**Cell membrane receptors on bovine mononuclear phagocytes involved in phagocytosis of *Mycobacterium avium* subsp *paratuberculosis***

Cleverson D. Souza, DVM, MS; Oral A. Evanson, BS; Srinand Sreevatsan, MVSc, MPH, PhD; Douglas J. Weiss, DVM, PhD

**Objective**—To determine cell membrane receptors involved in phagocytosis of *Mycobacterium avium* subsp *paratuberculosis* (MAP) organisms.

**Sample Population**—Monocytes were obtained from healthy adult Holstein dairy cows that were test negative for MAP infection on the basis of bacteriologic culture of feces and serologic test results.

**Procedures**—Monocytes or bovine macrophage cell line (BoMac) cells were incubated with MAP organisms for 30, 60, or 120 minutes with or without inhibitors of integrins, CD14, or mannose receptors. Phagocytosis was evaluated by light microscopy or by flow cytometry. CD11a/CD18, CD11b, and CD14 expression on monocytes and BoMac cells was evaluated by use of flow cytometry.

**Results**—Monocytes and BoMac cells rapidly phagocytized MAP organisms. However, compared with BoMac cells, monocytes had a greater total capacity to phagocytize MAP organisms. Addition of neutralizing anti-integrin antibodies (anti-CD11a/CD18 and anti-CD11b) substantially inhibited phagocytosis by monocytes during the first 60 minutes of incubation with MAP organisms, but were less effective at 120 minutes of incubation. Anti-CD11a/CD18 and anti-CD11b antibodies were less effective in inhibiting phagocytosis by BoMac cells. Addition of inhibitors of CD14 or mannose receptors also inhibited phagocytosis of MAP by monocytes. Addition of a combination of integrin and mannose inhibitors had an additive effect in reducing phagocytosis, but addition of integrin and CD14 inhibitors did not have an additive effect.

**Conclusions and Clinical Relevance**—Multiple receptors are involved in phagocytosis of MAP organisms. Although CD11/CD18 receptors appear to be the major receptors used by MAP at early time points, mannose receptors and CD14 also contribute substantially to phagocytosis. (*Am J Vet Res* 2007;68:975–980)
Effects of opsonization on phagocytosis of MAP organisms have been described. Addition of fresh autologous serum at concentrations as low as 0.1% resulted in an increase in phagocytosis of MAP organisms in vitro. Conversely, only a slight increase in phagocytosis of MAP was observed after addition of serum to BoMac cells. This difference may be the result of the difference in the number of complement receptors present on the surface of monocytes and BoMac cells.

From the results of previous studies, the importance of cell membrane receptor interactions and signal transduction has been documented. Addition of MAP organisms to bovine monocytes in vitro rapidly activates the mitogen-activated protein kinase pathway. Activation of mitogen-activated protein kinase-p38 leads to expression of the anti-inflammatory cytokine interleukin-10. Blocking the mitogen-activated protein kinase-p38 pathway decreased interleukin-10 expression, increased phagosome acidification, and enabled monocytes to kill MAP organisms. Therefore, the specific pathway used in entry may affect signal transduction, cytokine expression, phagosome trafficking, and antimicrobial activity and therefore may be critical to the eventual fate of the organism.

The cell membrane receptors involved in phagocytosis of MAP and initiation of cell signaling are incompletely understood. The purpose of the study reported here was to evaluate cell membrane receptors that interact with MAP organisms and are involved in phagocytosis. Monoclonal antibodies and α-methyl mannoside were used to block receptors on monocytes and BoMac cells, and phagocytosis was determined by use of light microscopy and flow cytometry.

**Materials and Methods**

**Bacterial strain and culture conditions**—**Mycobacterium avium** subsp paratuberculosis strain 1018 was obtained from a cow with naturally acquired paratuberculosis. Organisms were determined to be MAP on the basis of dependence on mycobactin J for growth and detection of species-specific DNA sequences by use of a PCR assay. Organisms were grown to a concentration of approximately 10^8 organisms/mL, washed, and resuspended in broth media containing a supplement, Tween 80, mycobactin J, and 5% fetal bovine serum. Viability of the organisms added to monocyte cultures varied between 82% and 95% as determined by propidium iodide exclusion. Before addition to monocyte cultures, organisms were washed and resuspended in RPMI medium. To remove clumps, the suspension of organisms was vortexed vigorously and remaining clumps were allowed to settle for 10 minutes.

**Cell isolation and culture**—Blood was collected from 4 healthy adult Holstein dairy cows that belonged to the University of Minnesota dairy herd. This herd has consistently tested negative for paratuberculosis, as determined by results of bacteriologic culture of feces and is considered to be paratuberculosis free. Mononuclear cells were isolated by use of Percoll density gradient centrifugation, as described. Isolated cells were washed in Dulbecco PBS solution and resuspended at 1 × 10^7 mononuclear cells/mL in RPMI 1640 medium containing 10% fetal bovine serum. For isolation of monocytes, 3 × 10^6 mononuclear cells were incubated on 22 × 22-mm glass coverslips or in 60 × 15-mm tissue culture plates for 90 minutes at 37°C to allow cells to adhere. Nonadherent cells were removed by repeated washing with RPMI 1640 medium warmed to 37°C. Adherent cells were cultured overnight at 37°C with 5% CO₂ in RPMI 1640 medium supplemented with 5% fetal bovine serum. Additionally, 5% fresh autologous serum was added to opsonized organisms.

The BoMac cells were obtained as a gift. These cells were used because they are derived from bovine macrophages, lack CD14 receptors, and have reduced numbers of integrin receptors. Therefore, BoMac cells provided an alternative approach to evaluation of the role of CD14 and integrin receptors in phagocytosis of MAP. The BoMac cells were grown in 60 × 15-mm plastic culture plates in RPMI-1640 medium supplemented with 5% fresh bovine serum and 5% fetal bovine serum. The medium was changed every 3 days. Cell viability was monitored by use of trypan blue exclusion. None of the treatments used altered monocyte or BoMac cell viability during the 120-minute incubation period.

**Cell membrane receptor inhibitors**—Anti-bovine CD11a/CD18 and anti-bovine CD11b antibodies were purchased commercially and were added separately or together to monocyte cultures. Preliminary dose-response studies were performed to determine the anti-body concentration that achieved maximal inhibition of phagocytosis (Figure 1). A concentration of 10 µg of antibody/10^6 monocytes was chosen for both antibodies. Anti-bovine CD14 antibody was provided as a gift and was added at a concentration of 10 µg of antibody/10^6 monocytes. This antibody has been shown to inhibit lipopolysaccharide-induced tumor necrosis factor-α production by bovine monocytes at this concentration. A dose-response study was not performed because of the limited amount of antibody available. The mannose receptor was blocked by addition of 10, 100, or 200 µM α-methyl mannoside. Results of previous studies indicate that at a concentration of 100 µM, α-methyl mannoside was able to prevent binding of the soluble 19-kd antigen of *M tuberculosis* to a human macrophage cell line.

**Determination of phagocytosis of MAP organisms by light microscopy**—The MAP organisms (multiplicity of infection, 10 bacilli/monocyte) were added to monocytes or BoMac cells attached to coverslips. After 30, 60, or 120 minutes of incubation at 37°C with 5% CO₂, coverslips were washed to remove nonphagocytized organisms and treated with trypsin-EDTA solution for 10 minutes to remove noningested bacteria. Thereafter, coverslips were stained with Ziehl-Neelsen carbolfuchsin stain that stains mycobacterial and other acid-fast organisms. The percentage of cells containing organisms was determined by counting a minimum of 300 cells by use of light microscopy. All tests were done in duplicate, and data from at least 3 separate experiments were evaluated.

**Flow cytometric evaluation of phagocytosis of MAP organisms**—The MAP organisms were labeled with fluorescein isothiocyanate and subsequently incu-
bated with bovine monocytes or BoMac cells (multiplicity of infection, 10 bacilli/monocyte). After 30, 60, or 120 minutes of incubation at 37°C with 5% CO₂, plates were washed to remove nonphagocytized organisms and cells were gently scraped into test tubes. Trypan blue was added just before analysis to neutralize the fluorescence of free organisms and organisms adherent to the monocyte surface. Cells were analyzed by use of a flow cytometer and mean fluorescence intensity of the monocyte or BoMac cell populations was determined. Negative controls consisted of cells incubated with unlabeled organisms and cells incubated with the phagocytosis inhibitor cytochalasin B (5 µg/mL) before addition of labeled organisms.

Flow cytometric detection of CD11a/CD18 and CD11b on bovine monocytes and BoMac cells and CD14 on monocytes—Cultured bovine monocytes and BoMac cells were harvested by use of a cell scraper. Cells were resuspended in Dulbecco PBS solution containing 2% sheep serum, 5mM sodium azide, and 0.1% glycine and then incubated with 2 µg of anti-bovine CD11a/CD18 antibody, 2 µg of anti-bovine CD11b antibody, or 5 µg of anti-CD14 antibody at 25°C for 30 minutes. After washing, cells were incubated with 10 µL of a 1:30 dilution of fluorescein isothiocyanate–labeled anti-mouse IgG. Cells were washed, and the mean fluorescence intensity of the entire cell population and the percentage of cells expressing CD11a/CD18, CD11b, or CD14 were determined by use of a flow cytometer.

Statistical analysis—All tests were done in duplicate or triplicate, and results of at least 3 separate experiments were evaluated. Results were expressed as mean ± SD. Differences between cell cultures incubated with and without the addition of inhibitors of cell membrane receptors were analyzed by use of the paired Student t test. Values of P < 0.05 were considered significant.

Results

Phagocytosis of MAP by monocytes and BoMac cells—Bovine monocytes or BoMac cells were incubat-
ed with MAP organisms for 30, 60, or 120 minutes, and phagocytosis was determined by light microscopy or flow cytometry. The percentage of monocytes phagocytizing MAP (78 ± 8%) was similar to the percentage of BoMac cells phagocytizing MAP organisms (80 ± 7%). Alternatively, when the mean fluorescence intensity of monocytes was compared with the mean fluorescence intensity of BoMac cells, monocytes had significantly greater mean fluorescence intensity at all time points (Figure 2).

Role of integrin receptors in phagocytosis of MAP organisms—The density of integrin receptors expressed on monocytes and BoMac cells was compared by use of flow cytometry after labeling with anti-CD11a/CD18 antibody or anti-CD11b antibody. Greater than 95% of monocytes expressed CD11a/CD18, and 89 ± 5% of monocytes expressed CD11b (Figure 3). Alternatively, only 28 ± 4% of BoMac cells expressed CD11a/CD18 and 23 ± 7% expressed CD11b. When the total fluorescence was evaluated, monocytes expressed 7.7-fold more CD11a/CD18 and 3.1-fold more CD11b, compared with BoMac cells. The role of integrin receptors in phagocytosis was evaluated by blocking CD11a/CD18 or CD11b with monoclonal antibodies before adding MAP organisms to monocytes or BoMac cells. Phagocytosis was evaluated by light microscopy and flow cytometry. The percent inhibition of phagocytosis was determined by comparing results to samples incubated with isotype-matched control antibodies. Addition of anti-CD11a/CD18 alone reduced the percentage of phagocytic monocytes by > 50% at 30 or 60 minutes of incubation (Figure 4). Addition of anti-CD11a/CD18 and anti-CD11b antibodies in combination resulted in an 82% decrease in the percentage of phagocytic monocytes at 30 minutes of incubation and a 72% decrease at 60 minutes. Addition of anti-CD11a/CD18 antibody with or without anti-CD11b antibody was less effective in blocking phagocytosis at 120 minutes. Anti-CD11a/CD18 antibody with or without anti-CD11b antibody was less effective in blocking phagocytosis of MAP by BoMac cells at all times, compared with monocytes.
Role of mannose receptors in phagocytosis of MAP organisms—Mannose receptors were evaluated by blocking their activity with the addition of α-methyl mannoside. When added at a concentration of 10μM, α-methyl mannoside resulted in minimal inhibition of phagocytosis, whereas concentrations of 100 and 200μM resulted in equivalent inhibition of phagocytosis (data not shown). Therefore, 100μM of α-methyl mannoside was used in subsequent studies. Addition of α-methyl mannoside to bovine monocytes resulted in 28% to 35% inhibition of phagocytosis at the various time points (Figure 5). Addition of α-methyl mannoside to BoMac cells also resulted in inhibition of MAP phagocytosis; however, mean values were lower at each time point and reached significance at 120 minutes.

Role of CD14 in phagocytosis of MAP organisms—Expression of CD14 was evaluated on monocytes and BoMac cells (Figure 3). CD14 was expressed on 84 ± 6% of monocytes. Alternatively, essentially no BoMac cells expressed CD14. Addition of anti-CD14 antibody to monocytes before addition of MAP organisms resulted in inhibition of phagocytosis at all time points (Figure 6).

Effect of inhibiting multiple cell membrane receptors—Effects of adding various combinations of inhibitors of integrin receptors, mannose receptors, and CD14 to monocytes before addition of MAP organisms were evaluated after 60 and 120 minutes of coincubation (Figure 7). The combination of integrin and mannose inhibitors resulted in 19% and 14% greater inhibition of phagocytosis at 60 and 120 minutes, respectively, compared with addition of integrin inhibitors alone. The combination of integrin and CD14 inhibitors failed to result in additive effects. Additionally, the combination of integrin, mannose, and CD14 inhibitors failed to have a greater inhibitory effect, compared with integrin and mannose inhibitors alone.

Discussion

Unlike some pathogenic organisms that have developed mechanisms to circumvent phagocytosis, mycobacteria rapidly enter cells with the apparent goal of evading antimicrobial activity. Results of our study indicate that multiple cell membrane receptors are involved in phagocytosis of MAP organisms. Under the experimental conditions of our study, integrin receptors appeared to be the major portals for phagocytosis of MAP during the first 30 minutes of incubation. Blocking mannose receptors or CD14 also partially inhibited phagocytosis. Therefore, all of these receptors appear to engage MAP organisms. This interaction may occur in a variety of ways. First, direct binding of the organism to several receptors may facilitate the phagocytic process. Secondly, multiple microbe-receptor interactions may induce cell-signaling events that converge to promote cell activation and enhance phagocytosis. Thirdly, induction of an inflammatory response may activate other cells and improve efficiency of phagocytosis.

Results of previous studies indicate that complement receptors are the major receptor for phagocytosis of M tuberculosis by human monocytes. Addition of anti-CD11a/CD18 antibody or anti-CD11b/CD18 antibody separately reduced M tuberculosis adherence by approximately 40%. However, when added in combination, M tuberculosis adherence was reduced by > 80%.

Mannose receptors are also incriminated in phagocytosis of some strains of mycobacteria. Inhibition of phagocytosis of M tuberculosis by human monocyte-derived macrophages was demonstrated by use of competitive inhibitors and anti-mannose receptor antibodies. Blocking of mannose receptors resulted in inhibition of phagocytosis of virulent strains of M tuberculosis but not an avirulent strain. Two ligands have been identified on M tuberculosis that bind to the mannose receptor. These include mannosylated lipoarabinomannan and 19-kd glycolipoprotein. Lipoarabinomannan is a major component of the cell wall of both pathogenic and nonpathogenic mycobacteria; however, pathogenic mycobacteria contain a mannose cap (ie, mannosylated lipoarabinomannan), whereas nonpathogenic mycobacteria contain an arabinosylated lipoarabinomannan. Mannosylated lipoarabinomannan preferentially interacts with the mannose receptor, whereas arabinosylated lipoarabinomannan interacts with TOLL-like receptors.

CD14 is best known as a receptor for lipopolysaccharide, which is abundant in the wall of gram-negative bacteria. However, CD14 may also be involved in the phagocytosis of M tuberculosis and M bovis organisms. Blocking anti-CD14 antibodies or soluble CD14 was shown to decrease phagocytosis of M tuberculosis by microglial cells when incubated in a serum-free environment. Phagocytosis of M bovis by porcine alveolar macrophages was blocked by up to 60% by addition of anti-CD14 antibody. The ligand for CD14 is uncertain, but it appears that lipoarabinomannan may bind it.

Other receptors that are potentially involved in phagocytosis of MAP organisms include scavenger receptors and Fcγ receptors. Scavenger receptors bind polyanionic macromolecules and particles, most notably lipopolysaccharides of gram-negative bacteria and lipoteichoic acid of gram-positive bacteria. Class A scavenger receptors have been incriminated in phagocytosis of virulent M tuberculosis by macrophages. Fcγ receptors would be expected to be involved only if individuals had circulating antibodies against the organism. This type of phagocytosis is unlikely in our study because the cows from which monocytes were derived were born and raised in a paratuberculosis-free herd.

The BoMac cells were studied because they lack CD14 receptors and have reduced CD11a/CD18 and CD11b receptors. The capacity of BoMac cells to ingest MAP organisms indicates that CD14 is not essential to the phagocytic process. Our results differed from those of a previous study. In the previous study, a maximum of 13% of BoMac cells phagocytized MAP organisms after 3 hours of coincubation, whereas in the present study, 80% of BoMac cells phagocytized MAP organisms after 60 minutes. The relative number of CD11a/CD18 and CD11b receptors expressed by the BoMac cells in that study and the present study was similar. We have previously observed variation in the capacity of BoMac cells to phagocytize MAP organisms (unpub-
lished observations). Although we are uncertain why this occurred, we suspect that it relates to the overall health of the cell cultures. In conclusion, multiple receptors including complement and noncomplement-dependent integrin receptors, mannose receptors, and CD14 are involved in phagocytosis of MAP organisms. Identification of cell membrane molecules involved in phagocytosis will facilitate identification of specific molecules on MAP that interact with the macrophage and induce cell-signaling events that suppress antimicrobial responses.

References

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