Anaplasma phagocytophilum Dense-Cored Organisms Mediate Cellular Adherence through Recognition of Human P-Selectin Glycoprotein Ligand 1[▽]

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Anaplasma phagocytophilum is an obligate intracellular bacterium that infects granulocytes to cause human granulocytic anaplasmosis. The susceptibilities of human neutrophils and promyelocytic HL-60 cells to *A. phagocytophilum* are linked to bacterial usage of P-selectin glycoprotein ligand 1 (PSGL-1) as a receptor for adhesion and entry. *A. phagocytophilum* undergoes a biphasic developmental cycle, transitioning between a smaller electron dense-cored cell (DC), which has a dense nucleoid, and a larger, pleomorphic electron lucent reticulate cell (RC), which has a dispersed nucleoid. The pathobiological roles of each form have not been elucidated. To ascertain the role of each form, we used electron microscopy to monitor bacterial binding, entry, and intracellular development within HL-60 cells. Only DCs were observed binding to and inducing uptake by HL-60 cells. By 12 h, internalized DCs had transitioned to RCs, which had initiated replication. By 24 h, large RC numbers were observed within individual inclusions. Reinfection had occurred by 36 h, as individual, vacuole-enclosed DCs and RCs were again observed. The abilities of DC- and RC-enriched *A. phagocytophilum* populations to bind and/or infect HL-60 cells or Chinese hamster ovary cells transfected to express PSGL-1 (PSGL-1 CHO) were compared. Only DCs bound PSGL-1 CHO cells and did so in a PSGL-1-blocking antibody-inhibitable manner. These results demonstrate that the respective roles of *A. phagocytophilum* DCs and RCs are consistent with analogous forms of other obligate intracellular pathogens that undergo biphasic development and hint that the PSGL-1-targeting adhesin(s) may be upregulated or optimally posttranslationally modified on DCs.

Anaplasma phagocytophilum is a member of the family *Anaplasmataceae* and an obligate intracellular bacterium that infects peripherally circulating neutrophils and bone marrow progenitors to cause the emerging and potentially fatal tick-transmitted disease human granulocytic anaplasmosis (HGA) (4, 11). Clinical manifestations of HGA range from subclinical or mild infection to severe or even fatal disease. Common symptoms include fever, malaise, myalgia, and headache. Laboratory findings consist of leukopenia, thrombocytopenia, and elevated levels of hepatic transaminases (4). The hallmark of HGA is the presence of intravacuolar colonies of *A. phagocytophilum*, termed morulae, within the cytosol of peripheral granulocytes.

Sialyl Lewis x (sLe^x)-modified P-selectin glycoprotein ligand 1 (PSGL-1) is the confirmed receptor utilized by *A. phagocytophilum* to bind and invade human neutrophils, bone marrow progenitors, and myeloid cell lines (12, 16). *A. phagocytophilum* binding to PSGL-1 requires cooperative recognition of the N-terminal primary amino acid sequence and the α 2,3-linked sialic acid and α 1,3-linked fucose of sLe^x (9, 44). This complex interaction is mediated either by multiple *A. phagocytophilum* adhesins or by a single adhesin having multiple binding domains (9, 44). The *A. phagocytophilum* adhesin(s) that medi-

ates attachment to human sLe^x-modified PSGL-1 has yet to be identified.

A. phagocytophilum undergoes a biphasic developmental cycle, transitioning between a smaller electron dense-cored cell (DC), which has a dense nucleoid, and a larger, pleomorphic electron lucent reticulate cell (RC), which has a dispersed nucleoid (14, 27–29, 31, 35, 41). The respective pathobiological role that each form plays has not been elucidated, but indirect insights into their potential roles are presented when the biphasic developmental cycles of other intravacuolar pathogens are considered. Biphasic development was first identified and is most well studied for *Chlamydia* spp., but it has also been observed for two *Anaplasmataceae* pathogens, *Anaplasma marginale* (26, 28) and *Ehrlichia chaffeensis* (31, 46), as well as *Coxiella burnetii*, the causative agent of Q fever (15, 25). Chlamydial elementary bodies (EB), which are analogous to DCs, are metabolically inert, environmentally resistant, and mediate attachment to and entry of eukaryotic host cells (2). Following attachment, EB are internalized via induced phagocytosis into a host cell-derived vacuole. EB remain within the pathogen-occupied vacuole, where they differentiate into noninfectious, metabolically active reticulate bodies (RB) and subsequently begin to divide by binary fission. Next, the progeny RB condense into EB and are released to reinitiate infection. Zhang and colleagues recently demonstrated that the *E. chaffeensis* DCs and RCs play analogous pathobiological roles to chlamydial EB and RB, respectively (46).

Obligate intracellular pathogen binding to host cells precedes entry and is therefore requisite for survival. An important observation that has been made for both the chlamydial

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EB and *E. chaffeensis* DC is that they specifically express outer membrane proteins that have been identified as adhesin candidates (32, 37, 42, 46), while the RC forms of each bacterium do not. Because *A. phagocytophilum* also transitions between the DC and RC, we hypothesized that adhesins targeting PSGL-1 are expressed on the DC. In this study, we monitored *A. phagocytophilum* development in human promyelocytic HL-60 cells over a 72-h time course following synchronous infection and tested bacterial populations enriched for DCs or RCs for their abilities to adhere to human PSGL-1 expressed on transfected Chinese hamster ovary (CHO) cell surfaces and to bind and infect HL-60 cells. Our results demonstrate that the *A. phagocytophilum* DC is the infectious form and that it mediates cellular adherence and specifically recognizes PSGL-1. These observations enhance our understanding of *A. phagocytophilum*-host cell interactions and represent a key step toward elucidating the PSGL-1-targeting adhesin(s).

MATERIALS AND METHODS

Cultivation of *A. phagocytophilum* and CHO cells. *A. phagocytophilum* strains HZ and NCH-1 were cultured in HL-60 cells (7). HZ was a gift from both Ralph Horwitz of New York Medical College (Valhalla, NY) and Yasuko Rikihisa of The Ohio State University (Columbus, OH). Untransfected CHO cells [CHO (–)] and transfected CHO cells coexpressing human PSGL-1, α 1,3-fucosyltransferase VII, and core 2 β 1,6-*N*-acetylglucosaminyltransferase (PSGL-1 CHO) were gifts from Rodger McEver (Oklahoma Medical Research Foundation) and were maintained as described previously (22).

Assessment of *A. phagocytophilum* binding to, invasion of, and intracellular development in HL-60 cells by electron microscopy. Host cell-free *A. phagocytophilum* organisms were recovered from twice the number of infected HL-60 cells and added to naïve HL-60 cells to initiate a synchronous infection. Aliquots (8.0×10^6 cells) were removed at 0.7, 4, 12, 24, 36, 48, and 72 h. The *A. phagocytophilum*-infected HL-60 cells were washed once in 0.1 N Sorenson phosphate buffer (SPB), pH 7.4, to remove any media and immediately fixed in 3.5% glutaraldehyde made in 0.1 N SPB for 1 h. Samples were subjected to four 15-min washes in 0.1 N SPB with 4% sucrose and stained with 1% OsO_4 in 0.1 N SPB for 1 h at 4°C. Samples were washed five times in 0.1 N SPB with 4% sucrose for 3 min each, followed by dehydration in a graded series of 50, 70, 80, 90, and 100% ethanol for 5 min each. Next, propylene oxide (PO) was used to wash each sample twice for 5 min each, after which a 1:1 mixture of PO and LX-112 resin mixture was added. Resin mixture consisted of dodecenyl succinic anhydride, nadic methyl anhydride, LX-112 resin, and 2,4,6-tri(dimethylamino-methyl) phenol (Ladd Research, Williston, VT). Samples were left overnight under a heat lamp. The next day, the PO–LX-112 resin mixture was removed and the samples were incubated in LX-112 resin mixture for 5 h under a heat lamp. Samples were centrifuged, the resin mixture was removed, fresh resin was added, and samples were allowed to harden at 60°C for 48 h. Samples were thin sectioned on a Reichert Jung Ultracut E microtome, poststained with uranyl acetate, and examined in a Tecnai BioTWIN 12 (Philips, Hillsboro, OR) or JEM-1230 (JEOL, Tokyo, Japan) transmission electron microscope.

Preparation of RC- and DC-enriched populations of *A. phagocytophilum*. RC- or DC-enriched populations of *A. phagocytophilum* organisms were prepared as follows. Bacteria were liberated from infected ($\geq 90\%$) HL-60 cells by syringe lysis, followed by differential centrifugation to separate bacteria from host cell debris (7). We empirically determined that an infection seeded from bacteria obtained from 10 times the number of infected cells that proceeds for 24 h yields a bacterial load that is comparable to an infection seeded from two times the number of infected cells that proceeds for 72 h. Accordingly, host cell-free bacteria obtained from 10 or 2 times the number of infected cells were added to uninfected cells to establish synchronous infections that proceeded for 24 or 72 h, respectively. To allow for bacterial adherence, *A. phagocytophilum* organisms were incubated with 2.0×10^6 HL-60 cells in 1 ml of Iscove's modified Dulbecco's Eagle medium (IMDM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (IMDM-10). Host cells were sedimented at $300 \times g$ for 5 min, washed twice with phosphate-buffered saline (PBS) to remove unbound bacteria, and incubated at 37°C in 5% CO_2 . To quantify the percentages of *A. phagocytophilum* host cell-free RC- and DC-enriched organisms at 24 and 72 h, respectively, the infected host cells were syringe lysed and the bacteria were recovered

by differential centrifugation and processed for electron microscopy as described above. Multiple sections for each sample were viewed, and several hundreds to thousands of individual RCs and DCs were counted.

Assessment of binding to and infection of HL-60 cells by RC- and DC-enriched populations of *A. phagocytophilum* using immunofluorescence microscopy and light microscopy. DC- or RC-enriched populations of *A. phagocytophilum* organisms were prepared as described above. At 24 or 72 h, RC- or DC-enriched *A. phagocytophilum* populations, respectively, were recovered from two times the number of infected cells and added to naïve HL-60 cells to establish a second synchronous infection, which was assessed for bacterial binding by immunofluorescence microscopy. Samples were monitored for infection by using light microscopic examination of samples that had been centrifuged onto glass slides using a Shandon Cytospin 4 cytocentrifuge (Thermo Scientific, Waltham, MA) at 24, 48, 72, and 96 h postinfection as described previously (7).

Measurement of PSGL-1 and sLe^x expression of CHO cells by flow cytometry. CHO cells were removed from adherent flasks with 0.5 mM EDTA (Irvine Scientific, Santa Ana, CA). Cells were treated with Fc block (5 $\mu\text{g}/\text{ml}$) (BD Pharmingen, San Diego, CA) for 5 min, followed by incubation with CSLEX (binds to sLe^x) (BD Pharmingen) (10), KPL1 (binds to the human PSGL-1 N terminus) (BD Pharmingen) (36), or murine immunoglobulin G1 (IgG1) or murine IgM isotype control antibodies (5 $\mu\text{g}/\text{ml}$). Following 30 min on ice, the cells were washed twice with PBS. Alexa Fluor 488-conjugated goat secondary antibody against either mouse IgM or IgG (Invitrogen) was added to a final concentration of 5 $\mu\text{g}/\text{ml}$. After 30 min on ice, the cells were washed with PBS, fixed with 1% paraformaldehyde, and analyzed on a BD Biosciences FACSscan using CellQuest Pro software (BD Pharmingen).

Assessment of *A. phagocytophilum* binding to CHO cells by immunofluorescence microscopy. PSGL-1 CHO or CHO (–) cells were grown on 12-mm-diameter round coverslips (Electron Microscopy Sciences, Hatfield, PA) in 24-well tissue culture plates (Greiner Bio-One, Monroe, NC) to near-confluent monolayers. The cells were washed twice with PBS. PSGL-1 CHO cells were pretreated with KPL1 or mouse IgG1 at 10 $\mu\text{g}/\text{ml}$ for 30 min on ice. Next, comparable amounts of RC-enriched (isolated 24 h post-synchronous infection as described above) or DC-enriched (isolated 72 h post-synchronous infection) *A. phagocytophilum* organisms were added at an approximate ratio, using host cell-free organisms liberated from twice the number of infected HL-60 cells as naïve CHO cells. Following a 30-min incubation at 25°C with rocking, the cells were washed three times with PBS to remove unbound organisms. Cells were fixed with methanol, and bound organisms were detected by immunofluorescence microscopy and enumerated (7).

Assessment of binding of DC- or RC-enriched *A. phagocytophilum* organisms to CHO cells using electron microscopy. Comparable amounts of DC- or RC-enriched host cell-free bacterial populations were liberated from 10 times the number of infected HL-60 cells and added to naïve CHO cells. Bacteria and CHO cells were incubated at a concentration of 2.0×10^6 CHO cells in 1 ml of PBS for 30 min at 25°C. The cells were centrifuged at $300 \times g$ and washed twice with PBS to remove unbound bacteria and processed for electron microscopy as described above.

Statistical analyses. Student's (paired) *t* test performed using the Prism 4.0 software package (GraphPad, San Diego, CA) was used to assess statistical significance. Statistical significance was set at a *P* value of <0.05 .

RESULTS

***A. phagocytophilum* binding to, invasion of, and replication within HL-60 cells.** To assess which *A. phagocytophilum* morphological form is responsible for binding and invasion of host myeloid cells and to monitor the kinetics of bacterial intracellular replication, differentiation between DC and RC, and reinfection, host cell-free *A. phagocytophilum* organisms were added to HL-60 cells. After removal of unbound bacteria, aliquots were processed at different time points over 72 h and observed by transmission electron microscopy. *A. phagocytophilum* DCs were $0.83 \pm 0.24 \mu\text{m}$ wide, had a dense nucleoid and a ruffled outer membrane, and were spheroid (Fig. 1A to H and 2). RCs were $0.69 \pm 0.36 \mu\text{m}$ by $1.04 \pm 0.44 \mu\text{m}$, had dispersed nucleoids and outer membranes that were somewhat smoother than those observed for DCs, and were pleomorphic. Representative electron micrographs depicting DCs and RCs

FIG. 1. *A. phagocytophilum* DCs and RCs. *A. phagocytophilum* organisms were cultivated in HL-60 cells and examined by transmission electron microscopy. Representative images of DCs (A to H) and RCs (I to P). (A to H) DCs were $0.83 \pm 0.24 \mu\text{m}$ wide, had a dense nucleoid and a ruffled outer membrane, and were spheroid. The arrow in panel A denotes an individual DC surrounded by a membranous projection within inclusions harboring other DCs (Fig. 1A). A similar observation was reported for *Ehrlichia* spp. (31). (I to P) RCs were $0.69 \pm 0.36 \mu\text{m}$ by $1.04 \pm 0.44 \mu\text{m}$, had a dispersed nucleoid and an outer membrane that was somewhat smoother than that observed for DCs, and were pleomorphic. Panels F and N are magnifications of the boxed areas in panels E and M, respectively. Bars, $0.5 \mu\text{m}$.

are presented in Fig. 1. We occasionally observed individual DCs surrounded by a membranous projection within inclusions harboring other DCs (Fig. 1A). A similar observation was reported for *Ehrlichia* spp. (31).

At 40 min and 4 h, only DCs were observed binding to, inducing their uptake by, or residing within host cells. At 40 min, 73.8% (214 of 290) of DCs were bound at the surfaces of HL-60 cells or inducing their own uptake by HL-60 cells, while only 26.2% (76 of 290) had internalized into host cell-derived vacuoles (Fig. 2A to C). By 4 h, 45.5% (30 of 55) had internalized (Fig. 2E and F). At 40 min and 4 h, internalized DCs

were tightly associated with the luminal faces of their host cell-derived vacuoles, which were barely larger than the DCs themselves (Fig. 2C, E, and F).

At 12 h, DCs were no longer associated with host cell surfaces (Fig. 3A). At this time point, the only DCs that were observed were individual bacteria harbored within tightly associated vacuoles comparable to those observed at 4 h (data not shown). The majority of pathogen-occupied vacuoles harbored one to a few RCs and were more spacious than those harboring DCs. Thus, bacterial replication had initiated and was concomitant with enlargement of the *A. phagocytophilum*-

FIG. 2. *A. phagocytophilum* adhesion to, invasion of, and replication within HL-60 cells. Host cell-free *A. phagocytophilum* organisms were incubated with HL-60 cells. After 40 min, unbound bacteria were removed and host cells were examined by transmission electron microscopy. (A to C) At 40 min, DC organisms were observed binding to and triggering their own uptake by HL-60 cells. (D to F) At 4 h, some DC organisms remained bound at the HL-60 cell surface, while others had been internalized into vacuoles that were in close proximity to the host cell surface. No RC organisms were observed interacting with HL-60 cell surfaces. Bars, 0.5 μ m. Representative results of one of two separate experiments are shown.

occupied vacuole. By 24 h, a considerable degree of replication had occurred, as evidenced by large vacuoles containing numerous RCs that were more elongated than the RCs observed at 12 h (Fig. 3B). Some vacuoles contained *A. phagocytophilum* organisms that had begun to condense to DCs (Fig. 3B). By 36 h, multiple inclusions harboring individual DCs or RCs that were tightly associated with the luminal walls of their vacuoles were once again observed (Fig. 3C and D), which reflects the occurrence of reinfection by *A. phagocytophilum* organisms that had been released between 24 and 36 h. This premise is further supported by immunofluorescent detection of extracellular and HL-60 surface-bound *A. phagocytophilum* organisms at all post-24-h time points (data not shown). Also at 36 h, individual host cells contained mature and nascent *A. phagocytophilum* inclusions (Fig. 3D). Consequently, the infection of the HL-60 culture proceeded asynchronously for the remainder of the time course. This was apparent in cells observed at 48 h and 72 h, at which time the host cells were heavily infected with multiple inclusions that harbored numerous DCs or RCs (Fig. 3E and F). Also observed were inclusions that contained bacteria that were transitioning from RCs to DCs (Fig. 3E). At no time point was fusion of morulae observed.

Not all *A. phagocytophilum* organisms that initially bound to HL-60 cell surfaces remained attached or successfully entered

host cells. At 40 min, 62% of the HL-60 cells had attached *A. phagocytophilum* organisms (data not shown). By 4 h, the percentage of HL-60 cells with bound bacteria had dropped to 29%. By 24 h, approximately 25% of the cells were infected, which suggests that those organisms that had remained bound at 4 h successfully invaded.

Quantification of the numbers of *A. phagocytophilum* organisms observed per morula and the relative abundance of DC and RC observed over the course of infection. To better characterize the developmental cycle over the course of infection, the numbers of *A. phagocytophilum* DCs or RCs per morula were counted for each time point. The greatest diversity in the number of bacteria per inclusion was observed at 24 h (Fig. 4A). At 36 h, the largest numbers of vacuoles containing individual DCs or RCs or two RCs were observed, which signified that reinfection had occurred and the newly internalized bacteria were transitioning or had recently transitioned into RCs (Fig. 3C and D and 4A). As a prerequisite step for isolating *A. phagocytophilum* populations enriched for DCs or RCs and assessing their differential cellular adherence and recognition of PSGL-1, we determined the optimal time points for recovering such bacterial populations by counting the total number of DCs or RCs from all electron micrographs generated from the time course analysis. It is important to keep in mind that

FIG. 3. Intracellular development of *A. phagocytophilum* in HL-60 cells. Host cell-free *A. phagocytophilum* organisms were incubated with HL-60 cells. After 40 min, unbound bacteria were removed and host cells were examined at 12 (A), 24 (B), 36 (C and D), 48 (E), and 72 h (F) postinfection by transmission electron microscopy. Arrowheads in panels B and E indicate morulae harboring *A. phagocytophilum* organisms that are transitioning from RCs to DCs. Arrows in panels D and F denote inclusions that contain DCs. Bars, 1.0 μ m. Representative results of one of two separate experiments are shown.

the true numbers of DCs or RCs are underrepresented by this method, as they were determined from two-dimensional cross-sections of infected cells. The greatest relative abundance of RCs with a minimal amount of DCs prior to reinfection occurred at 24 h (Fig. 4B). Though RCs outnumbered DCs at all postreplication time points, the greatest number of DCs was observed at 72 h.

DC-enriched *A. phagocytophilum* organisms bind and infect HL-60 cells more effectively than RC-enriched bacteria. Our electron microscopy time course studies revealed that DC, but not RC, organisms bound and were internalized into HL-60 cells (Fig. 2 to 4). We therefore rationalized that the *A. phagocytophilum* DC is more effective than the RC at adhering to and invading host cells. To assess this, we isolated DC- and RC-enriched *A. phagocytophilum* populations from infected HL-60 cells. Following a synchronous infection, host cell-free *A. phagocytophilum* populations were recovered at 24 and 72 h, visualized by electron microscopy, and enumerated. *A. phagocytophilum* populations isolated at 24 h consisted of $70.1\% \pm 3.4\%$ RCs and $29.9\% \pm 3.4\%$ DCs (Table 1). At 72 h, 53% more DC organisms were present, as host cell-free bacterial populations contained $45.8\% \pm 14.7\%$ DCs. Accordingly, *A. phagocytophilum* populations recovered from infected HL-60 cells at 24 h and 72 h are denoted below as RC- and DC-enriched populations, respectively. Next, comparable amounts

of DC- and RC-enriched *A. phagocytophilum* organisms were isolated and incubated with naïve HL-60 cells. After the removal of unbound organisms, the numbers of adhered bacteria or morulae per cell were determined. Significantly more DC-enriched organisms bound to HL-60 cells than RC-enriched bacteria (Fig. 5A). Likewise, significantly more morulae were observed at 72 and 96 h postinfection within HL-60 cells that had been incubated with DC-enriched bacteria than cells that had been incubated with RC-enriched organisms (Fig. 5B).

***A. phagocytophilum* DC-enriched organisms more effectively bind PSGL-1 CHO cells than RC-enriched organisms.** Because significantly more binding to and infection of HL-60 cells were observed for DC-enriched *A. phagocytophilum* organisms than RC-enriched bacteria and because susceptibility of human neutrophils and HL-60 cells to *A. phagocytophilum* binding and infection is directly attributable to surface expression of PSGL-1 (12, 16, 33), we next compared the abilities of comparable amounts of DC- and RC-enriched organisms to bind to the surfaces of PSGL-1 CHO cells (22, 43). In addition to PSGL-1, these cells coexpress $\alpha 1,3$ -fucosyltransferase VII and core 2 $\beta 1,6$ -N-acetylglucosaminyltransferase, which are required to glycosylate PSGL-1 such that it is decorated with sLe^x, binds to P-selectin, and supports *A. phagocytophilum* binding (9, 16, 22, 44). Flow cytometry analyses confirmed that PSGL-1 CHO cells express PSGL-1 and sLe^x on their cell

FIG. 4. Number of *A. phagocytophilum* (Ap) organisms observed per morula and the relative abundances of DCs and RCs observed over the course of infection. Values presented were determined by enumerating the numbers of *A. phagocytophilum* organisms within individual morulae observed by transmission electron microscopy (A) and the total numbers of RCs and DCs in Fig. 2 and 3 (B). (A) Numbers of organisms observed per morula between 12 and 72 h postinfection. Morulae containing 1 to 2, 3 to 5, 6 to 10, 11 to 15, and ≥ 16 organisms were enumerated and are presented as percentages of the total number of morulae. (B) Total numbers of DCs and RCs observed binding to or internalized within HL-60 cells. Representative results of one of two separate experiments are shown.

surfaces (Fig. 6A and B). Significantly more DC-enriched *A. phagocytophilum* organisms than RC-enriched bacteria bound to PSGL-1 CHO cells in the presence of isotype control antibody (Fig. 7). Monoclonal antibody (MAb) KPL1, which in-

FIG. 5. Binding and infection of RC- and DC-enriched *A. phagocytophilum* (Ap) organisms in HL-60 cells. Comparable amounts of host cell-free *A. phagocytophilum* organisms were isolated at 24 h (RC-enriched) or 72 h (DC-enriched) following a synchronous infection and incubated with naïve HL-60 cells for 40 min. (A) After removal of unbound bacteria, aliquots were examined by indirect immunofluorescence microscopy and the mean numbers of bound *A. phagocytophilum* organisms per cell were enumerated. (B) The remainders of each infected HL-60 culture were grown for 96 h, during which aliquots were removed at 24-h intervals and assessed for infection, as determined by light microscopic detection of morulae. Results shown are the means of four separate experiments. Statistically significant values are indicated (*, $P < 0.05$).

terferes with *A. phagocytophilum* binding by blocking access to the PSGL-1 N-terminal peptide (16, 33, 34, 36), reduced binding by DC- and RC-enriched populations to PSGL-1 CHO cells to the background binding levels observed for CHO (–) cells.

TABLE 1. Quantification of *A. phagocytophilum* RCs and DCs

Experiment no.	<i>A. phagocytophilum</i> organisms isolated at ^a :					
	24 h			72 h		
	Total no. of organisms	% RCs (70.1 $\pm$ 3.4)	% DCs (29.9 $\pm$ 3.4)	Total no. of organisms	% RCs (54.2 $\pm$ 14.7)	% DCs (45.8 $\pm$ 14.7)
1	630	66.2	33.8	837	45.0	55.0
2	1,036	71.6	28.4	1,348	45.6	53.5
3	518	72.6	27.4	2,506	71.1	28.9

^a Mean values are shown in parentheses.

FIG. 6. Analysis of PSGL-1 CHO and CHO (–) cells for PSGL-1 and sLe^x expression. PSGL-1 CHO and CHO (–) cells were incubated with anti-PSGL-1 MAb KPL-1 (A), anti-sLe^x MAb CSLEX1 (B), or isotype-matched controls. Alexa Fluor 488-conjugated secondary antibodies detected bound primary antibodies. Representative results of one of two separate experiments are shown.

Confirmation that the *A. phagocytophilum* DC specifically recognizes PSGL-1. Because DC-enriched organisms bound most effectively to HL-60 cells and PSGL-1 CHO cells relative to RC-enriched bacteria (Fig. 5 and 7), we hypothesized that the *A. phagocytophilum* DC specifically recognizes PSGL-1 while the RC does not. Because RC-enriched bacteria more effectively bound to PSGL-1 CHO cells than to CHO (–) cells in the presence of isotype control antibody (Fig. 7), we further hypothesized that the contaminating DC in the RC-enriched population mediated PSGL-1 recognition. To verify this, PSGL-1 CHO cells were incubated with comparable amounts of DC- or RC-enriched *A. phagocytophilum* populations and visualized by transmission electron microscopy. For both populations, $\geq 91.5\% \pm 3.5\%$ of the *A. phagocytophilum* organisms that bound to the surfaces of PSGL-1 CHO cells were the DC form (Fig. 8A, B, and D). The few non-DC forms attached to PSGL-1 CHO surfaces did not resemble RCs. Instead, they resembled either bacterial membrane ghosts (Fig. 8C) or what appeared to be transitional forms that were intermediate in size relative to DCs and RCs and exhibited outer membranes that were more ruffled than the RC, but not as ruffled as the DC (Fig. 8E). These results support that the DC is the *A. phagocytophilum* morphological form that mediates binding to PSGL-1 and indicate that late-stage transition organisms are also capable of binding PSGL-1.

FIG. 7. Binding of RC- and DC-enriched *A. phagocytophilum* (Ap) organisms to PSGL-1 CHO and CHO (–) cells. Comparable amounts of host cell-free *A. phagocytophilum* organisms isolated at 24 h (RC-enriched) and 72 h (DC-enriched) post-synchronous infection were incubated with PSGL-1 CHO cells in the presence of anti-PSGL-1 MAb KPL-1 or isotype control or with CHO (–) cells. After removal of unbound bacteria, *A. phagocytophilum* binding was assessed by indirect immunofluorescence microscopy. Representative results of one of three separate experiments are shown. Statistically significant values are indicated (**, $P < 0.01$; ***, $P < 0.001$).

DISCUSSION

Our data demonstrate that the *A. phagocytophilum* DC mediates cellular adhesion, recognizes human PSGL-1, and invades human myeloid cells, while the RC does not. In the hundreds of transmission electron micrographs of HL-60 or PSGL-1 CHO cells with bound or newly internalized *A. phagocytophilum* examined, DCs were almost always observed interacting with the host cell surfaces or within nascent inclusions. We never observed RCs attached to host cell surfaces or within nascent vacuoles. Similar observations have been reported for *A. phagocytophilum* and *E. chaffeensis* interactions with ISE6 tick embryonic cells and DH82 histiocytoma cells, respectively (27, 46). In rare instances, we observed membrane ghosts or what appeared to be late-transition stage organisms exhibiting characteristics of both DC and RC binding to host cell surfaces. The latter observation is not surprising given that *A. phagocytophilum* differentiation is a continual process and syringe lysis of infected cells would conceivably release DC, RC, and transition stage organisms for subsequent incubation with host cells. The abilities of DC and transition stage organisms and the inability of RC to bind to human PSGL-1 presented on eukaryotic cell surfaces hints that expression and/or posttranslational modification of the adhesin(s) requisite for PSGL-1 recognition occurs during *A. phagocytophilum* differentiation from RC to DC. This expression pattern is analogous to that for putative adhesins of other obligate vacuolar pathogens that exhibit biphasic development, such as *E. chaffeensis*, *C. trachomatis*, and *C. pneumoniae* (32, 37, 38, 42, 46).

The percentage of HL-60 cells with bound *A. phagocytophilum* organisms declined from 62% at 40 min to 29% at 4 h, which suggests that not all *A. phagocytophilum* DC or late transition stage organisms that initially bind to the host cell surfaces are capable of remaining bound. Also, *A. phagocytophilum* entry into human myeloid cells in vitro is variable and

FIG. 8. *A. phagocytophilum* (Ap) DCs, but not RCs, bind to PSGL-1 CHO cells. Comparable amounts of host cell-free DC-enriched or RC-enriched populations were incubated with PSGL-1 CHO cells. After removal of unbound bacteria, the PSGL-1 CHO cells were examined by transmission electron microscopy. (A) Percentage of bound *A. phagocytophilum* organisms that are in either the DC stage or other (membrane ghosts or transition) stages that were observed bound to the surfaces of PSGL-1 CHO cells. One hundred PSGL-1 CHO cell-associated *A. phagocytophilum* organisms were counted per population per experiment. Results shown are the means of two separate experiments. Statistically significant values are indicated (**, $P < 0.01$; ***, $P < 0.001$). (B to E) Representative transmission electron micrographs showing a DC (B and D), a membrane ghost (C), or a putative transition stage organism (E) interacting with the PSGL-1 CHO cell surface.

can take hours. Indeed, we observed that only 26.2% of DCs had entered HL-60 cells by 40 min, while 73.8% remained surface bound. By 4 h, 45.5% of the DCs had entered. Using fluoresceinated bacteria, we and others have previously shown

that it takes up to 4 h for an appreciable number of bound *A. phagocytophilum* organisms to invade HL-60 cells or neutrophils (5, 8, 19). We surmise that inefficient binding and/or invasion is related to suboptimal expression or posttranslational modification of adhesins or other virulence factors that are requisite for adhesion and/or entry by bacteria that have not yet fully differentiated into infectious DC. AnkA, a type IV secretion system effector, is delivered into HL-60 cells within minutes following *A. phagocytophilum* binding (18, 23). We observed newly internalized DCs remaining tightly associated with the inner face of the inclusion membrane during initial postinvasion hours. This may allow *A. phagocytophilum* to continue to deliver AnkA and/or other type IV effectors into the host cell cytosol following entry.

HL-60 cells are nonphagocytic (6, 30), yet we observed HL-60 plasma membrane extensions engulfing bound organisms, which indicates that cytoskeletal rearrangement is evoked upon *A. phagocytophilum* binding, presumably to PSGL-1. Our visualization of this process is in agreement with published electron micrographs depicting *A. phagocytophilum* invasion of ISE6 cells and *E. chaffeensis* invasion of DH82 cells (27, 46) but contradicts the supposition that the bacterium enters via receptor-mediated endocytosis, not phagocytosis (24, 45). PSGL-1 is a signaling molecule in leukocytes (40). *A. phagocytophilum* engagement of PSGL-1 activates the tyrosine kinase, Syk, a step that is essential for PSGL-1-dependent entry (34, 39, 40). PSGL-1 is present and interacts with Syk in lipid rafts at the myeloid cell surface (1). PSGL-1 association with Syk is mediated through ezrin and moesin (3, 40), which also function as membrane-actin cytoskeleton linkers and play roles in the formation of protrusive membrane structures such as filopodia and membrane ruffles (20). Thus, we propose that *A. phagocytophilum* binding of PSGL-1 promotes actin cytoskeletal rearrangement through Syk-ezrin/moesin-mediated signaling, which culminates in induced phagocytic—rather than endocytic—bacterial uptake. Further support for our premise comes from observations that treatment with methyl- β -cyclodextrin, which disrupts lipid rafts by chelating cholesterol (13), prevents Syk recruitment to interact with PSGL-1 (1) and prevents *A. phagocytophilum* entry (24).

Our data demonstrate that *A. phagocytophilum* RCs are the replicative form. At 4 h, individual DCs were observed within nascent inclusions. By 12 h, individual or multiple RCs were present within inclusions, thereby signifying that replication had initiated. The *A. phagocytophilum* genome codes for *ftsZ* (APH_1292) (21), which suggests that replication occurs by binary fission. By 24 h, the number of RCs within morulae had increased considerably. During this burst of replication, the morula membrane increases in size, which partially accommodates the large numbers of RCs it harbors. However, intravacuolar space does become limited. Indeed, RCs within mature inclusions harboring large bacterial numbers are more elongated than the individual or few spheroid RCs within nascent vacuoles, perhaps as a means of conforming to the available intravacuolar space.

A. phagocytophilum development within HL-60 cells is asynchronous. By 24 h, condensation of RC into DC has initiated within some, but not all, morulae. After replication to a considerable number of RCs within individual morulae, *A. phagocytophilum* organisms condense into DCs and are subsequently

released in a timely manner to initiate new infections. This premise is supported by observations that multiple vacuoles harboring individual organisms were once again observed at 36 h and that RCs outnumbered DCs at all time points after RCs were first observed. Whether these developmental kinetics are reminiscent of the development within neutrophils and whether *A. phagocytophilum* DCs are released from host cells in a lytic or nonlytic fashion remain to be confirmed.

A. phagocytophilum development within individual morulae within HL-60 cells is highly synchronous, an observation that is consistent with that of *A. phagocytophilum* development within ISE6 cells and *E. chaffeensis* development within DH82 cells (27, 46). Interestingly, the chlamydial developmental cycle within inclusions is asynchronous (2). Thus, these two obligate intravacuolar bacterial pathogens, each of which undergoes biphasic development, are prompted by distinct signals to transition from DC (or EB) to RC (or RB). It has been proposed that chlamydial RB detachment from the vacuolar face of the inclusion membrane caused by overcrowding within the inclusion provides the signal for RB to condense to EB (17). Such a model does not apply to *A. phagocytophilum*. Rather, the synchronous development of *A. phagocytophilum* within inclusions suggests that the entire intravacuolar population simultaneously receives signals that prompt differentiation. Also in contrast to what has been observed for *Chlamydia* spp. (2) is that we never observed fusion between morulae. Even as HL-60 cells became heavily infected, morulae in direct contact with each other remained distinct. This is in agreement with our previous observation that independent morulae containing transgenic *A. phagocytophilum* organisms expressing either green fluorescent protein or mCherry within the same HL-60 cell do not fuse (34).

A. phagocytophilum organisms isolated at 72 h bind more effectively to and yield more productive infections of HL-60 cells than bacteria isolated at 24 h. This is due to the presence of more adherence-competent and infectious DC organisms being present at 72 h than at 24 h. Nevertheless, the mean standard deviations of bound organisms per cell and the percentages of infected HL-60 cells were considerably greater for DC-enriched populations (Fig. 5). We attribute this to the fact that at 24 h, the relative amounts of RCs and DCs were consistent for each of the three *A. phagocytophilum* populations isolated and used as inocula, while the RC-to-DC ratio varied among the three inoculum populations isolated at 72 h (Table 1). The variability at 72 h is a result of asynchronous development occurring later in infection. The marginal binding of *A. phagocytophilum* organisms of RC-enriched populations to HL-60 and PSGL-1 CHO cells is due to the contaminating DC, a premise that is supported by the fact that electron microscopy detected only DC binding to PSGL-1 CHO cells following incubation with RC-enriched populations.

Neoexpression of fucosylated PSGL-1 in CHO cells is an effective model for studying *A. phagocytophilum* interactions with PSGL-1. Intriguingly, we never observed morulae within PSGL-1 CHO cells even at 24 h postinfection (data not shown). This is in striking contrast to the ability of the bacterium to not only bind but also to enter and replicate within B lymphoblastoid BJAB cells transfected to express fucosylated PSGL-1 (16) and hints that certain host cell factors and/or

signaling platforms may be required to optimally support *A. phagocytophilum*.

In closing, the respective roles of the *A. phagocytophilum* DCs and RCs are adherence/infection and vacuolar replication, an observation that is consistent with the life cycles of other obligate intravacuolar pathogens that undergo biphasic development. Moreover, the *A. phagocytophilum* DC is responsible for recognizing PSGL-1, the dominant receptor for infection of human myeloid cells. Future characterization of the DC surface proteome will be vital to identifying the adhesin(s) that mediate PSGL-1 recognition. Deciphering the adherence mechanism by which *A. phagocytophilum* engages PSGL-1 as a prerequisite for host cell entry will characterize a vital step in *A. phagocytophilum* pathogenesis and potentially lead to the development of novel, nonantibiotic-based approaches for treating or preventing HGA.

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