Major Surface Protein 2 of *Anaplasma phagocytophilum* Facilitates Adherence to Granulocytes

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Anaplasma phagocytophilum is an obligate intracellular bacterium that infects myeloid cells in the mammalian host. Msp2 (p44) is the major immunodominant outer-membrane protein of these bacteria. We hypothesized that Msp2 acts as an adhesin for *A. phagocytophilum* entry into granulocytes. This potential role was investigated by blocking binding with Msp2 monoclonal antibodies and by antagonizing binding and propagation with recombinant Msp2 (rMsp2) in vitro. With HL-60 cells, fresh human peripheral blood neutrophils, and a cell line devoid of the fucosylated platelet selectin glycoprotein ligand 1 (PSGL-1) receptor for *A. phagocytophilum* or one that was transfected to express this ligand, Msp2 monoclonal antibody and rMsp2 used as the antagonist caused concentration-dependent reductions in bacterial adhesion ($P < 0.007$ and $P < 0.02$, respectively) and propagation ($P < 0.05$ and $P < 0.001$), although inhibition of adhesion or propagation was moderate and incomplete. Likewise, rMsp2 bound to surfaces of the transfected cell at a level similar to that of extracellular *A. phagocytophilum* and significantly ($P < 0.05$) beyond that of nontransfected cells. Moreover, a dose-dependent reduction ($P < 0.019$) in PSGL-1 monoclonal antibody binding to HL-60 cells was elicited with rMsp2. We conclude that Msp2s of *A. phagocytophilum* are involved in bacterial adhesion to ligands on host myeloid cells before intracellular infection.

Anaplasma phagocytophilum is a tick-borne obligate intracellular bacterium that infects humans as well as horses, dogs, ruminants, and other animals (10, 11, 23, 29, 33). The bacterium infects and propagates chiefly within vacuoles of blood neutrophils in mammals (10, 11, 18, 34). The clinical manifestations of infection range from mild to fatal and are associated with opportunistic infections with neutropenia or functional neutrophil defects (3, 4, 17, 29). *A. phagocytophilum* infection of neutrophils is inhibited by antibodies to sCD15 (sialylated Lewis^X), and neutrophil binding occurs via fucosylated platelet selectin glycoprotein ligand 1 (PSGL-1) (also called CD162) (15, 19). Although *A. phagocytophilum* enters after binding to fucosylated PSGL-1 on neutrophils, the identity of the bacterial adhesin is not known.

A major immunodominant surface-exposed protein antigen of *A. phagocytophilum* is phylogenetically most similar to major surface protein-2 (Msp2) of *Anaplasma marginale* (5, 8, 12, 20, 27, 36, 37). Although this 44-kDa protein antigen in *A. phagocytophilum* has been given several names, we use Msp2 because the terminology is already established for *A. marginale* (25, 30, 32). Similar to those of *A. marginale*, *A. phagocytophilum* Msp2s are encoded by a multigene family whose members exhibit various transcription and expression characteristics (2, 8, 21, 27, 37, 38). Because of abundant surface expression, we hypothesized that Msp2 might be an *A. phagocytophilum* adhesin for neutrophil binding. Thus, we tested the ability of monoclonal antibodies (MAb) and recombinant Msp2 (rMsp2) to block or antagonize binding to and in vitro propagation in granulocytes. Additionally, by blocking PSGL-1 MAb binding to granulocytes with rMsp2 and by blocking *A. phagocytophilum* adhesion to BJAB lymphoblastoid cells transfected to express the known *A. phagocytophilum* cellular ligand, fucosylated PSGL-1, we investigated whether Msp2 binds to PSGL-1.

MATERIALS AND METHODS

MAb. Msp2 MAb were developed from mice immunized with *A. phagocytophilum* (Webster strain) in Ribi adjuvant (Ribi Immunochem, Hamilton, Mont.). Splenocytes were fused to myeloma SP2/0 cells by standard methods, and clones were selected by fluorescent antibody tests using *A. phagocytophilum*-infected HL-60 cells. Clones reactive with the 44-kDa Msp2 were selected by protein immunoblotting, and each was subcloned three times before antibody purification (12). Two MAb designated 20B4 and 5B5 (both immunoglobulin G2a kappa [IgG2aκ]) reacted with Msp2s of seven different strains of *A. phagocytophilum* from California, Minnesota, Wisconsin, and New York.

MAb KPL-1 (IgG1aκ) that reacts with an extracellular domain of PSGL-1 (also called CD162) was purchased from BD Pharmingen, San Diego, Calif. Control IgG1aκ and IgG2aκ MAb purified from mouse ascites were purchased from Sigma (Chemical Co., St. Louis, Mo.).

rMsp2 and AnkA. rMsp2 was prepared from a bacteriophage clone (20H13) first identified in an *A. phagocytophilum* (BDS strain) λ Express phage (Stratagene, La Jolla, Calif.) genomic library (8, 9). λ Plaques that reacted with the 20B4 Msp2 MAb were selected, and the recombinant inserts were excised in vivo into the pBK-CMV vector that was then used to transform *Escherichia coli* XL1-Blue MRF′. Phagemids of transformed *E. coli* clones that reacted with the 20B4 Msp2 MAb in protein immunoblot experiments were purified, and the recombinant inserts were sequenced. Potential open reading frames were identified, and a full-length open reading frame predicted to encode a protein of appropriate molecular size was selected, amplified by PCR, and subcloned using an Affinity™ LIC cloning and protein purification kit (Stratagene). The selected clone had a sequence identical to that of an *msp2* gene transcribed at a low level after seven passages in vitro in the *A. phagocytophilum* Webster strain (8). After being subcloned into the pCAL-n-EK vector, transformed *E. coli* BL21(DE3)pLysS cells were tested for rMsp2 expression by protein immunoblotting with MAb 20B4 and polyclonal rabbit anti-*A. phagocytophilum*. The rMsp2-calmodulin binding protein fusion was purified from transformed *E. coli* on calmodulin affinity resin columns (Stratagene); the recombinant protein re-

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tained reactivity with both Msp2 MAb 20B4 and rabbit polyclonal anti-*A. phagocytophilum* in protein immunoblot experiments.

Recombinant AnkA was used as a control because of its constitutive, non-membrane expression in *A. phagocytophilum* (9). Recombinant AnkA (rAnkA) was produced using an identical protocol after amplification of the gene from *A. phagocytophilum* Webster strain genomic DNA. The rAnkA protein retained reactivity with AnkA MAb IE3 and rabbit polyclonal anti-AnkA in protein immunoblot experiments (9).

Growth of *A. phagocytophilum*. *A. phagocytophilum* Webster strain was propagated in the HL-60 promyelocytic cell line in RPMI 1640 medium supplemented with between 1 and 20% fetal bovine serum and 2 mM L-glutamine (2, 14). Prior to the experiments, the concentration of infected cells was adjusted by adding uninfected HL-60 cells. Cell viability (trypan blue exclusion) prior to and at the end of each experiment was always at least 95%.

To assess whether Msp2 MAb can block propagation of *A. phagocytophilum* in HL-60 cells, MAb 20B4 and isotype-matched control MAb were added in 10-fold-increasing concentrations from 0.1 to 0.2 µg/ml through 10 to 20 µg/ml; experiments were conducted in triplicate 1-ml cultures. Cultures were examined at day 4 or 5, and the proportion of infected cells was determined on Romanowsky-stained slides. In separate experiments, rMsp2, rAnkA, or bovine serum albumin (BSA) was added to RPMI 1640 medium in concentrations ranging from 0.05 to 5 µg/ml. To ensure viability of cells during the experiments, the tissue culture medium, with or without MAb, recombinant proteins, or control protein, was changed every 2 days.

Extracellular *A. phagocytophilum*. Extracellular *A. phagocytophilum* was prepared from heavily infected HL-60 cells that were disrupted by three to five passages through a 26-gauge syringe needle. Cellular debris was removed by centrifugation at $750 \times g$ for 10 min, and extracellular bacteria were harvested from the supernatant by centrifugation ($2,500 \times g$ for 10 min). The extracellular bacteria were labeled with the lipophilic fluorescent compound PKH-67 (Sigma Chemical Co.) and checked for purity using fluorescence microscopy.

BJAB and PSGL-1/FucT-VII-transfected BJAB cells. BJAB and PSGL-1/FucT-VII-transfected cells were kindly provided by Karen R. Snapp, Northwestern University Medical School. BJAB and transfected cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine at 37°C in 5% CO_2 (19). To confirm that the transfected BJAB cells expressed PSGL-1, transfected and nontransfected cells were analyzed by Western blotting using PSGL-1 MAb KPL-1 (IgG1a).

***A. phagocytophilum* propagation assay.** HL-60 cell cultures were adjusted to contain either 3 or 5% infected cells by supplementation with uninfected cells. Bacterial propagation in HL-60 cells in the presence or absence of antibodies, recombinant proteins, and control proteins was assessed microscopically with 200 cells in Romanowsky-stained cytocentrifuged preparations after 4 to 5 days of culture. All analyses were performed in triplicate or quadruplicate. The proportion of infected cells in experimental cultures was expressed as a percentage of infected cells in control cultures supplemented with the appropriate isotype-matched control MAb or control protein (rAnkA and BSA).

To determine whether infection of primary peripheral blood neutrophils was also affected by these treatments, extracellular bacteria were first reacted with Msp2 MAb or isotype-matched MAb or with rMsp2, rAnkA, or BSA for 30 min at 37°C in 5% CO_2. The treated extracellular bacteria were then incubated overnight with 10^6 neutrophils in RPMI 1640 medium supplemented with 5% fetal bovine serum and 2 mM L-glutamine. The cells were harvested by cytocentrifugation onto glass slides and fixed in cold acetone. Bacterial infection of neutrophils was assessed by staining with fluorescein-labeled Msp2 MAb 20B4 for 1 h at room temperature, washing in PBS, and then counterstaining with Evans blue. Fluorescence microscopy and quantitation were performed using a Vistra Systems Fluorimager IS (Sunnyvale, Calif.) with Molecular Dynamics Image QuaNT software (version 4.2a). Fluorescence intensities were compared among groups and replicates to determine differences in binding.

***A. phagocytophilum* adhesion and early infection assays.** Washed fluorescent extracellular *A. phagocytophilum* was first preincubated for 1 h with different concentrations (5 or 10 µg/ml) of Msp2 MAb 20B4 or 5B5 or with IgG2aκ control MAb. The antibody-treated bacteria were incubated with 2×10^5 HL-60 cells or neutrophils for 1 h at 37°C in 5% CO_2. The cells were then washed two times with RPMI 1640 medium with 1% fetal bovine serum. Cells were resuspended in 100 µl of tissue culture medium, cytocentrifuged onto glass slides, and mounted with PBS-glycerol under coverslips. The cells were examined microscopically and measured fluorometrically. Alternately, cells were mixed with a 5-µg/ml concentration of rMsp2, rAnkA, BSA, or medium for 1 h at 37°C in 5% CO_2 and then washed as described above prior to treatment with approximately 1.3×10^7 fluorescent extracellular *A. phagocytophilum* bacteria for 1 h as de-

scribed above. The cells were then washed again, prepared on slides, and analyzed as described above.

PSGL-1 binding assay of rMsp2 and extracellular *A. phagocytophilum*. To determine whether Msp2 binds to fucosylated PSGL-1 on exposed cell surfaces, extracellular *A. phagocytophilum* or rMsp2 was incubated with 2×10^5 BJAB cells that lack PSGL-1 and fucosyltransferase or with PSGL-1/FucT-VII-transfected BJAB cells for 1 h at 37°C in 5% CO_2. The cells were harvested by centrifugation, washed two times with RPMI 1640 tissue culture medium to remove unbound bacteria, cytocentrifuged onto glass slides, and fixed in cold acetone. Using fluorescence microscopy and fluorimager quantitation, binding of bacteria or rMsp2 to BJAB and PSGL-1/FucT-VII-transfected cells was assessed by staining with Msp2 MAb 20B4 and fluorescein-labeled anti-mouse Ig (Kierkegaard and Perry Laboratories, Gaithersburg, Md.). Net fluorescence intensity was calculated by adjusting each measurement for background fluorescence (fluorescence in cell cultures not treated with extracellular *A. phagocytophilum* or rMsp2) and was expressed as the difference in fluorescence between identical manipulations on PSGL-1/FucT-VII-transformed and nontransformed BJAB cells.

Inhibition assay of *A. phagocytophilum* binding to PSGL-1-expressing BJAB cells. To determine whether the binding of *A. phagocytophilum* to PSGL-1 in PSGL-1/FucT-VII-transfected BJAB cells is inhibited by anti-Msp2 MAb, extracellular bacteria labeled with PKH-67 were first reacted with various concentrations of Msp2 MAb or isotype-matched MAb for 1 h at 37°C in 5% CO_2. The cells were then incubated with 2×10^5 BJAB and PSGL-1/FucT-VII-transfected BJAB cells. After washing, the cells were cytocentrifuged onto glass slides for microscopic analysis and fluorescence quantitation.

Alternately, BJAB and PSGL-1/FucT-VII-transfected BJAB cells were mixed with different concentrations (1 or 5 µg) of rMsp2, BSA as the control protein, or medium only for 1 h at 37°C in 5% CO_2 and then washed prior to treatment with fluorescence-labeled extracellular *A. phagocytophilum* for 1 h. The cells were washed again, prepared on slides, and analyzed as described above.

Blocking of PSGL-1 MAb binding to HL-60 cells by rMsp2. To determine whether rMsp2 binds directly to PSGL-1 on the surfaces of HL-60 cells, a modified fluorescent antibody method was used. Teflon-coated 12-well slides containing 10^4 uninfected HL-60 cells per well were fixed in cold anhydrous acetone for 10 min. The slides were air dried, and the HL-60 cells were incubated for 1 h at 37°C in a humid chamber with various concentrations (0.02 to 3 µg/ml) of rMsp2, rAnkA, or BSA. After three washes with PBS, anti-PSGL-1 MAb KPL-1 (BD Pharmingen) (25 µg/ml) was added and the cells were incubated for another hour at 37°C in a humid chamber followed by three washes with PBS. Inhibition of KPL-1 binding was analyzed in slides incubated with fluorescein isothiocyanate-labeled anti-mouse Ig (Kierkegaard & Perry Laboratories) for 1 h at 37°C, followed by a 5-min wash in PBS with 0.005% Evans blue and two additional PBS washes. Slides were examined by fluorescence microscopy and quantitative fluorescence as described above.

Statistical analyses. All analyses were conducted using one-tailed, paired equal-variance, or two-sample unequal-variance Student's *t* tests, as appropriate. A *P* value of <0.05 was considered significant.

Human subjects. Human peripheral blood neutrophils were obtained from healthy adult volunteers with the approval of the Johns Hopkins Medicine Internal Review Board under compliance with all federal and institutional guidelines and policies.

RESULTS

Blocking of *A. phagocytophilum* propagation with Msp2 MAb. A significant antibody concentration-dependent reduction in *A. phagocytophilum* in cells propagated overnight or for up to 5 days was observed in five replicated experiments using HL-60 cells (Table 1) and in three replicated experiments using fresh peripheral blood neutrophils (Fig. 1). Although the degree of reduced propagation was variable, HL-60 cell cultures supplemented with various concentrations (10 to 20 µg/ml) of MAb 20B4 or 5B5 were significantly smaller and contained as little as 63% ($P < 0.05$) of the number of infected cells observed in cultures incubated with identical concentrations of isotype-matched control MAb. In three of five experiments, a significant reduction in propagation was also noted in cultures incubated with ≤1 µg of Msp2 MAb/ml. The effect of

TABLE 1. Msp2 MAb 20B4 inhibits *A. phagocytophilum* growth in HL-60 cells over 4 or 5 days[a]

MAb concn (μg/ml)	% of control infected cells in:			Mean
	Expt 1	Expt 2	Expt 3	
10–20	66[b]	58[b]	64[b]	63
1–2	71[b]	104	76	84
0.1–0.2	86	97	80	88

[a] Results shown are mean percentages of infected cells in triplicate or quadruplicate cultures compared to those in cultures supplemented with isotype-matched control MAb (IgG2aκ). Similar results were obtained using Msp2 MAb 5B5 (data not shown).

[b] $P < 0.05$.

TABLE 2. Growth of *A. phagocytophilum* in HL-60 cells is reduced when the cells are supplemented with rMsp2[a]

Protein concn (μg/ml)	% of control infected cells after supplementation with:		
	rMsp2[b]	rAnkA[b]	BSA
5	25[c]	86	85
0.5	42[c]	114	103
0.005	61[c]	116	106

[a] Results shown are mean percentages of infected cells in triplicate or quadruplicate cultures compared to those in cultures supplemented with matched concentrations of control proteins.

[b] Purified recombinant N-terminal fusions with calmodulin binding protein.

[c] $P < 0.02$.

increasing MAb concentrations approached maximum inhibition at approximately 60% in HL-60 cells and 78% in neutrophils (Fig. 1).

Blocking of *A. phagocytophilum* propagation with rMsp2. To determine whether the effects of overnight and 4- to 5-day bacterial-propagation blocking with Msp2 MAb were due to direct interference with bacterial binding and invasion and not destruction after opsonization, the results were confirmed by antagonism of Msp2 binding with soluble rMsp2. Since no other recombinant surface proteins for *A. phagocytophilum* are available for controls, we used *A. phagocytophilum* rAnkA because this cytosolic protein is unlikely to be involved in adhesion; in addition, we used BSA as a nonspecific protein control. Although not of great magnitude, an rMsp2 dose-dependent antagonism of *A. phagocytophilum* propagation in HL-60 cells (Table 2) and antagonism of overnight binding and propagation in neutrophils (Fig. 2) was consistently observed, similar to the results of experiments conducted with blocking MAb. Overall, significant ($P < 0.02$) reductions in *A. phagocytophi-*

lum propagation were noted even in the presence of small quantities (50 ng/ml; Table 2) of rMsp2.

Msp2 MAb and rMsp2 block adhesion of *A. phagocytophilum* to HL-60 cells and neutrophils. To investigate whether *A. phagocytophilum* adheres to HL-60 cell and neutrophil surfaces via Msp2, experiments were conducted that assayed for binding and entry at 1 h prior to bacterial replication. Extracellular bacteria treated with Msp2 MAb 20B4 were significantly ($P < 0.003$) less likely to be bound to the surfaces of HL-60 cells than those treated with isotype-matched control MAb (Fig. 3), although the effect was less dramatic, but still significant ($P < 0.033$), with neutrophils and MAb 5B5. Likewise, incubation of HL-60 cells with ≥ 0.5 μg of rMsp2/ml significantly ($P < 0.02$) inhibited the binding of extracellular *A. phagocytophilum* compared with incubation with an identical concentration of rAnkA (Fig. 4) or BSA (data not shown) or no supplemental protein. To ensure that fluorescence quantitation resulted from bacterial adhesion and not binding of nonspecific fluorescent debris, the presence of bacteria and lack of fluorescent debris were empirically confirmed by fluorescence microscopy in all cultures (data not shown).

rMsp2 blocks binding of PSGL-1 MAb to HL-60 cells. If Msp2 binds directly to fucosylated PSGL-1 on the surfaces of
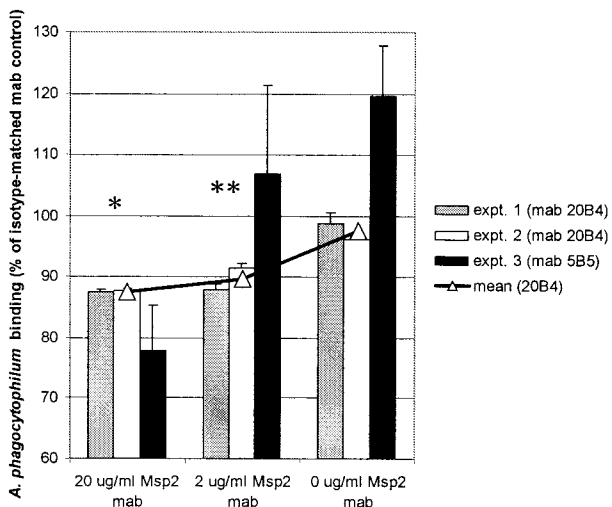

FIG. 1. MAb to Msp2 reduce binding to, entry into, and early propagation in neutrophils of fluorescence-labeled *A. phagocytophilum* in a dose-dependent manner after overnight incubation. Results are shown as proportions of overall fluorescence intensity bound when *A. phagocytophilum* was incubated with Msp2 MAb (mab) 20B4, 5B5, or no supplemental MAb compared to those seen with cultures with control IgG2aκ MAb. *, $P < 0.001$; **, $P < 0.001$ for MAb 20B4 only. Error bars represent standard errors of the means (SEM).
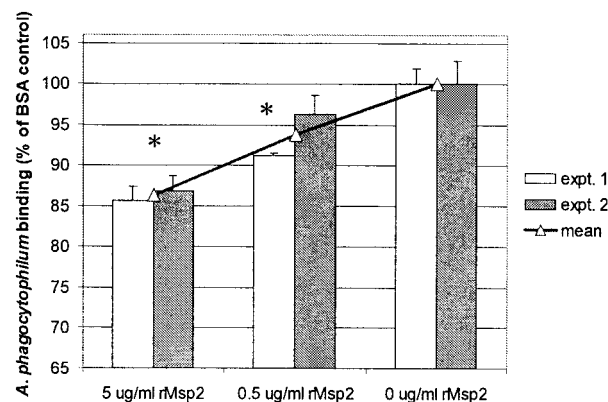

FIG. 2. rMsp2 reduces binding to, entry into, and early propagation in neutrophils of fluorescence-labeled *A. phagocytophilum* in a dose-dependent manner. Results are shown as proportions of overall fluorescence intensity bound when *A. phagocytophilum* was incubated with rMsp2 or control protein BSA compared to that seen with cultures without any supplemental proteins. *, $P < 0.007$. Error bars represent SEM.
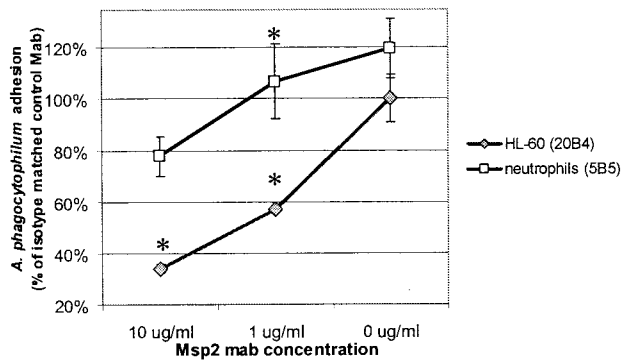
FIG. 3. Msp2 MAb reduces adhesion of fluorescence-labeled *A. phagocytophilum* to HL-60 cells and neutrophils in a dose-dependent manner after 1 h of incubation. (Top panel) Quantitative results are shown as proportions of overall fluorescence of Msp2 MAb-treated cultures compared with those of isotype-matched control MAb-treated cultures. *, $P < 0.003$. Error bars represent SEM. (Bottom panel) Binding of fluorescence-labeled *A. phagocytophilum* to HL-60 cells (a to c) and neutrophils (d to f) is partially inhibited in the presence of Msp2 MAb. (a and d) No fluorescent *A. phagocytophilum*; (b and e) fluorescent *A. phagocytophilum* only; (c and f) fluorescent *A. phagocytophilum* and 10 μg of Msp2 MAb 5B5/ml.
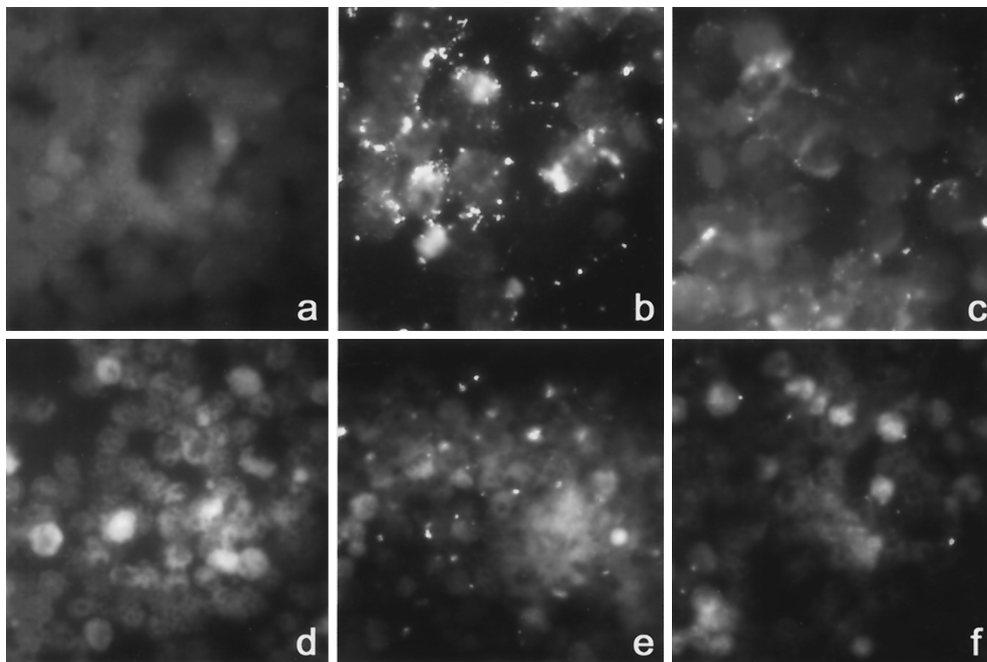
HL-60 cells and neutrophils, it would be predicted to block binding of MAb KPL-1 that is known to recognize an extracellular exposed PSGL-1 domain. In quantitative fluorometry experiments, a dose-dependent reduction of KPL-1 MAb binding to fucosylated PSGL-1 on the surfaces of HL-60 cells was demonstrated (Fig. 5). In contrast, no reduction in fluorescence was observed between HL-60 cells incubated with either rAnkA or BSA compared to the fluorescence observed in the absence of supplemental protein, suggesting that rMsp2 binds directly to fucosylated PSGL-1. The blocking of binding to fucosylated PSGL-1 by rMsp2 was particularly strong ($P < 0.03$) when high rMsp2 concentrations (3 μg/ml) were used. However, even 10-fold lower concentrations (0.3 μg/ml) yielded significant reductions in binding.

Extracellular *A. phagocytophilum* and rMsp2 bind to PSGL-1/FucT-VII-transfected BJAB cells. To confirm that *A. phagocytophilum* binds to host cells via fucosylated PSGL-1 and specifically by Msp2, an assay for binding of extracellular *A. phagocytophilum* to nontransfected BJAB cells not expressing PSGL-1 and PSGL-1/FucT-VII-transfected BJAB cells was used (19). Fucosylated PSGL-1 was confirmed to be expressed by PSGL-1/FucT-VII-transfected BJAB cells by immunoblot-
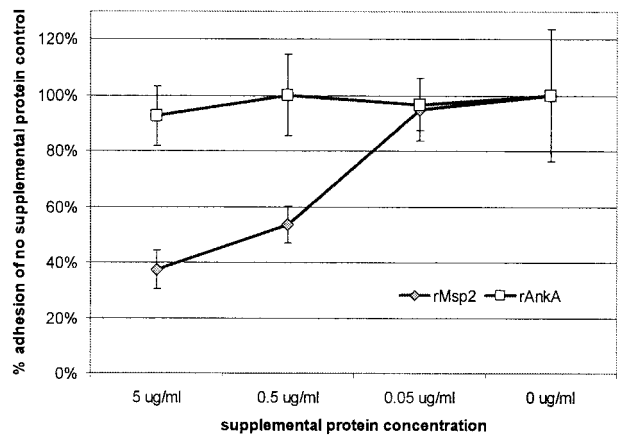

FIG. 4. rMsp2 inhibits adhesion of fluorescence-labeled *A. phagocytophilum* to HL-60 cells after 1 h of incubation. Results are shown as proportions of overall fluorescence of rMsp2-treated and rAnkA-treated cultures (*$P < 0.02$ between rMsp2 and rAnkA) compared with those of control cultures containing no supplemental protein. Results of one of three experiments with similar results are shown.
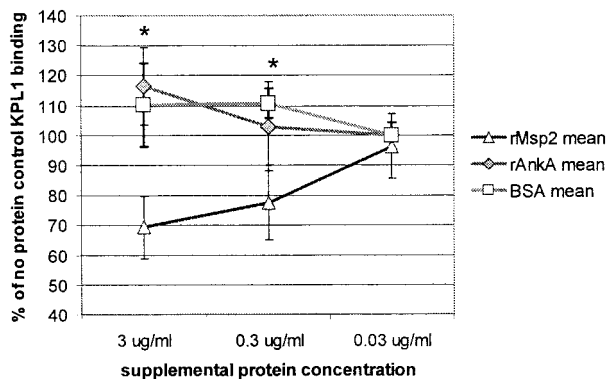
FIG. 5. rMsp2 blocks binding of PSGL-1 antibodies to HL-60 cells in a dose-dependent manner after overnight incubation. Results are shown as proportions of overall fluorescence observed in rMsp2-, rAnkA-, and BSA-treated cultures compared with those of cultures treated with no supplemental protein (* $P < 0.03$). Error bars represent SEM; the lowest concentration of rAnkA and BSA was 0 μg/ml.

ting under reducing conditions when reacted with MAb KPL-1 (data not shown); PSGL-1 was not detected in nontransfected BJAB cells. Moreover, transfected but not nontransfected cells can be infected with extracellular *A. phagocytophilum* in culture, as shown previously (19) (data not shown). Also, as demonstrated previously (19), after only 1 h of in vitro incubation extracellular bacteria bound to the surfaces of both untransfected and PSGL-1/FucT-VII-transfected BJAB cells but significantly ($P < 0.001$) more bound to the PSGL-1/FucT-VII-transfected cells for both concentrations of bacteria tested (Fig. 6). To determine whether Msp2 can mediate PSGL-1 binding directly, rMsp2 was incubated with BJAB and PSGL-
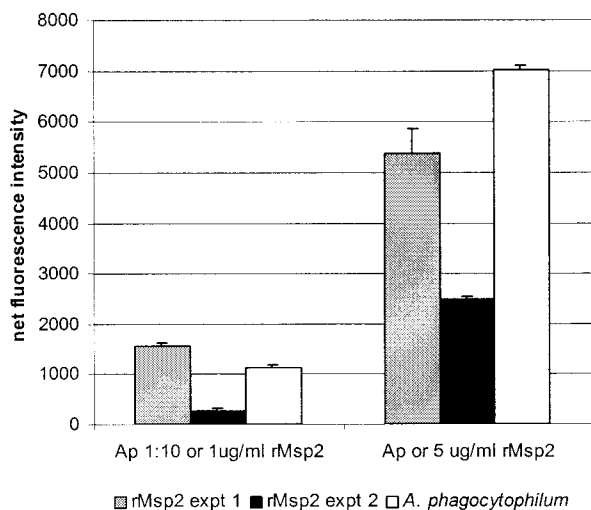

FIG. 6. Extracellular *A. phagocytophilum* and rMsp2 bind to PSGL-1/FucT-VII-transfected BJAB cells to a similar degree and in a dose-dependent relationship. Fluorescence intensity was normalized for nonspecific fluorescence (in cultures lacking *A. phagocytophilum* and rMsp2) and is shown as the quantity of fluorescence beyond that of similar manipulations using nontransformed BJAB cells as a control ($P < 0.007$). rMsp2 experiments were repeated four times with similar results; the results of two separate experiments are shown. Ap, extracellular *A. phagocytophilum* left undiluted or diluted 1:10. Error bars represent SEM.
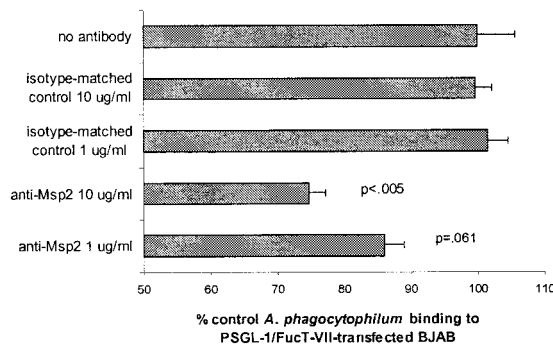

FIG. 7. Msp2 MAb incubation with fluorescence-labeled *A. phagocytophilum* blocks adhesion to PSGL-1/FucT-VII-transfected BJAB cells in a dose-dependent manner. Results are derived from two separate experiments and are shown as the mean percentages of fluorescence in Msp2 MAb- or isotype-matched control-treated *A. phagocytophilum* cultures versus that in cultures with no added antibody (defined as 100% adhesion). *P* values shown represent comparisons to the same concentration of isotype-matched control MAb. Error bars represent SEM.

1/FucT-VII-transfected BJAB cells. As with extracellular bacteria, rMsp2 bound to both cells, but binding to the surfaces of PSGL-1/FucT-VII-transfected BJAB cells was dose dependent and took place at significantly ($P < 0.003$) higher levels than binding to nontransfected BJAB cells incubated with control protein (Fig. 6).

Inhibition of *A. phagocytophilum* binding by Msp2 MAb and rMsp2. As shown with HL-60 cells and neutrophils, an antibody concentration-dependent reduction in *A. phagocytophilum* binding was observed in replicated experiments using PSGL-1/FucT-VII-transfected BJAB cells. Extracellular bacteria supplemented with 10 μg of MAb 20B4/ml showed a significant ($P < 0.05$) reduction of binding to PSGL-1/FucT-VII-transfected BJAB cells compared to cells supplemented with isotype-matched control MAb (Fig. 7). In cultures supplemented with 1 μg of MAb 20B4/ml, a slight reduction of *A. phagocytophilum* binding was also noted. In similarity to the results of these experiments and those using HL-60 cells and neutrophils, significant reductions in *A. phagocytophilum* binding to PSGL-1/FucT-VII-transfected BJAB cells were noted in the cultures incubated with 1 μg of rMsp2/ml ($P < 0.002$) and 5 μg of rMsp2/ml ($P < 0.001$) compared to those incubated with the same concentrations of control protein (Fig. 8).

DISCUSSION

Many infections that are initiated by the attachment of pathogens such as bacteria and viruses to host target cells often require the interaction of specialized surface adhesins (6, 7). For intracellular pathogens, the microorganisms adhere as a prerequisite to penetration of the host cell membranes prior to infection and disease. The adhesins of gram-negative and gram-positive bacteria, such as fimbrial adhesins in *E. coli* or afimbrial adhesins located on outer-surface membranes of *Helicobacter pylori*, are very well documented (16, 24, 28, 35). Although recent work has identified the cellular ligand by which *A. phagocytophilum* binds to granulocytes, the identity of the bacterial adhesin is not yet known. Major immunodomi-
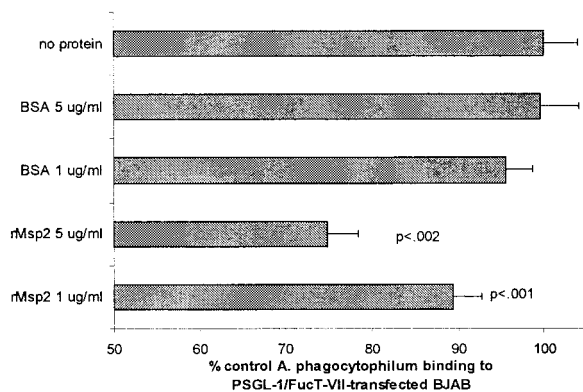
FIG. 8. rMsp2 blocks adhesion of fluorescence-labeled *A. phagocytophilum* to PSGL-1/FucT-VII-transfected BJAB cells. Results represent fluorescence intensity as percentages of cultures not incubated with rMsp2. The means and SEM of two separate experiments are shown. *P* values shown represent comparisons to the same concentration of BSA control protein.

nant outer-membrane proteins of various bacteria are good candidates for adhesins in many cases; thus, we hypothesized that this is also true for *A. phagocytophilum*.

Msp2 is the immunodominant protein on the surface of *A. phagocytophilum* (2, 12, 36). The structure of Msp2, beyond its molecular size (40 to 49 kDa) and predicted amino acid sequence and composition, is not known. The gene sequences of the multiple paralogs in the genome possess highly conserved regions that flank a hypervariable domain predicted to contain most T- and B-cell epitopes (8, 27, 37). Strong evidence exists to support a role for *A. marginale* Msp2 in bacterial antigenic variation and long-term persistence of infection in reservoir hosts (5, 13, 31).

This research provides several lines of evidence that together support the hypothesis that Msp2 is an adhesin for *A. phagocytophilum*. First, we observed a dose-dependent, moderate (ranging from 15 to 70%) inhibition of propagation and adhesion to cell surfaces of *A. phagocytophilum* when cultures were grown in HL-60 cells or neutrophils in the presence of anti-Msp2 MAb 20B4 and 5B5. Destruction of opsonized bacteria is an unlikely explanation for these results, since a similar dose-dependent inhibition of propagation and adhesion was demonstrated when rMsp2 was used as an antagonist. In addition, it could be argued that reduction of binding after incubation of cells with rMsp2 results from membrane patching or internalization. However, this is unlikely, given that rMsp2 is easily detected on the membranes of cells at intervals of time similar to those examined here. Second, rMsp2 was bound to BJAB cells transfected to express fucosylated (FucT-VII) PSGL-1, a known ligand of granulocytes for *A. phagocytophilum*, in a dose-dependent manner and to a degree similar to that of extracellular *A. phagocytophilum*. Moreover, Msp2 MAb and rMsp2 blocked or antagonized binding of extracellular *A. phagocytophilum* to the surfaces of PGSL-1/FucT-VII-transfected BJAB cells in a manner similar to that observed for HL-60 cells and neutrophils. Third, binding of MAb to PSGL-1 on the surfaces of HL-60 cells is inhibited by rMsp2 in a dose-dependent manner. Accordingly, these data together strongly support the hypothesis that *A. phagocytophilum* binds

to the surfaces of granulocytes via Msp2 with or without contributions of other bacterial components.

It is well recognized that *A. phagocytophilum* Msp2s, similar to those of *A. marginale*, are encoded by a multigene family of paralogs or paralogous pseudogenes that may differ in transcription and expression over time (8, 21, 37, 38). Whether all variants of *A. phagocytophilum* Msp2 are able to act as adhesins or whether variants encode specific cellular adhesins still needs to be investigated (38). In contrast, on the basis of the rationale that host receptors do not differ considerably, Abraham et al. contend that bacterial adhesins should also be highly conserved (1). The observation that propagation and adhesion of *A. phagocytophilum* were abolished by a single Msp2 MAb that reacts broadly with geographically diverse strains of *A. phagocytophilum* seems to be consistent with this requirement. These MAb may bind to conserved regions that flank (or to conserved motifs within) Msp2 hypervariable domains. However, it would be expected that propagation and/or adhesion should be completely inhibited by either Msp2 MAb or rMsp2, a prediction not entirely consistent with the moderate increments of inhibition observed here. Thus, these findings suggest several possibilities: (i) alternative Msp2 binding sites exist, potentially related to the hypervariable domains not recognized by the MAb; (ii) the adhesin is a complex of surface-exposed molecules that involve more than just Msp2, as is known for the related *A. marginale*; (iii) *A. phagocytophilum* Msp2 has the capacity to bind to other host ligands; (iv) a specific Msp2 conformation is maintained despite ongoing antigenic changes, a situation well recognized for the highly variable *env* gene products of human immunodeficiency virus that maintain conformation and binding to the CD4 receptor of T lymphocytes (26); and (v) *A. phagocytophilum* bacteria propagated in vitro are a heterogeneous population, members of which express a variety of different Msp2s that are ineffectively antagonized with the Msp2 MAb and the single rMsp2 used.

Blocking of adhesion and infection by antibodies and recombinant proteins does not exclude the possibility that the adhesin is a complex of physically associated proteins in the membrane of the bacterium. This situation is known to exist in the related *A. marginale*, in which a complex of at least six distinct major surface proteins (MSP-1a, MSP-1b, MSP-2, MSP-3, MSP-4, and MSP-5) are associated with the three potential adhesins, MSP1a, MSP1b, and MSP2 (32). Blocking of *A. phagocytophilum* adhesion could result from steric hindrance if antibody or recombinant protein antagonists bind in proximity to a separate receptor on Msp2 or in a complex analogous to that of *A. marginale*. Regardless of the mechanism, it is clear that rMsp2 alone also blocks binding of *A. phagocytophilum* and specific PSGL-1 MAb to the recognized host cell ligand PSGL-1, confirming the close association of PSGL-1 and Msp2 and lending further support to the hypothesis. Of interest is the finding by Nelson et al. that antiserum which blocks adhesion to HL-60 cells in vitro differentially identifies a 135-kDa antigen in an *A. phagocytophilum* genomic library, providing more evidence that a complex of proteins may comprise the adhesin of this organism (C.M. Nelson, J. Ahn, M. J. Herron, and J. L. Goodman, Abstract, *Bartonella* as an Emerging Pathogen Group 2001 Joint Conference, American Society of Rickettsiology, abstr. 54, 2001). Whether the actual binding occurs by blocking direct Msp2-fucosylated PSGL-1 interactions or by

blocking the interaction of PSGL-1 with other Msp2-associated proteins is not known but is an active area of investigation.

Although the adhesion-blocking MAb react broadly with many *A. phagocytophilum* strains, recent studies in our laboratory indicate that MAb 20B4 reacts with epitopes expressed in several, but not all, recombinant hypervariable domain peptides expressed from a set of 22 *msp2* paralogs identified in the *A. phagocytophilum* genome (unpublished data). This suggests that epitopes or conformation is conserved in the hypervariable domains, despite the changing expression of individual hypervariable Msp2s. The lack of consistent and robust inhibition of adhesion and propagation with Msp2 MAb and a single rMsp2 would be consistent with these data, since the *A. phagocytophilum* used in these experiments is not clonal and is probably heterogeneous with reference to Msp2 expression.

That alternative host cell receptors can be exploited by the bacterium is suggested from the work of Goodman et al. and Herron et al., who demonstrated the important role of sCD15 and α-(1,3) fucosyltransferase (FucT-VII)-modified PSGL-1 in the binding of *A. phagocytophilum* (15, 19). It was also noted that a specific cell line derived from HL-60 cells that was deficient in FucT-VII could become infected, albeit at a reduced rate. Thus, alternate *A. phagocytophilum* receptors must be present. The data presented here are consistent with that observation, since absolute elimination of infection was not achieved with any concentration of antibody or antagonist. It is possible that the loss of fucosylation or PSGL-1 expression in host cells selects for a subpopulation of *A. phagocytophilum* that expresses Msp2 variants capable of binding to different or modified host cell receptors.

A final possible consideration follows from the recognition that *A. phagocytophilum* demonstrates regulated *msp2* transcription in vivo (21, 38) and multiple simultaneous transcripts can be detected in vitro (8) that are continuously altered with in vitro passage (D. Scorpio, submitted for publication). Such a heterogeneous population of bacteria would be likely to contain at least minor populations unable to react with blocking antibodies or to compete for binding with rMsp2 and could also explain the lack of complete binding and propagation inhibition.

The *A. phagocytophilum* 44-kDa Msp2, like that of *A. marginale*, is involved in adhesion of the bacterium to the surface of its host cell. A number of unanswered questions still exist, including those involving the presence and identity of other potential membrane interactors that could be part of an adhesin complex and the issues of whether the natural diversity of Msp2 can contribute to continued host cell binding even with an aggressive anti-Msp2 immune response and whether any of this information might help in the design of appropriate strategies to improve control of *A. phagocytophilum* infections. One may speculate that a pool of antibodies or Msp2 antagonists might be highly effective in blocking the propagation of infection in vivo and that these data would predict failure for the use of a single gene or protein paralog construct. Likewise, the diversity of Msp2, the immunodominant antigen of *A. phagocytophilum*, might confound serologic diagnosis when a limited spectrum of recombinant proteins is used (22). Thus, this information can not only provide a better fundamental understanding of the biology of *A. phagocytophilum* but can also help the rational design of vaccines or reagents used for the serologic diagnosis of human granulocytic ehrlichiosis.

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