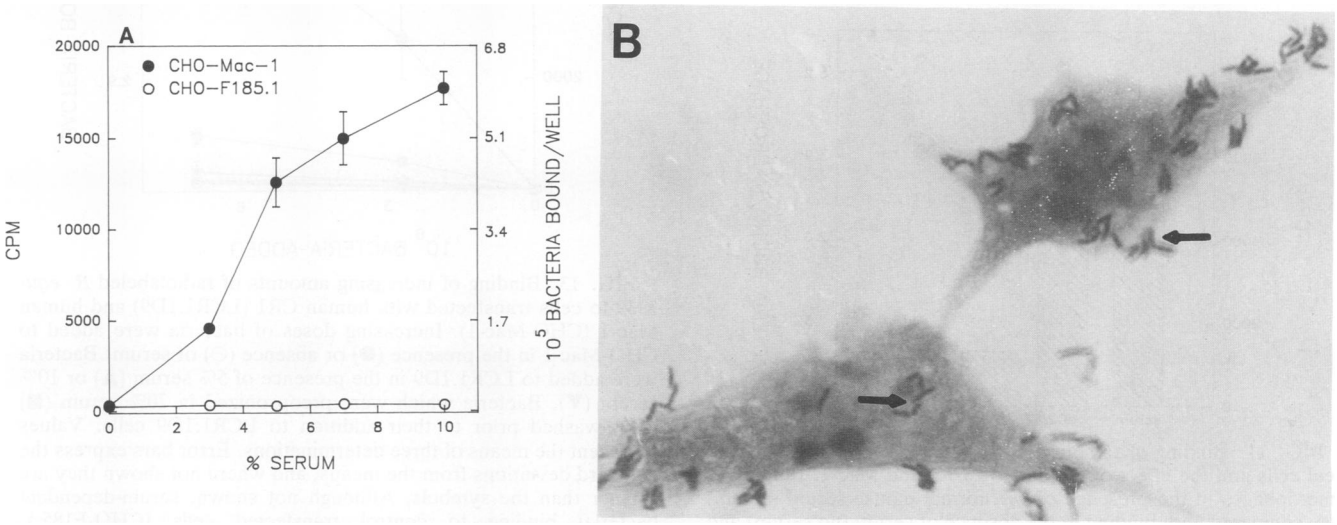

FIG. 9. (A) Binding of radiolabeled *R. equi* 6939 to transfected CHO cells. CHO-Mac-1 cells were stably transfected with human Mac-1, and CHO-F185.1 cells were transfected with a truncated form of ICAM-1. Approximately 4.4×10^6 bacteria were added to approximately 10^5 cells in either the absence or presence of increasing amounts of normal human serum. Binding proceeded for 45 min at 37°C, and monolayers were then washed to remove unbound bacteria. Determinations were performed in triplicate, and values are expressed as the means and standard deviations. (B) Light microscopy of Giemsa-stained CHO cells transfected with human Mac-1 showing bound *R. equi* 6939 cells. The CHO cells were fixed with 2.5% glutaraldehyde and stained with Giesma. Cell-associated bacteria are indicated by arrows. Magnification, ×1,000.

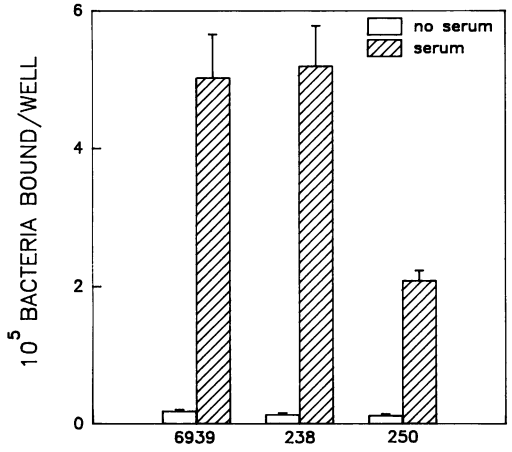

FIG. 10. Binding of clinical isolates of radiolabeled *R. equi* (isolates 238 and 250) and the type strain (ATCC 6939) to Mac-1-transfected CHO cells. A total of 5×10^6 to 8×10^6 bacteria were added to approximately 10^5 cells in either the presence or absence of normal human serum. Values were determined in triplicate, and error bars represent the standard deviations from the means. Although not shown, binding to CHO-F185.1 control-transfected cells was evaluated, and the values obtained were indistinguishable from those for binding to CHO-Mac-1 transfectants under serum-free conditions.

of rhodococci to murine peritoneal cells (Fig. 11). Bacterial binding to L-929 cells transfected with human CR1 (LCR1.1D9) was also evaluated. Cells stably transfected with CR1 were positive for receptor expression by flow cytometry (100% positive, MFI of 32.8) and rosetted C3b-opsonized erythrocytes (Fig. 12). In the presence of 5% human serum, a concentration of serum adequate to mediate binding of *R. equi* to the Mac-1 transfectants (Fig. 9A) and primary macrophages (Fig. 2), bacterial binding to the CR1-transfected cells was indistinguishable from serum-independent binding ($n = 5$), even at high bacterial inputs (Fig. 13). The addition of 10% serum, however, did result in measurable bacterial binding, although this binding was considerably reduced relative to binding to the Mac-1-transfected cells. Under conditions in which Mac-1-transfected cells bound 8 bacteria per cell, the CR1 transfectants bound 0.5 bacterium per cell (Fig. 13). Thus, in contrast to the Mac-1 transfectants, the presence of biologically active CR1 alone

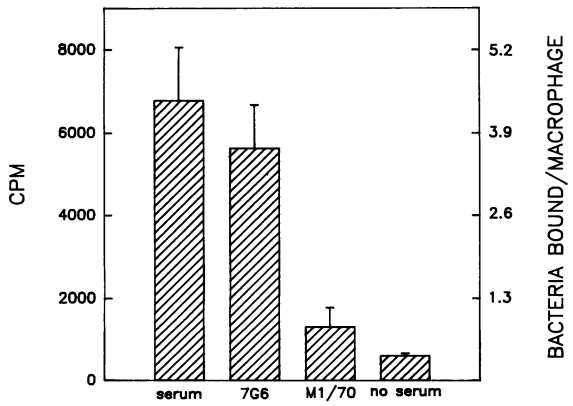

FIG. 11. Binding of radiolabeled *R. equi* 6939 to murine peritoneal cells and the effect of antibody to CR1 and Mac-1. Binding to macrophages in the presence of 5% normal mouse serum (serum) was compared with binding in the absence of serum (no serum) and to binding in the presence of blocking MAb to CR1 (7G6) or MAb to Mac-1 (M1/70). Under conditions with antibody, the antibody was preincubated with the cell monolayer for 15 min before the addition of bacteria and remained in the wells throughout the experiment. Purified M1/70 and 7G6 were used at a final concentration of 20 μg/ml. Normal mouse serum (5%) was present in wells containing the antibodies.

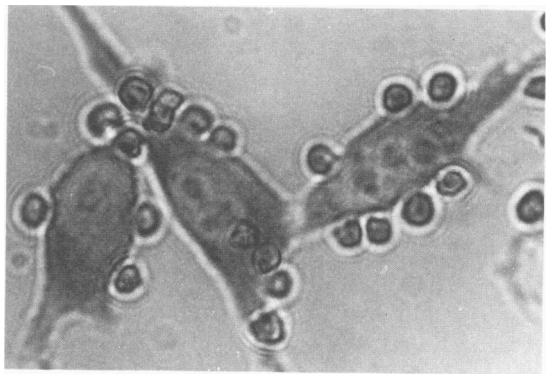

FIG. 12. Cells stably transfected with human CR1 form rosettes with EAC3b. Monolayers of LCR1.1D9 cells were incubated with EAC3b. Giemsa-stained monolayers were examined by light microscopy. Magnification, ×1,000. No rosettes were observed when LCR1.1D9 cells were incubated with EA (data not shown).

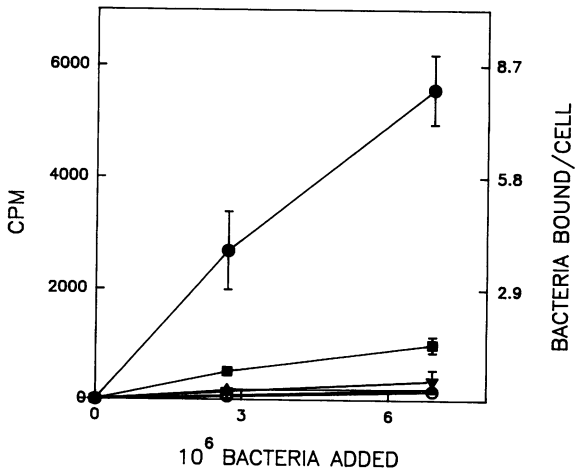

FIG. 13. Binding of increasing amounts of radiolabeled *R. equi* 6939 to cells transfected with human CR1 (LCR1.1D9) and human Mac-1 (CHO-Mac-1). Increasing doses of bacteria were added to CHO-Mac-1 in the presence (●) or absence (○) of serum. Bacteria were added to LCR1.1D9 in the presence of 5% serum (▲) or 10% serum (▼). Bacteria which were preopsonized in 20% serum (■) were washed prior to their addition to LCR1.1D9 cells. Values represent the means of three determinations. Error bars express the standard deviations from the means, and where not shown they are smaller than the symbols. Although not shown, serum-dependent bacterial binding to control transfected cells (CHO-F185.1, Lneo.1E3) was indistinguishable from binding under serum-independent conditions. This figure is representative of four separate experiments in which bacterial binding to LCR1.1D9 and CHO-Mac-1 at multiple doses in 10% serum was 3.5 ± 2.8 ($n = 11$)- and 60.6 ± 33 ($n = 9$)-fold higher, respectively, than serum-independent values. The binding of bacteria which were preopsonized in 20% serum was 12.0 ± 10.4 ($n = 10$)-fold higher than serum-independent values.

on a cell was not sufficient to mediate bacterial binding when bacteria were added to these cells in the presence of complement. Experimental conditions were adjusted to increase rhodococcus binding to CR1. Bacteria were preopsonized in 20% fresh serum and washed prior to being added to the CR1 transfectants. This method of opsonization increased bacterial adhesion to LCR1.1D9 cells to 1.4 bacteria per cell (Fig. 13). Only bacteria which were preopsonized in this manner bound efficiently to cellular CR1.

DISCUSSION

We have examined the interaction of the facultative intracellular bacterium *R. equi* with its host cell, the macrophage. We report that complement fixed to the surface of the bacteria is the primary ligand which mediates bacterial adhesion to macrophages. In the absence of serum, binding to macrophages is minimal. The presence of fresh nonimmune serum dramatically increases the binding of rhodococci to both murine and human macrophages. Serum that was heated to inactivate complement and serum immunologically depleted of C3 were unable to mediate bacterial adhesion. Complement fixation depended on an intact ACP, a mechanism which does not rely on antibody, and the serum used had no detectable antibody to *R. equi* as determined by ELISA.

We also report that Mac-1 is the primary macrophage receptor involved in bacterial recognition. Our evidence for this is the following. (i) MAb to Mac-1 inhibited bacterial binding to macrophages by more than 70%. (ii) Bacterial binding was limited to cells expressing complement receptors. The human epithelioid cell line, HEp-2, and several fibroblastoid cells, including murine L-929 cells (24), CHO cells, COS-7 cells (data not shown), and primary human foreskin fibroblasts, all failed to bind bacteria, whereas monocytes and macrophages, cells which express Mac-1, bound bacteria avidly. (iii) Transfection of cloned Mac-1 into fibroblastoid cells converted them into cells which bind *R. equi* avidly. (iv) In the presence of normal serum, bacteria bound directly to purified Mac-1 adsorbed onto plastic. Therefore, the single leukocyte receptor, Mac-1, is adequate and sufficient to bind *R. equi*, provided that the organism is opsonized with complement. Furthermore, the molecular specificity of this interaction may well determine the cellular tropism of *R. equi* and define the macrophage as the singular definitive host cell for this intracellular bacterium.

Complement receptor-mediated entry into macrophages has been documented for *Leishmania major*, *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Legionella pneumophila*, *Listeria monocytogenes*, and *Cryptococcus neoformans* (10, 33, 39, 44, 49, 50). In the presence of exogenous complement, all of these intracellular organisms are able to bind to macrophages in a complement-dependent manner. It is clear, however, that *Listeria monocytogenes*, *Legionella pneumophila*, and *Mycobacterium* spp. are also able to bind to other noncomplement receptors on mammalian cells, since each of these organisms has been shown to infect cells which do not express complement receptors (14, 16, 31, 43, 59). Furthermore, *Leishmania* spp. and *Histoplasma capsulatum* can bind to macrophages in the absence of exogenous complement (6, 55), indicating that their infectivity does not depend exclusively on complement. We demonstrate that in contrast to these organisms, *R. equi* binds only to cells expressing receptors for complement in vitro and binds only in the presence of a functionally opsonic complement system. This dependence on complement receptors by *R. equi* for cell attachment is unique, and it alone may explain the in vivo observation that *R. equi* infection is limited to macrophages.

Since complement opsonization plays a key role in directing rhodococci to macrophages, we addressed the mechanism of complement fixation. Complement can be activated by the classical complement pathway, which requires antibody, or by the ACP, which is usually antibody independent. Lipopolysaccharide, a major outer membrane component of gram-negative bacteria, has been shown to mediate ACP activation and complement-mediated opsonization (2, 15, 17). Though lacking LPS, some gram-positive bacteria have also been shown to activate the ACP (10, 57). Complement fixation by the ACP, however, is frequently not opsonic for encapsulated bacteria (58). Only antibody-directed complement fixation is opsonic for these organisms, presumably because it allows C3 deposition on the capsular surface, making it accessible to phagocyte complement receptors (5, 56). Using immunofluorescence and flow cytometry, we demonstrate that *R. equi* fixes complement by the ACP. C3 deposition on bacteria occurred in human serum depleted of the classical complement pathway protein C2 or C4. We also demonstrate that unlike many other encapsulated bacteria, this ACP fixation to encapsulated *R. equi* is fully opsonic. Bacteria opsonized in C2- or C4-deficient sera were fully capable of binding to either purified Mac-1 or to cloned Mac-1 transfected into nonmacrophage cells. Thus, rhodococcus is able to activate the ACP, resulting in C3 fixation to the bacterial surface in an opsonic form accessible to cellular complement receptors.

Our data with Mac-1-transfected cells largely parallel data obtained with primary macrophages. CHO cells transfected with Mac-1 bind serum-opsonized bacteria, whereas control transfected CHO cells do not. Bacterial binding to these transfectants is almost completely abolished by MAb to Mac-1. Similarly, binding to murine macrophages was also dependent on complement and was markedly inhibited (at least 70%) by MAb to murine Mac-1. These results indicate that Mac-1 is able to mediate bacterial binding and probably accounts for the majority of *R. equi* binding to macrophages. The residual amount of *R. equi* binding to macrophages, which was not inhibitable by MAb to Mac-1, might be attributable to other macrophage complement receptors, such as the CR1 or CR4. These receptors each probably account for only a minor fraction of bacterial adhesion, which may be below our limits of detection. Our evidence for the subordinate role for these receptors is as follows. (i) Serum-opsonized *R. equi* failed to bind to affinity-purified p150,95 adhered to plastic (data not shown). (ii) Bacterial adhesion was virtually unaffected by a blocking MAb to CR1. (iii) *R. equi* bound poorly to cells transfected with CR1 in the presence of complement, under conditions in which they bound avidly to Mac-1-transfected cells. Failure to bind *R. equi* was not due to a lack of functional CR1 expression on these transfected cells, because these cells bound to erythrocytes preopsonized with C3b. The failure of the CR1 transfectants to bind *R. equi* may be due to the factor I cofactor activity of CR1, which would result in the conversion of bound C3b to iC3b. CR1 has been shown to bind iC3b with a 100-fold lower affinity than that with which it binds to C3b (26). Preopsonization of *R. equi*, followed by washing to remove exogenous complement regulatory proteins from the assay, partially restored *R. equi* binding to the CR1 expressing cells.

Studies involving the intracellular protozoan *Leishmania major* have suggested that the mode of entry into host cells

affects the intracellular fate of the organism. We demonstrated a correlation between complement fixation by *Leishmania major* and subsequent intracellular survival of the parasite (40). We hypothesize that macrophages have "permissive" versus "restrictive" receptors and that ligand binding to permissive complement receptors could result in the establishment of a successful intracellular infection. Our observation that the intracellular bacterium *R. equi* binds to complement receptors is consistent with this hypothesis. We are currently investigating whether the mode of entry by *R. equi* affects intracellular survival in resident macrophages.

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REFERENCES

1. **Beller, D. I., T. A. Springer, and R. D. Schreiber.** 1982. Anti-Mac-1 selectively inhibits the mouse and human type three complement receptor. J. Exp. Med. **156:**1000–1009.
2. **Bjornson, A. B., and H. S. Bjornson.** 1977. Activation of complement by opportunistic pathogens and chemotypes of *Salmonella minnesota*. Infect. Immun. **16:**748–753.
3. **Brown, E., and E. Hendler.** 1989. Rhodococcus peritonitis in a patient treated with peritoneal dialysis. Am J. Kidney Dis. **14:**417–418.
4. **Brown, E. J., J. F. Bohnsack, and H. D. Gresham.** 1988. Mechanism of inhibition of immunoglobulin G-mediated phagocytosis by monoclonal antibodies that recognize the Mac-1 antigen. J. Clin. Invest. **81:**365–375.
5. **Brown, E. J., S. W. Hosea, C. H. Hammer, C. G. Burch, and M. M. Frank.** 1982. A quantitative analysis of the interactions of anti-pneumococcal antibody and complement in experimental pneumococcal bacteremia. J. Clin. Invest. **69:**85–98.
6. **Bullock, W. E., and S. D. Wright.** 1987. Role of the adherence promoting receptors CR3, LFA-1, and p150,95, in the binding of *Histoplasma capsulatum* by human macrophages. J. Exp. Med. **165:**195–210.
7. **Diamond, M. S., J. Garcia-Aguilar, J. K. Bickford, A. L. Corbi, and T. A. Springer.** 1993. The I domain is a major recognition site on the leukocyte integrin Mac-1 (CD11b/CD18) for four distinct adhesion ligands. J. Cell Biol. **120:**1031–1043.
8. **Diamond, M. S., D. E. Staunton, A. R. de Fougerolles, S. A. Stacker, J. Garcia-Aguilar, M. L. Hibbs, and T. A. Springer.** 1990. ICAM-1 (CD54): a counter receptor for Mac-1 (CD11b/CD18). J. Cell Biol. **111:**3129–3139.
9. **Doig, C., M. J. Gill, and D. L. Church.** 1991. *Rhodococcus equi*—an easily missed opportunistic pathogen. Scand. J. Infect. Dis. **23:**1–6.
10. **Drevets, D. A., and P. A. Campbell.** 1991. Roles of complement and complement receptor type 3 in phagocytosis of *Listeria monocytogenes* by inflammatory mouse peritoneal macrophages. Infect. Immun. **59:**2645–2652.
11. **Dustin, M. L., and T. A. Springer.** 1989. T cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. Nature (London) **341:**619–624.
12. **Emmons, W., B. Reichwein, and D. L. Winslow.** 1991. *Rhodococcus equi* infection in the patient with AIDS: literature review and report of an unusual case. Rev. Infect. Dis. **13:**91–96.
13. **Fearon, D. T.** 1984. Cellular receptors for fragments of the third component of complement. Immunol. Today **5:**105–110.
14. **Filley, E. A., and G. A. W. Rook.** 1991. Effect of mycobacteria on sensitivity to the cytotoxic effects of tumor necrosis factor. Infect. Immun. **59:**2567–2572.
15. **Frank, M. M., J. F. May, T. Gaither, and L. Ellman.** 1971. In vitro studies of complement function in sera of C4 deficient guinea pigs. J. Exp. Med. **134:**176–187.
16. **Gaillard, J.-L., P. Berche, J. Mounier, S. Richard, and P. Sansonetti.** 1987. In vitro model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like line Caco-2. Infect. Immun. **55:**2822–2829.
17. **Gewurz, H., H. S. Shin, and S. E. Mergenhagen.** 1968. Interactions of the complement system with endotoxic lipopolysaccharide: consumption of the six terminal components. J. Exp. Med. **128:**1049–1057.
18. **Gorman, C., R. Padmanabhan, and B. H. Howard.** 1983. High efficiency DNA-mediated transformation of primate cells. Science **221:**551–553.
19. **Griffin, F. M.** 1987. Activation of macrophage C3 receptors by a unique lymphokine. Lymphokines **14:**431–441.
20. **Harvey, R. L., and J. C. Sunstrom.** 1991. *Rhodococcus equi* infection in patients with and without human immunodeficiency virus infection. Rev. Infect. Dis. **13:**139–145.
21. **Hassan, H. F., J. R. Cutilli, and S. D. Douglas.** 1990. Isolation of highly purified human blood monocytes for *in vitro* HIV-1 infection studies of monocyte/macrophages. J. Immunol. Methods **130:**283–285.
22. **Hietala, S. K., and A. A. Ardans.** 1987. Interaction of *Rhodococcus equi* with phagocytic cells from *R. equi* exposed and non-exposed foals. Vet. Microbiol. **14:**307–320.
23. **Hillerdal, G., I. Riesenfeldt-Orn, A. Pedersen, and E. Ivanicova.** 1988. Infection with *Rhodococcus equi* in a patient with sarcoidosis treated with corticosteroids. Scand. J. Infect. Dis. **20:**673–677.
24. **Hondalus, M. K., C. R. Sweeney, and D. M. Mosser.** 1992. An assay to quantitate the binding of *Rhodococcus equi* to macrophages. Vet. Immunol. Immunopathol. **32:**339–350.
25. **Johnson, J. A., J. F. Prescott, and R. J. Markham.** 1983. The pathology of experimental *Corynebacterium equi* infection in foals following intrabronchial challenge. Vet. Pathol. **20:**440–449.
26. **Kalli, K. R., J. M. Ahearn, and D. T. Fearon.** 1991. Interaction of iC3b with recombinant isotypic and chimeric forms of CR2. J. Immunol. **147:**590–594.
27. **Kaufman, Y., E. Tseng, and T. A. Springer.** 1991. Cloning of the murine lymphocyte function-associated molecule-1 α-subunit and its expression in COS cells. J. Immunol. **147:**369–374.
28. **Kinoshita, T., J. Takeda, K. Hong, H. Kozono, H. Sakai, and K. Inoue.** 1988. Monoclonal antibodies to mouse complement receptor type 1 (CR1). J. Immunol. **140:**3066–3072.
29. **Kishimoto, T. K., R. S. Larson, A. L. Corbi, M. L. Dustin, D. E. Staunton, and T. A. Springer.** 1989. The leukocyte integrins: LFA-1, Mac-1, and p150,95. Adv. Immunol. **46:**149–182.
30. **Klickstein, L. B., T. J. Bartow, V. Miletic, L. D. Rabson, J. A. Smith, and D. T. Fearon.** 1988. Identification of distinct C3b and C4b recognition sites in the human C3b/C4b receptor (CR1, CD35) by deletion mutagenesis. J. Exp. Med. **168:**1699–1717.
31. **Kuhn, M., and W. Goebel.** 1989. Identification of an extracellular protein of *Listeria monocytogenes* possibly involved in intracellular uptake by mammalian cells. Infect. Immun. **57:**55–61.
32. **Lasky, J. A., N. Pulkingham, M. A. Powers, and D. T. Durack.** 1991. *Rhodococcus equi* causing human pulmonary infection: review of 29 cases. South. Med. J. **84:**1217–1220.
33. **Levitz, S. M., and A. Tabuni.** 1991. Binding of *Cryptococcus neoformans* by human cultured macrophages: requirements for multiple complement receptors and actin. J. Clin. Invest. **87:**528–535.
34. **McGowan, K. L., and M. F. Mangano.** 1991. Infections with *Rhodococcus equi* in children. Diagn. Microbiol. Infect. Dis. **4:**347–352.
35. **Micklem, K. J., and R. B. Sim.** 1985. Isolation of complement-fragment: C3b-binding proteins by affinity chromatography. The identification of p150,95 as an iC3b-binding protein. Biochem. J. **231:**233–236.
36. **Moore, A. E., L. Sabachewsky, and H. W. Toolan.** 1955. Culture characteristics of four permanent lines of human cancer cells. Cancer Res. **15:**598–602.
37. **Mosser, D. M., S. K. Burke, E. E. Coutavas, J. F. Wedgewood, and P. J. Edelson.** 1986. Leishmania species: mechanism of

complement activation by five strains of promastigotes. Exp. Parasitol. **62**:394–404.
38. **Mosser, D. M., and P. J. Edelson.** 1984. Activation of the alternative complement pathway by leishmania promastigotes: parasite lysis and attachment to macrophages. J. Immunol. **132**:1501–1505.
39. **Mosser, D. M., and P. J. Edelson.** 1985. The mouse macrophage receptor for iC3b (CR3) is a major mechanism in the phagocytosis of leishmania promastigotes. J. Immunol. **135**:2785–2789.
40. **Mosser, D. M., and P. J. Edelson.** 1987. The third component of complement (C3) is responsible for the intracellular survival of *Leishmania major*. Nature (London) **327**:329–331.
41. **Mosser, D. M., T. A. Springer, and M. S. Diamond.** 1992. Leishmania promastigotes require opsonic complement to bind to the human leukocyte integrin Mac-1 (CD11b/CD18). J. Cell Biol. **116**:511–520.
42. **Myones, B. L., J. G. Dalzell, N. Hogg, and G. D. Ross.** 1988. Neutrophil and monocyte cell surface p150,95 has iC3b receptor (CR4) activity resembling CR3. J. Clin. Invest. **82**:640–651.
43. **Oldham, L. J., and F. G. Rodgers.** 1985. Adhesion, penetration, and intracellular replication of *Legionella pneumophila*: an *in vitro* model of pathogenesis. J. Gen. Microbiol. **131**:697–706.
44. **Payne, N. R., and M. A. Horwitz.** 1987. Phagocytosis of *Legionella pneumophila* is mediated by human monocyte complement receptors. J. Exp. Med. **166**:1377–1389.
45. **Prescott, J. F.** 1991. *Rhodococcus equi*: an animal and human pathogen. Clin. Microbiol. Rev. **4**:20–34.
46. **Ross, G. D.** 1981. Detection of complement receptors and Fc receptors on macrophages. Man. Macrophage Methodol. **13**: 209–227.
47. **Sanchez-Madrid, F., P. Simon, S. Thompson, and T. A. Springer.** 1983. Mapping of antigenic and functional epitopes on the α- and β-subunits of two related mouse glycoproteins involved in cell interactions, LFA-1 and Mac-1. J. Exp. Med. **158**:586–602.
48. **Sanchez-Madrid, F. A., A. M. Krensky, C. F. Ware, E. Robbins, J. L. Strominger, S. J. Burakoff, and T. A. Springer.** 1982. Three distinct antigens associated with human T-lymphocyte cytolysis, LFA-1, LFA-2, and LFA-3. Proc. Natl. Acad. Sci. USA **79**:7489–7493.
49. **Schlesinger, L. S., C. G. Bellinger-Kawahara, N. R. Payne, and M. C. Horowitz.** 1990. Phagocytosis of *Mycobacterium tuberculosis* is mediated by human monocyte complement receptors and complement component C3. J. Immunol. **144**:2771–2780.
50. **Schlesinger, L. S., and M. A. Horowitz.** 1990. Phagocytosis of leprosy bacilli is mediated by complement receptors CR1 and CR3 on human monocytes and complement component C3 in serum. J. Clin. Invest. **85**:1304–1314.
51. **Shapiro, J. M., B. M. Romney, M. D. Weiden, C. S. White, and K. M. O'Toole.** 1992. *Rhodococcus equi* endobronchial mass in a patient with AIDS. Thorax **47**:62–63.
52. **Sirera, G., J. Romeu, B. Clotet, P. Velasco, J. Arnal, F. Rius, and M. Fox.** 1991. Relapsing systemic infection due to *Rhodococcus equi* in a drug abuser seropositive for human immunodeficiency virus. Rev. Infect. Dis. **13**:509–510.
53. **Springer, T. A., G. Galfre, D. S. Secher, and C. Milstein.** 1979. Mac-1: a macrophage differentiation antigen identified by monoclonal antibody. Eur. J. Immunol. **9**:301–303.
54. **Stacker, S. A., and T. A. Springer.** 1991. Leukocyte integrin p150,95 (CD11c/CD18) functions as an adhesion molecule binding to a counter-receptor on stimulated endothelium. J. Immunol. **146**:648–655.
55. **Talamas-Rohana, P., S. D. Wright, M. R. Lehnartz, and D. G. Russell.** 1990. Lipophosphoglycan from *Leishmania mexicana* promastigotes binds to members of the CR3, p150,95, and LFA-1 family of leukocyte receptors. J. Immunol. **144**:4817–4824.
56. **Verbrugh, H. A., P. K. Petersen, B. T. Nguyen, S. P. Sisson, and Y. Kim.** 1982. Opsonization of encapsulated *Staphylococcus aureus*: the role of specific antibody and complement. J. Immunol. **129**:1681–1687.
57. **Wilkelstein, J. A., and A. Tomasz.** 1978. Activation of the alternative complement pathway by pneumococcal cell wall teichoic acid. J. Immunol. **120**:174–182.
58. **Wilkinson, B. J., S. P. Sisson, Y. Kim, and P. K. Peterson.** 1979. Localization of the third component of complement on the cell wall of encapsulated *Staphylococcus aureus* M: implications for the mechanism of resistance to phagocytosis. Infect. Immun. **26**:1159–1163.
59. **Wong, M. C., E. P. Ewing, C. S. Callaway, and W. L. Peacock.** 1980. Intracellular multiplication of *Legionella pneumophila* in cultured human embryonic lung fibroblasts. Infect. Immun. **28**:1014–1018.
60. **Wong, W. W., L. B. Klickstein, J. A. Smith, J. H. Weis, and D. T. Fearon.** 1985. Identification of a partial cDNA clone for the human receptor for complement fragments C3b/C4b. Proc. Natl. Acad. Sci. USA **82**:7711–7715.
61. **Wright, S. D., P. E. Rao, W. C. Van Voorhis, L. S. Craigmyle, K. Iida, M. A. Talle, E. F. Westberg, G. Goldstein, and S. C. Silverstein.** 1983. Identification of the C3bi receptor on human monocytes and macrophages by using monoclonal antibodies. Proc. Natl. Acad. Sci. USA **80**:5699–5703.
62. **Wright, S. D., and S. C. Silverstein.** 1982. Tumor-promoting phorbol esters stimulate C3b and C3b′ receptor-mediated phagocytosis in cultured human monocytes. J. Exp. Med. **156**:1149–1164.
63. **Yager, J. A.** 1987. The pathogenesis of *Rhodococcus equi* pneumonia in foals. Vet. Microbiol. **14**:225–232.
64. **Zink, M. C., J. A. Yager, J. F. Prescott, and M. A. Fernando.** 1987. Electron microscopic investigation of intracellular events after ingestion of *Rhodococcus equi* by foal alveolar macrophages. Vet. Microbiol. **14**:295–305.
65. **Zink, M. C., J. A. Yager, and N. L. Smart.** 1986. *Corynebacterium equi* infections in horses, 1958–1984. Can. Vet. J. **27**:213–217.