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APPROACHES TO IDENTIFY SURFACE PROTEINS OF ANAPLASMA PHAGOCYTOPHILUM DENSE-CORED ORGANISMS AS ADHESINS TO HUMAN P-SELECTIN GLYCOPROTEIN LIGAND-1

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A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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In closing I dedicate this dissertation to:

the TROESE family

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List of Abbreviations

Å	angstrom
Ab	antibody
ABC	ammonium bicarbonate
ACN	acetonitrile
Ap	*Anaplasma phagocytophilum*
A. phagocytophilum	*Anaplasma phagocytophilum*
ApVar-1	*A. phagocytophilum*-Variant 1
Asp55	*Anaplasma* surface protein of 55-kDa
Asp62	*Anaplasma* surface protein of 62-kDa
BLAST	basic local alignment search tool
BME	β-mercaptoethanol
BSA	bovine serum albumin
CELLO	subCELlular LOcalization predictor program
CHO	chinese hamster ovary
CO_2	carbon dioxide
DAPI	4',6-diamidino-2-phenylindole
DC	dense-cored cell of *A. phagocytophilum*
DNA	deoxyribonucleic acid
DTSSP	dithiobis(sulfosuccinimidylpropionate) crosslinker
DTT	dithiothreitol
EB	elementary bodies of *Chlamydia*
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
g	relative centrifugal force (RCF), or g-force
h	hour
HGA	human granulocytic anaplasmosis
HGE	human granulocytic ehrlichiosis
HRP	horseradish peroxidase
HVR	hypervariable region
Ig	immunoglobulin
IL	interleukin
IMDM	iscove's modified dulbecco's eagle medium
IMDM-10	iscove's modified dulbecco's eagle medium with 10% FBS
iNOS	inducible nitric oxide

IP	immunoprecipitation
IFA	indirect immunofluorescence assay
IFNγ	interferon-γ (gamma)
KCl	potassium chloride
kDa	kilodalton
LAMP-1	lysosomal-associated membrane protein 1
MAb	monoclonal antibody
Mb	megabases
$MgCl_2$	magnesium chloride
min	minute
ml	milliliter
mM	millimolar
MS	mass spectrometry
Msp2(P44)	major surface protein 2 (44-kDa protein)
MudPIT	multi-dimensional protein identification technology
MyD88	myeloid differentiation factor 88
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
NaF	sodium fluoride
Na_3VO_4	sodium orthovanadate
NCBI	national center for biotechnology information
ng	nanogram
NHS	n-hydroxysuccinimide
OMP	outer membrane protein
O/N	overnight
Opa	opacity-associated proteins of *Neisseria*
O_2^-	superoxide
1D	one-dimensional
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PHOX	phagocyte NADPH oxidase
PMSF	phenylmethylsulfonyl fluoride
PO	propylene oxide
Pos.	positive
PSGL-1	p-selectin glycoprotein ligand-1
RB	reticulate bodies of *Chlamydia*

RC	reticulate cell of *A. phagocytophilum*
rhPSGL-1	recombinant human p-selectin glycoprotein ligand-1
RIPA	radioimmunoprecipitation assay
RT	room temperature
SCV	small cell-variant of *Coxiella*
SCID	severe combined immunodeficiency
SDS	sodium dodecyl sulfate
sec	seconds
sLe^x	sialyl Lewis x
SPB	sorenson phosphate buffer
SPG	sucrose-phosphate-glutamate
Sulfo-SDAD	sulfo-NHS-SS-Diazirine crosslinker
TBS-t (0.5)	tris-buffered saline with 5% tween-20
TEM	transmission electron microscopy
T4SS	type IV secretion system
TIGR	the institute for genomic research
TLR	toll-like receptor
µg	microgram
µl	microliter
°C	degrees Celsius

Abstract

APPROACHES TO IDENTIFY SURFACE PROTEINS OF *ANAPLASMA PHAGOCYTOPHILUM* DENSE-CORED ORGANISMS AS ADHESINS TO HUMAN P-SELECTIN GLYCOPROTEIN LIGAND-1

By Matthew James Troese

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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Anaplasma phagocytophilum is an obligatory intracellular bacterium that infects neutrophils to cause human granulocytic anaplasmosis. 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 and myeloid cell lines. As an obligate intracellular pathogen, the binding of *A. phagocytophilum* to a host cell receptor is a prerequisite step for entry and replication, and thus its survival. However, the bacterial adhesins mediating this process have yet to be identified. In this study, we sought to

identify surface proteins of *A. phagocytophilum* as putative adhesins. *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. We determined that the respective roles of the *A. phagocytophilum* DCs and RCs are adherence/infection and vacuolar replication, respectively, which is a finding that is consistent with the life cycles of other obligate intravacuolar pathogens that undergo biphasic development. Most importantly, we demonstrated the *A. phagocytophilum* DC is responsible for recognizing human PSGL-1. To identify surface proteins as putative adhesins we tested a variety of approaches. Three different computer prediction programs were compared, resulting in identification of 16 to 130 potential membrane proteins. As a more direct means to identify *A. phagocytophilum* surface proteins as PSGL-1 adhesins, several affinity capture approaches were tested. We used commercially available recombinant human PSGL-1 (rhPSGL-1) to try and capture adhesins by crosslinking and affinity purification. We were unsuccessful, but nevertheless gained insight into the binding properties of *A. phagocytophilum*. We next chose to take a broader approach to identify outer membrane proteins of the adherent DC by biotinylation. In the process we developed new density-gradient centrifugation approaches which successfully purified an RC-enriched population as well as a mixed population of RC and DC organisms. Results from this work demonstrate that *A. phagocytophilum* DC organisms are responsible for binding PSGL-1. Additionally, the results obtained thus far of gradient-purified bacteria will serve as a foundation for future experiments in identifying surface and developmental form specific proteins.

CHAPTER 1: Introduction

History and Ecology of *Anaplasma phagocytophilum*

The family *Anaplasmataceae* consists of obligate intracellular bacterial pathogens that are distinctive in their ability to replicate in vacuoles within the cytoplasm of a host cell. Located within the order *Rickettsiales*, five members within the family *Anaplasmataceae*, consisting of *Anaplasma phagocytophilum*, *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, *Ehrlichia canis* and *Neorickettsia sennetsu* are capable of causing disease in humans. Human infection by these pathogens is generally referred to as "ehrlichiosis." Although members of the family *Anaplasmataceae* were first recognized as early as 1910, reported cases on human ehrlichiosis published from the CDC as a notifiable disease has only occurred since 1999 (1, 183).

First identified in 1932 as a veterinary agent, the tick-transmitted obligate intracellular bacterium *A. phagocytophilum* has become an increasingly recognized threat since 1994, when it was discovered to infect humans (34, 43). With the support of DNA sequencing and genetic analysis of 16S rRNA and *groESL* genes, three former agents, *Ehrlichia phagocytophila*, *Ehrlichia equi* and human granulocytic ehrlichiosis (HGE) agent that were originally thought unique have since been reclassified in 2001 as the single

agent, *A. phagocytophilum* (42). *A. phagocytophilum* infection in humans is now termed human granulocytic anaplasmosis **(HGA)**.

A. phagocytophilum is transmitted by *Ixodes* ticks, specifically by *I. scapularis* in the Northeast and upper Midwest United States, *I. pacificus* in the Western United States, *I. ricinus* in Europe and *I. persulcatus* in Asia (56, 170). In the United States, *A. phagocytophilum* is maintained in a transmission cycle between its tick vector and small mammals, the primary of which is the white-footed mouse (*Peromyscus leucopus*) (56, 169). Acquisition of *A. phagocytophilum* by ticks occurs during a blood meal of an infected mammalian host, as transovarial transmission does not occur. Nonetheless, once a tick becomes infected transstadial transmission does occur as the tick molts from the larva, nymph and to the adult stage (56). The overall life cycle of *Ixodes* ticks and transmission potential of *A. phagocytophilum* is outlined in **Figure 1A**. Humans are not the intended host of ticks, but are susceptible to infection if fed upon by an infected tick. Upon tick attachment, transmission of *A. phagocytophilum* to a murine host can occur within 24 hours (40).

Epidemiology

The majority of reported HGA cases within the United States occur primarily in northern California, the upper Midwest, and the Northeast (119). This correlation is also seen in canines (**Fig. 1B**) as evidenced by antibodies to *A. phagocytophilum* (24, 129). The number of HGA cases reported to the CDC was an average of 0.23 cases per 100,000 people in 2006 (118). However, exposure to this pathogen in endemic areas is presumably

Figure 1. Life cycle of *Ixodes* ticks and threat of *A. phagocytophilum* exposure. (**A**) Life cycle of *Ixodes* species of tick. Adult females lay uninfected eggs, as transovarial transmission does not occur with *A. phagocytophilum*. The uninfected larval ticks hatch from eggs and acquire their first blood meal. Once a tick acquires *A. phagocytophilum* from a host, it will remain infected throughout its life cycle as transstadial transmission does occur. Although not the intended host of ticks, humans, canines and other mammals can become infected if fed upon by an infected tick. (**B**) Potential threat of exposure to *A. phagocytophilum.* Map indicates by county exposure to *A. phagocytophilum* as evidenced by antibodies detected in canines. Canines are more frequently exposed to tick bites than humans, allowing them to serve as sentinels in determining risk of human exposure and infection within particular geographic areas. Gray shading indicates no results (<10), and white indicates no canines reported as positive (0%). Remaining colors are as follows: 0.01 - 0.49% (pale green), 0.5 - 1.0% (lichen), 1.1 - 5.0% (kelly green) and 5.1 - 40% (dark moss) (24).

A.

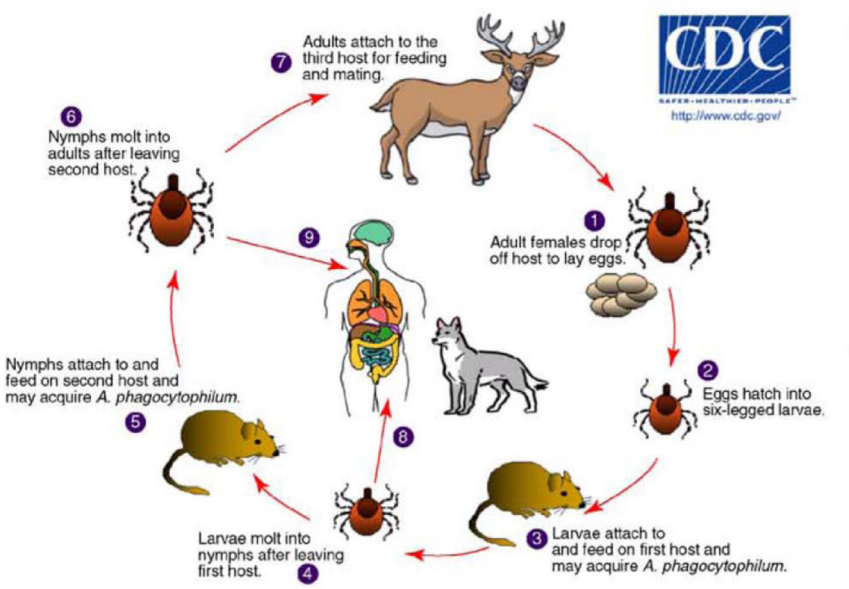


Image from: Nicholson, W.L., K.E. Allen, J.H. McQuiston, E.B. Breitschwerdt and S.E. Little. 2010. The increasing recognition of rickettsial pathogens in dogs and people. Trends in Parasitology 26(4):205-212.

B.

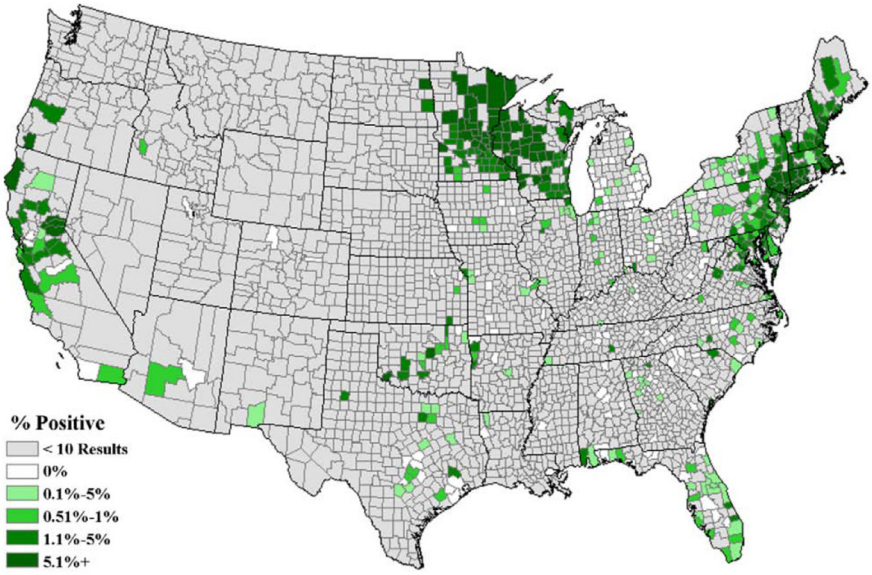


Image from: Bowman, D., S.E. Little, L. Lorentzen, J. Shields, M.P. Sullivan and E.P. Carlin. 2009. Prevalence and geographic distribution of *Dirofilaria immitis*, *Borrelia burgdorferi*, *Ehrlichia canis*, and *Anaplasma phagocytophilum* in dogs in the United States: Results of a national clinic-based serologic survey. Veterinary Parasitology 160(1-2):138-148.

much greater as human serological studies in Wisconsin and New York suggest exposure is as high as 15% to 36%, respectively (4, 12). An average of 35 cases per 100,000 people was reported over a three-year period in Connecticut, making *A. phagocytophilum* the second most common tick-transmitted pathogen in this state (82). Serological data clearly demonstrates transmission of this pathogen is high; however the occurrence of diagnosed disease after exposure is generally less frequent.

Clinical features and treatment

Clinical manifestations of HGA range in severity from subclinical or mild infection to severe or even fatal disease. Non-specific symptoms of *A. phagocytophilum* include malaise, fever, myalgia, and headache. Rarely does rash occur along with infection. Laboratory tests typically identify irregularities, which include thrombocytopenia, leukopenia, anemia and elevated liver enzymes. Pathology includes inflammation of the liver, slight lymphoid depletion, hyperplasia and hemophagocytosis. Severe complications include prolonged fever, shock, confusion, seizures, pneumonitis, acute renal failure, hemorrhages and rhabdomyolysis (41). Although many infections are asymptomatic and may be self-limiting, it is estimated half of all symptomatic individuals require hospitalization, with up to 7% admitted to intensive care (10, 43). HGA has an estimated mortality rate of about 1%. Mortality has occurred from opportunistic infections and the severity of disease generally increases with age and immunosuppressed individuals (10).

The hallmark of HGA infection is the presence of intravacuolar colonies termed morulae, located within the cytoplasm of peripheral granulocytes (149). The term morulae

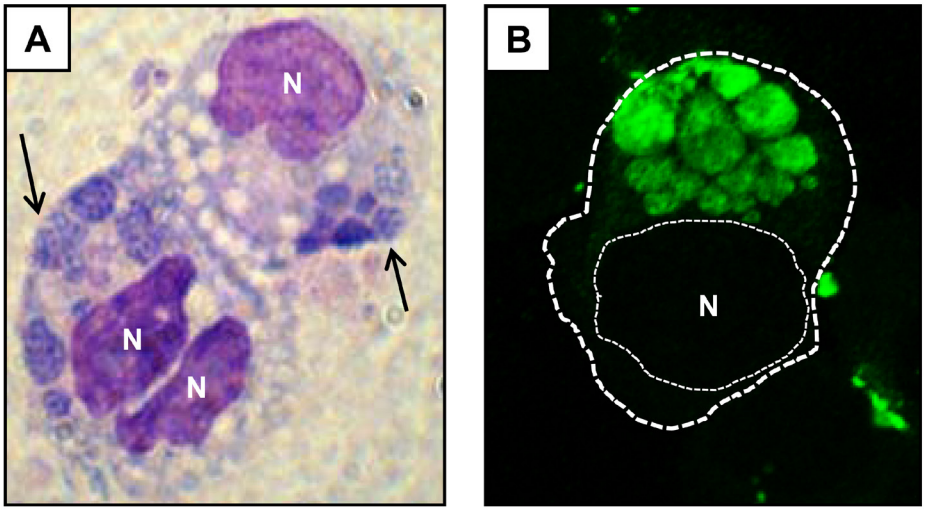
extends from the punctuate staining of *A. phagocytophilum*, which looks like mulberries when viewed under a light microscope with Giemsa stains (**Fig. 2A**). Morulae can be detected in blood smears of 25-75% of patients with HGA within the first week of infection, although sometimes only 0.1% of neutrophils will be infected (170). Polymerase chain reaction (PCR) also has a high detection rate of 67-90% (44). If HGA is identified or suspected, treatment with doxycycline will usually resolve infection within 1-2 days (11, 13). After treatment, detection of *A. phagocytophilum* by Giemsa stains and PCR will decrease. Nonetheless, some individuals have reported to display symptoms of fatigue, fever and chills 1-3 years after treatment (142).

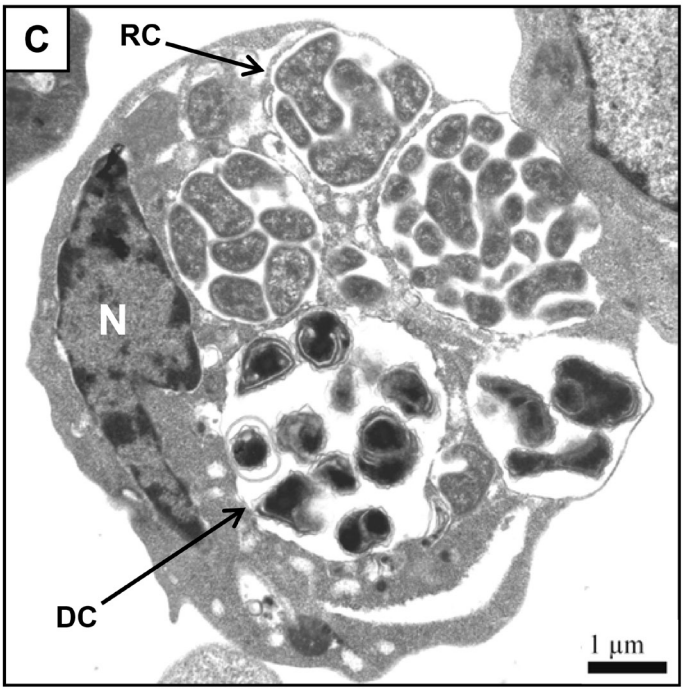
In addition to *A. phagocytophilum*, the causative agent of Lyme disease - *Borrelia burgdorferi* - is also transmitted by *Ixodes spp.* ticks. As a consequence it is not surprising that coinfection of ticks and simultaneous transmission of *A. phagocytophilum* and *B. burgdorferi* to humans can occur (98, 127). Coinfection contributes to more clinical manifestations than those observed with *B. burgdorferi* or *A. phagocytophilum* infection by themselves (92). In vitro studies show coinfection results in higher cytokine and chemokine production, resulting in impaired barrier functions of human brain microvascular endothelial cells (59). If coinfection is suspected, treatment with doxycycline is successful in treating both infections (44).

Intracellular survival and pathogenesis

A remarkable characteristic of *A. phagocytophilum* is its ability to replicate within neutrophils, the primary effector cell of microbial killing (158). *A. phagocytophilum*

Figure 2. Examples of *A. phagocytophilum* morulae in tissue culture cells. (**A**) Giemsa stain. Morulae (smaller dark staining purple vacuoles) can be seen within the cytoplasm of the host cells. Within some morulae the punctuate appearance of individual organisms (indicated by arrows) can be seen. (**B**) Immunofluorescence. Organisms within morulae are detected with antiserum to Msp2(P44), the most abundant surface protein of *A. phagocytophilum*. Thick and thin dashed lines delineate the host cell and host cell nucleus membranes, respectively. (**C**) Transmission electron microscopy (8,200x). Morulae and individual organisms are easily distinguished. Both the electron dense-cored cell (DC) and pleomorphic electron lucent reticulate cell (RC) can be distinguished. All images are of *A. phagocytophilum* growing in HL-60 cells. N indicates host cell nucleus.





survival is largely dependent on neutrophils as their depletion in mice will reduce the bacterial load (20). Infection of mouse neutrophils also results in secretion of the chemokine interleukin-8 (IL-8), which promotes additional neutrophils to migrate to sites of infection, thereby further promoting *A. phagocytophilum* survival (5). It has also been more recently demonstrated that *A. phagocytophilum* can infect human endothelial and megakaryocytic cell lines (64, 73).

Infection of human neutrophils is linked to its surface expression of platelet selectin glycoprotein ligand-1 (PSGL-1), a selectin ligand (58, 74). The promyelocytic leukemia cell line **HL-60 expresses PSGL-1 and supports *A. phagocytophilum* infection** (57). This cell line represents the standard for in vitro cultivation of *A. phagocytophilum*. After binding to PSGL-1, *A. phagocytophilum* enters the cell and resides within a host cell-derived vacuole. Within this vacuole *A. phagocytophilum* will replicate by binary fission. **The terms morulae, vacuole and inclusion are synonymous with each other**. During intracellular development *A. phagocytophilum* undergoes a biphasic developmental cycle consisting of two distinct morphological forms. These consist of a smaller adherent/infectious dense-cored cell (DC) and a larger noninfectious reticulate cell (RC). The focus of Chapter 2 is to determine the pathobiological roles of these two forms. The two forms can only be distinguished by transmission electron microscopy (TEM) (**Fig. 2C**), as no form specific proteins have been identified to differentiate between RCs and DCs by indirect immunofluorescence assay (IFA). During intracellular replication *A. phagocytophilum* has adapted to utilizing host components for survival, such as acquiring host cell amino acids for use, as well as incorporating host cell cholesterol into its

membrane (45, 193). This dependence on the host cell has reduced the genome to 1.47 Megabases (Mb).

To survive within such a hostile intracellular environment, *A. phagocytophilum* utilizes multiple strategies. Upon entry into human myeloid cells, the *A. phagocytophilum* occupied vacuole recruits Rab GTPases that are associated with recycling endosomes (78). Rabs are responsible for organelle identity and regulate endosome maturation. By disguising its self as a recycling endosome, the *A. phagocytophilum* vacuole prevents its maturation to a mature endosome and avoids lysosomal fusion. This has been further demonstrated by exclusion of early endosome markers from the occupied-vacuole, such as the transferrin receptor, Rab 5, and early endosome antigen 1 (121). The *A. phagocytophilum* vacuole also does not acquire Lysosomal-associated membrane protein 1 (LAMP-1) associated with late endosome and does not acquire vacuole-type H^+ ATPases, which are required for vacuolar acidification (30, 185).

Typically when bacteria are phagocytosed into neutrophils, toxic oxygen intermediates are delivered into the phagosome. This delivery is controlled by the assembled nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex on the phagosome membrane. Yet, *A. phagocytophilum* inhibits transcription of genes encoding for components of the NADPH oxidase complex, preventing formation and thus delivery of toxic oxygen intermediates into the phagosome (31). In addition, the ability to scavenge exogenous superoxide (O_2^-) and rapidly detoxify O_2^- is another strategy *A. phagocytophilum* employs for its survival (28).

The type IV secretion system (T4SS) encoded by *A. phagocytophilum* is also directly responsible for promoting its survival through delivery of bacterial proteins into the host cell (150). Three T4SS proteins have been identified in *A. phagocytophilum*; AnkA, Ats-1 and AptA (101, 132, 165). AnkA, which contains ankyrin repeats is the best studied and characterized of these proteins. Within minutes of organisms binding to a naïve cell and before bacterial entry occurs, AnkA is injected into the cytoplasm of the host cell where it is phosphorylated by the Abl-1 tyrosine kinase (81, 101). After injection, AnkA enters the host cell nucleus and modifies gene transcription by directly binding to chromatin (54). Although few bacterial virulence factors have been identified, *A. phagocytophilum* can inhibit host cell apoptosis through multiple pathways (30, 95, 148). *A. phagocytophilum* infection has also been documented to deacetylate histones (52). Changes in histone modification presumably have a direct effect on gene expression by changing chromatin structure, resulting in compacted chromatin, which limits access to transcriptional activation. All of these effects on the host cell presumably occur to promote intracellular survival and allow for increased replication time in neutrophils, as peripheral blood neutrophils generally only survive for several days.

Immunopathology

During human infection the percentage of infected neutrophils can be exceptionally low; nevertheless the pathology can be severe. This discrepancy between the low level of bacteria during infection and high histopathology has always pointed to immune effectors as the primary cause of disease. Cytokines produced during human, mouse, horse and dog

infections are similar (97). The most prominent cytokine produced during infection is interferon-γ (IFNγ). Infection models have demonstrated that IFNγ knockout mice do not develop histopathological lesions, regardless of a 5-8 fold increase in the level of organisms. In comparison, when IL-10 knockout mice which lack the ability to repress IFNγ production are infected with *A. phagocytophilum* the bacterial load is identical to a wild type control, but the level of histopathology is considerably more severe (110). This demonstrates that histopathologic injury is not directly dependent on the bacterial load, but instead depends on host cell immune responses.

The immune system pathways that are critical for controlling and eventually clearing *A. phagocytophilum* infections have not been fully elucidated. When *A. phagocytophilum* is repeatedly passaged in severe combined immunodeficiency (SCID) mice, the virulence of *A. phagocytophilum* can increase and can eventually kill the SCID mouse (20). This suggests that intact immune systems are more beneficial than disadvantageous. In addition, when various components of the innate immune system were studied in knockout mice, it was demonstrated that inducible nitric oxide (iNOS), phagocyte NADPH oxidase (PHOX), tumor necrosis factor, toll-like receptor (TLR)2, TLR4 and myeloid differentiation factor 88 (MyD88) do not contribute to controlling infection (182). Moreover, some of these intact innate immune features during infection actually contributed to increased inflammation and hepatic injury (157). One feature that is important for eventual elimination is $CD4^+$ T cells. But oddly, this clearance occurs in the absence of perforin, Fas/FasL and Th1 cytokines such as IL-12 and IFNγ, which are effectors generally required for eliminating intracellular pathogens (20). The unusual

feature of *A. phagocytophilum* to infect neutrophils and its means to avoid pathways that successfully eliminates other intracellular pathogens likely points towards an equally distinctive and untraditional means required for controlling infection that has yet to be fully understood.

Bacterial adhesion-receptor interactions

Bacterial adhesins are the "tip of the sword" when it comes to interacting with host cell receptors, and are a necessity for the initial steps of colonization and infection. For obligate intracellular pathogens, adhesion and entry into a host cell is more than just an arbitrary event, it is a requirement for survival. Bacterial adhesins are typically classified as fimbrial or afimbrial. Fimbrial adhesins are those that protrude from the surface of bacteria, such as various types of pili. Afimbrial adhesins are those that do not extend beyond the organism and are surface bound (90, 139).

Bacterial adhesins typically recognize specific structural requirements of their respective receptors, which usually restricts the organism to specific tissues or cell types (48). Nevertheless, there are exceptions such as the family of variable outer membrane opacity-associated (Opa) proteins of *Neisseria* that recognize multiple human cell surface receptors (181). Adhesins can bind two types of receptors, protein and carbohydrate (49). Carbohydrate moieties can be located on both glycoproteins and glycolipids (39, 49). The respective bacterial adhesins for a given receptor can also be protein or carbohydrate. In fact, the majority of confirmed or suspected bacterial adhesins from many different species have been identified as proteinaceous (7, 33, 90, 109, 139, 143, 152, 186, 199). Even

though most adhesins are protein, the importance of bacterial glycosylation and its role in adhesion has also been established (18, 37, 53, 75, 176). Characterization of adhesins is crucial to understanding how bacteria initially colonize and infect various cells and tissue, and the host cell responses they elicit. Adhesins are also ideal targets for the development of vaccines to prevent infection and colonization by bacteria (35, 93, 94, 191).

A. phagocytophilum adhesins are likely surface exposed proteins

Several observations suggest that the *A. phagocytophilum* adhesin(s) are located within the outer membrane and are directly surface exposed. Foremost, organisms that have been incubated with an excess of membrane impermeable crosslinkers (crosslink surface proteins), exhibit decreased binding to HL-60 cells, which implies that the crosslinkers are either binding to or sterically hindering the adhesin(s) (unpublished data). In addition, *A. phagocytophilum* organisms that have been fixed with paraformaldehyde (PFA) still bind specifically to PSGL-1 (194), which demonstrates that the adhesin(s) presumably are surface exposed, as fixed organisms could not upregulate or remodel protein surface structures. Also, *A. phagocytophilum* organisms bind equally well to HL-60 cells at 4, 23, and 37°C, which demonstrates that binding is not temperature dependent. Furthermore, when other obligate intracellular bacterial pathogens that undergo biphasic development such as *Chlamydia* are considered, the infectious form is metabolically inert due to compacted chromatin (3). This would imply the infectious DC form of *A. phagocytophilum* does not possess the ability to upregulate surface proteins in response to environmental stimuli. Indeed, when *A. phagocytophilum* organisms are isolated from

mammalian HL-60 cells and incubated with mammalian and bovine endothelial cell lines, they are susceptible to infection (124). Additionally, we have observed by TEM that the infectious DC form of *A. phagocytophilum* isolated from HL-60 cells binds to mouse mast cells (unpublished data). This strongly implicates the adhesin(s) for multiple cells types are likely expressed on the DC surface.

To further characterize the nature of the *A. phagocytophilum* adhesin(s), former Carlyon lab member Jonathan Sims extensively tested the binding properties required for adhesion to the confirmed human receptor, PSGL-1. Considering the adhesin-receptor interactions discussed in the previous section, organisms were pretreated with enzymes or oxidative agents and then tested for their ability to bind PSGL-1. When organisms were treated with lipase to remove lipids, sodium periodate to degrade carbohydrates and pronase to degrade proteins, only pretreatment with pronase caused a significant reduction in bacterial binding (unpublished data). These experiments strongly suggest that the adhesin(s) is proteinaceous in nature. Taken as a whole, these experiments and observations point to the *A. phagocytophilum* adhesin(s) as surface exposed proteins.

Major surface protein 2 (Msp2[P44]): The Dominant surface protein

Although the surface of *A. phagocytophilum* acts as a crucial interface for adherence to host cells, it has not yet been characterized in depth. One of the few confirmed and most studied surface proteins of *A. phagocytophilum* is the Msp2(P44) family that encode 42- to 49-kilodalton (kDa) transmembrane proteins that carry immunodominant B-cell epitopes. Msp2(P44) proteins share a common structure

consisting of a central hypervariable region (HVR) flanked by conserved N- and C-terminal regions. The HVRs exhibit strong predictability of being surface exposed, thus providing potential sources for surface phenotype diversity (84, 126, 200). The *A. phagocytophilum* Msp2(P44) proteins are orthologous to those encoded by the *Anaplasma marginale* Msp2 family (134). *A. marginale* remains within its ruminant host for the animals lifetime, and Msp2 antigenic variation is directly linked to the bacterium's persistence in vivo (46, 134). Likewise, the *A. phagocytophilum* Msp2(P44) proteins are believed to afford antigenic variation (43, 56), and a study that followed the *msp2*(*p44*) transcriptional profile of a clonal population over the course of equine infection supports this hypothesis (105).

Diversity in Msp2(P44) results from RecF-mediated gene conversion of a single genomic *msp2*(*p44*) expression site by partially homologous sequences (14-16, 106, 107). The *A. phagocytophilum* genome contains a large reserve (113 in the sequenced strain, HZ) of *msp2*(*p44*) genes or gene fragments lacking 5'- or 3'-terminal sequences (45). The gene fragments have been referred to as "functional pseudogenes" (105) because, rather than being nonfunctional fragments on their way to elimination, they recombine into the *msp2*(*p44*) expression site to yield full-length *msp2*(*p44*) paralogs (15, 16, 105, 106). Proteomic characterization of Msp2(P44) paralogs revealed the protein is posttranslationally modified into multiple isoforms and is glycosylated (155, 174). Msp2(P44) is also constitutively expressed throughout the developmental cycle (56) and can function as a porin (80).

Although some evidence suggests that Msp2(P44) acts as an adhesin (136, 184), the data has never been overwhelmingly convincing. The biggest argument against Msp2(P44) functioning as an adhesin is the constant variation of the surface exposed HVR, which would have to remain constant to routinely bind the same receptor. During mammalian infection, the immune response to Msp2(P44) is very well conserved and has been tested as a suitable antigen for laboratory diagnosis (85, 200). When mice are vaccinated with lysates of purified organisms or passively administered antibodies and then challenged by syringe- or tick-transmission, the mice are provided considerable but not complete immunity to infection from *A. phagocytophilum* (166). The mice that were immunized with lysates of purified organisms generate strong antibody responses to Msp2(P44). Given the strong antibody response to Msp2(P44) and the abundance of the protein on the surface of *A. phagocytophilum*, infection should have been largely preventable if Msp2(P44) was a true adhesin. A more probable explanation is antibodies to Msp2(P44) coat the surface of the organism and sterically hinder true adhesin(s), thus resulting in incomplete protection.

Other surface proteins of *A. phagocytophilum*

The only other study to date that set out to identify additional surface proteins of *A. phagocytophilum* was conducted by Yan Ge and Yasuko Rikihisa (55). Using a membrane impermeable biotin reagent, 14 putative *A. phagocytophilum* surface proteins were labeled, recovered, and identified by mass spectrometry (MS). Biotinylation has become a valuable and reliable technique to identify the cell surface protein composition of many

organisms (25, 88, 138, 141, 153). From this approach, only 2 of the 14 identified proteins were further confirmed as surface exposed. The two newly identified surface proteins are designated Asp55 and Asp62. This study demonstrates biotinylation can serve as a reasonable approach to use for identification of *A. phagocytophilum* surface proteins.

Although this study was the first to further characterize the surface of *A. phagocytophilum*, the data from this experiment does have several limitations. First, both a mixture of the adherent/infectious DC and non-adherent/noninfectious RC forms were used as the substrate for biotinylation. Consequently, it cannot be determined whether the identified proteins are expressed on only one or both developmental forms. As we will demonstrate in Chapter 2, we believe the adhesin(s) are likely upregulated on the adherent/infectious DC form. Second, recovered biotinylated proteins were resolved by 1 dimensional sodium dodecyl sulfate - polyacrylamide gel electrophoresis (1D SDS-PAGE), which yielded many protein bands of interest from which only 7 were chosen for analysis. Since only a select few bands were analyzed, many surface proteins may not have been identified. Third, it has been demonstrated that membrane impermeable biotin reagents can gain access through porins to label inner membrane proteins (25). Some of the identified proteins in this study possessed characteristics of integral surface membrane proteins, while others did not. The dominant surface protein of *A. phagocytophilum* Msp2(P44), has been shown to function as a porin (80), which may have potentially allowed for labeling and recovery of inner membrane proteins. In Chapter 5, we use biotinylation as a means to identify surface proteins of *A. phagocytophilum*, but do so

using improved methods to address the above limitations and to identify a more comprehensive list of surface proteins on the adherent/infectious DC form.

A. phagocytophilum adhesion to sLe^x-modified PSGL-1

As an obligate intracellular pathogen, the binding of *A. phagocytophilum* to a host cell receptor is a prerequisite step for entry and replication, and thus its survival. However, the bacterial adhesin(s) mediating this process have yet to be identified, and therefore presents a significant gap in our understanding of *A. phagocytophilum* pathogenesis. The hallmark characteristic of *A. phagocytophilum* infection is neutrophil colonization. It has also been documented *A. phagocytophilum* can infect human bone marrow progenitor cells (89). The uncommon tropism for neutrophils as well as neutrophil precursor cells suggest that a specific receptor is targeted by *A. phagocytophilum*. The receptor has since been identified as platelet selectin glycoprotein ligand-1 (PSGL-1) and the tetrasaccharide sialyl Lewis x (sLe^x), which modifies the PSGL-1 N-terminus (58, 74). PSGL-1 is highly expressed on neutrophils, where its function is to adhere to inflamed endothelial cells (117). Human PSGL-1 is a homodimer consisting of two 120-kDa subunits connected by a disulfide bond and is coated with O-glycans (**Fig. 3A**). The region critical for *A. phagocytophilum* binding is located on the N-terminal region of PSGL-1. The promyelocytic leukemia cell line HL-60 expresses sLe^x-modified PSGL-1 and is the standard for supporting *A. phagocytophilum* infection in vitro (57).

Three specific determinants are essential for *A. phagocytophilum* binding. The carbohydrate components include α2,3-linked sialic acid and α1,3-linked fucose of sLe^x.

The protein component is the amino acid sequence YEYLDYDFLPE, which is located in the PSGL-1 N-terminus (**Fig. 3B**) (144, 194). Antibody blocking or chemical removal of any one of these three determinants will significantly reduce *A. phagocytophilum* binding (58, 74, 154, 194). When sLe^x and the PSGL-1 N-terminal peptide region are independently coupled to a microsphere, *A. phagocytophilum* binding still occurs. This indicates that sLe^x does not have to be presented directly on PSGL-1 (194).

From this data several adhesin scenarios are proposed for *A. phagocytophilum* adhering to PSGL-1 (**Fig. 4**). First, *A. phagocytophilum* may express one adhesin with multiple domains which bind the PSGL-1 N-terminal peptide, fucose, and sialic acid on one PSGL-1 molecule (**Fig. 4A**). Second, one adhesin with multiple domains could simultaneously bind the PSGL-1 N-terminal peptide on one PSGL-1 molecule and fucose and sialic acid presented on another glycoprotein (**Fig. 4B**). Third, *A. phagocytophilum* could also express several adhesins each of which individually bind the PSGL-1 N-terminal peptide, fucose, and sialic acid on one PSGL-1 molecule (**Fig. 4C**). Fourth, individual adhesins could bind the PSGL-1 N-terminal peptide on one PSGL-1 molecule and fucose and sialic acid presented on another glycoprotein (**Fig. 4D**). The scenarios in which adhesins bind sLe^x directly presented on PSGL-1 (**Fig. 4A and C**) are preferred as they result in better binding (194). Higher levels of sLe^x-modified PSGL-1 expression also support better binding (194).

Although these scenarios represent basic binding models, *A. phagocytophilum* adhesion could be more complex. We cannot exclude the possibility a single adhesin as represented in the models could be in reality a complex of 2 or more proteins. Regardless

Figure 3. Model of human PSGL-1 and the determinants recognized by *A. phagocytophilum*. (**A**) Human PSGL-1 is a homodimer consisting of two 120-kDa subunits connected by a disulfide bond. PSGL-1 is decorated with various serine- and threonine-linked oligosaccharides (O-glycans). The region critical for *A. phagocytophilum* binding is located on the N-terminus of PSGL-1 indicated by the black dashed box. (**B**) Model of the PSGL-1 N-terminal peptide region that is essential for *A. phagocytophilum* binding. Critical residues include the amino acid region highlighted in pink of the peptide backbone, as well as α2,3-sialic acid (blue diamond) and α1,3-fucose (red triangle) of the tetrasaccharide sLe^x.

A.

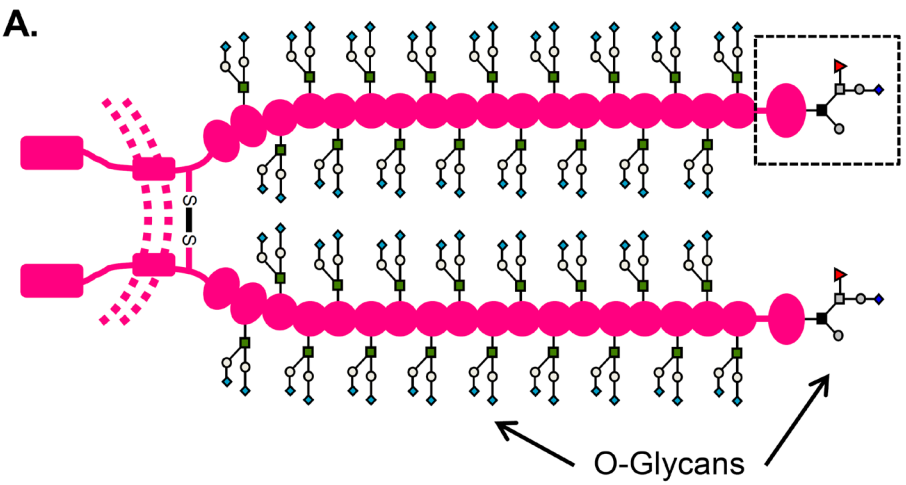


Image adapted from: McEver, R.P. and R.D. Cummings. 1997. Role of PSGL-1 binding to selectins in leukocyte recruitment. Journal of Clinical Investigation 100(3): 485-492.

B.

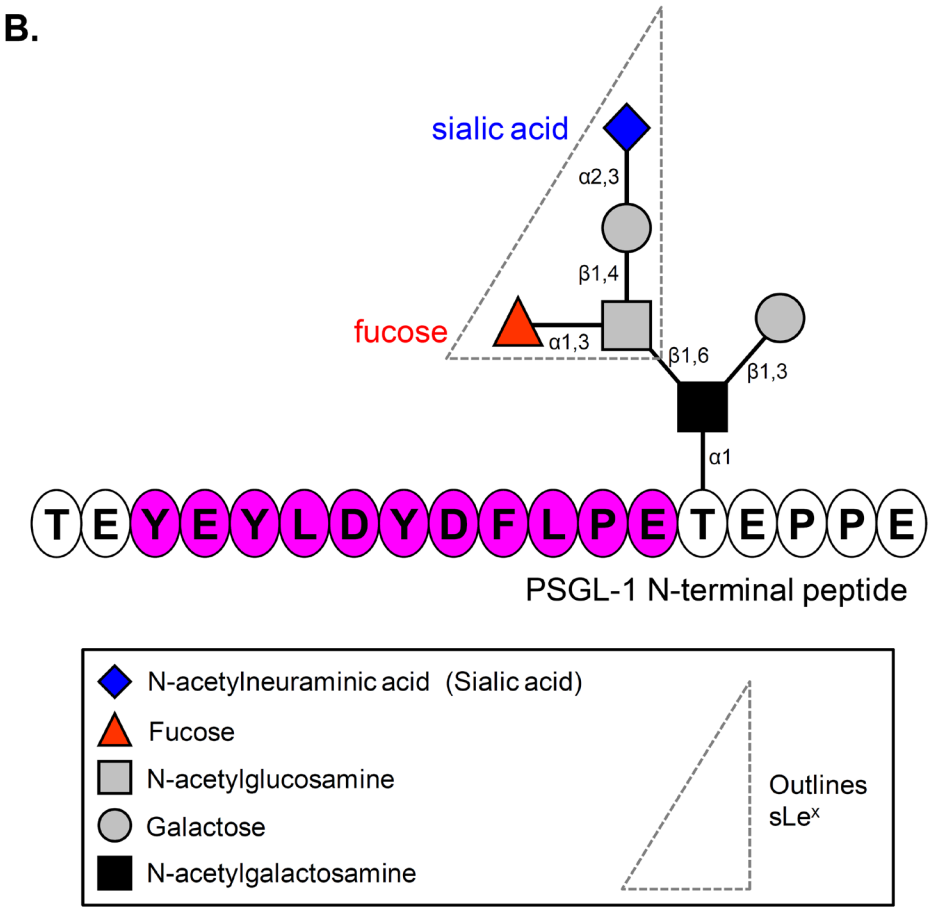
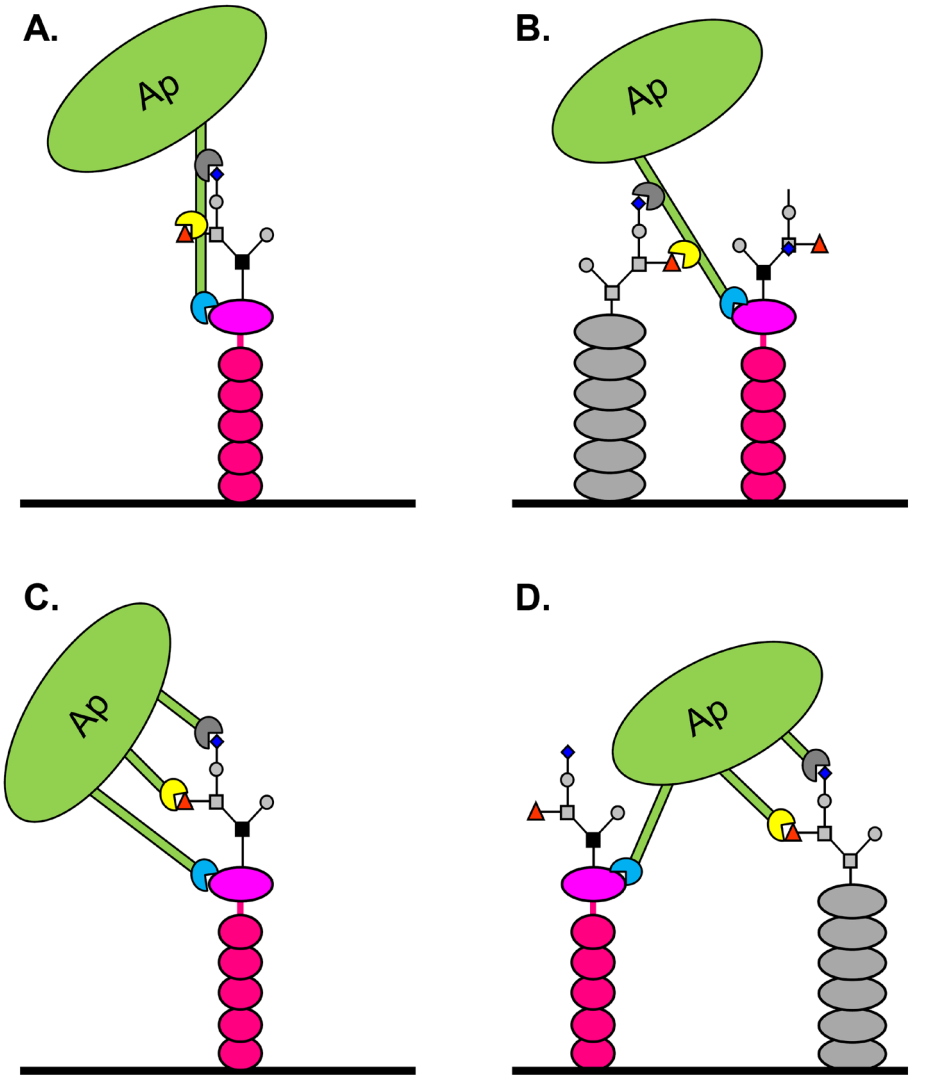


Figure 4. *A. phagocytophilum* adhesion scenarios. *A. phagocytophilum* binding to sLe^x-modified PSGL-1 may occur by several different mechanisms. (**A**) *A. phagocytophilum* may express one adhesin with multiple domains that bind the PSGL-1 N-terminal peptide, fucose, and sialic acid on one PSGL-1 molecule. (**B**) One adhesin could simultaneously bind the PSGL-1 N-terminal peptide on one PSGL-1 molecule and fucose and sialic acid presented on another glycoprotein. (**C**) *A. phagocytophilum* could express several adhesins each of which individually bind the PSGL-1 N-terminal peptide, fucose, and sialic acid on one PSGL-1 molecule. (**D**) Individual adhesins could bind the PSGL-1 N-terminal peptide on one PSGL-1 molecule and fucose and sialic acid presented on a another glycoprotein.



of how adhesion occurs, identification of surface proteins as putative adhesin(s) will allow greater insight into *A. phagocytophilum* pathogenesis. As individuals continue to participate in outdoor activities, exposure to this pathogen will continue to increase, making the characterization of surface proteins of *A. phagocytophilum* crucial to understanding their role in adhesion, which may serve as potential drug and vaccine targets to treat or prevent HGA.

Research objectives

As an obligate intracellular pathogen, the binding of *A. phagocytophilum* to a host cell receptor is a prerequisite step for entry and survival. We know that the susceptibilities of human neutrophils and promyelocytic HL-60 cells to *A. phagocytophilum* are linked to bacterial usage of sLe^x-modified PSGL-1 as the receptor for adhesion and entry. However, the bacterial adhesin(s) mediating this process have yet to be identified. The overall goal and work presented here seeks to identify surface proteins of *A. phagocytophilum* as putative adhesins. To undertake this objective, we first determined when during *A. phagocytophilum* development the PSGL-1 targeting adhesins are expressed. We next tested several affinity-based approaches to try to capture PSGL-1 adhesin(s). Lastly, we developed new density-gradient centrifugation approaches to purify *A. phagocytophilum* organisms.

CHAPTER 2: *A. phagocytophilum* Dense-Cored Organisms Mediate Cellular Adherence through Recognition of Human P-Selectin Glycoprotein Ligand-1

INTRODUCTION

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 (58, 74). *A. phagocytophilum* binding to PSGL-1 requires cooperative recognition of the N-terminal primary amino acid sequence and the α2,3-linked sialic acid and α1,3-linked fucose of sLe^x (29, 194). This complex interaction is mediated either by multiple *A. phagocytophilum* adhesins or by a single adhesin having multiple binding domains (29, 194). The *A. phagocytophilum* adhesin(s) that mediates 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 (69, 123-125, 140, 151, 185). 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* species, but it has also been observed for two *Anaplasmataceae* pathogens, *Anaplasma marginale* (122, 124) and *Ehrlichia chaffeensis* (140, 199), as well as *Coxiella burnetii*, the causative agent of Q fever (70, 116). Chlamydial elementary bodies (EB), which are analogous to DCs, are metabolically inert, environmentally resistant, and mediate attachment to and entry of eukaryotic host cells (3). Following attachment, EBs are internalized via induced phagocytosis into a host cell-derived vacuole. EBs 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 (199).

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 EB and *E. chaffeensis* DC is that they specifically express outer membrane proteins that have been identified as adhesin candidates (143, 162, 186, 199), 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. To test this hypothesis, 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.

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 ± 0.24 μm wide, had a dense nucleoid and a ruffled outer membrane, and were spheroid (**Fig. 5A to H and 6**). RCs were 0.69 ± 0.36 μm by 1.04 ± 0.44 μm, 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 are presented in **Figure 5**. We occasionally observed individual DCs surrounded by a membranous projection within inclusions harboring other DCs (**Fig. 5A**). A similar observation was reported for *Ehrlichia* species (140).

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. 6A to C**). By 4 h, 45.5% (30

of 55) had internalized (**Fig. 6E 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. 6C, E, and F**).

At 12 h, DCs were no longer associated with host cell surfaces (**Fig. 7A**). 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*-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. 7B**). Some vacuoles contained *A. phagocytophilum* organisms that had begun to condense to DCs (**Fig. 7B**). 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. 7C 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. 7D**). 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

Figure 5. ***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 ± 0.24 µm 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. (**I to P**) RCs were 0.69 ± 0.36 µm by 1.04 ± 0.44 µm, 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. Scale bars, 0.5 µm.

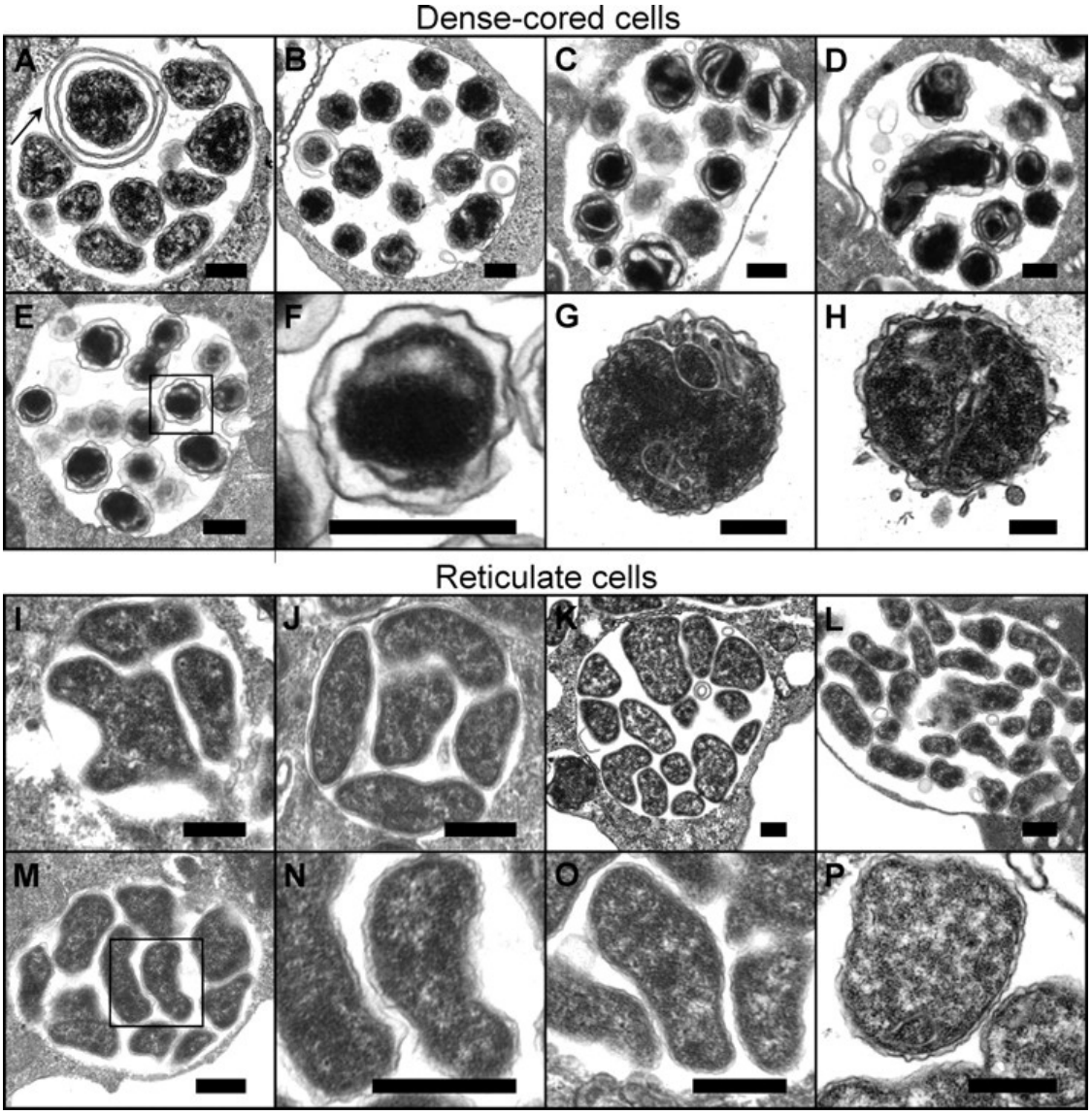


Figure 6. ***A. phagocytophilum*** **adhesion to, invasion of, and replication within HL-60 cells.** Host cell-free *A. phagocytophilum* organisms were incubated with HL-60 cells. After 40 min, unbound bacteria were removed and host cells were examined by transmission electron microscopy. (**A to C**) 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. Scale bars, 0.5 µm. Representative results of one of two separate experiments are shown.

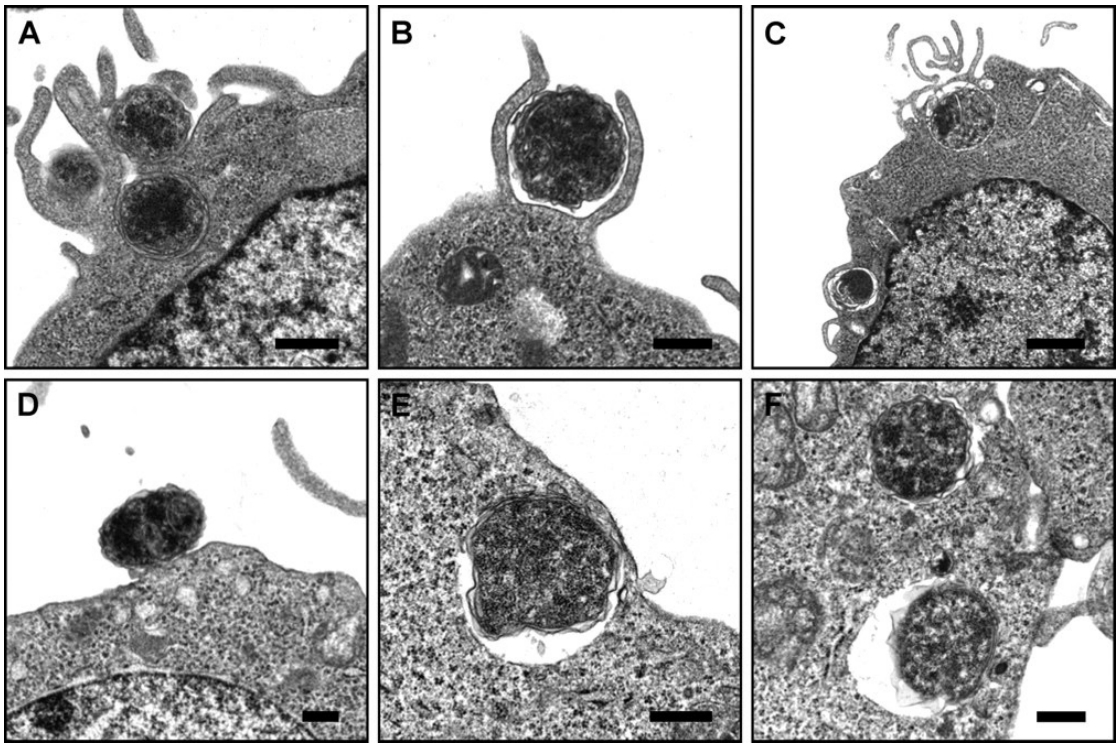
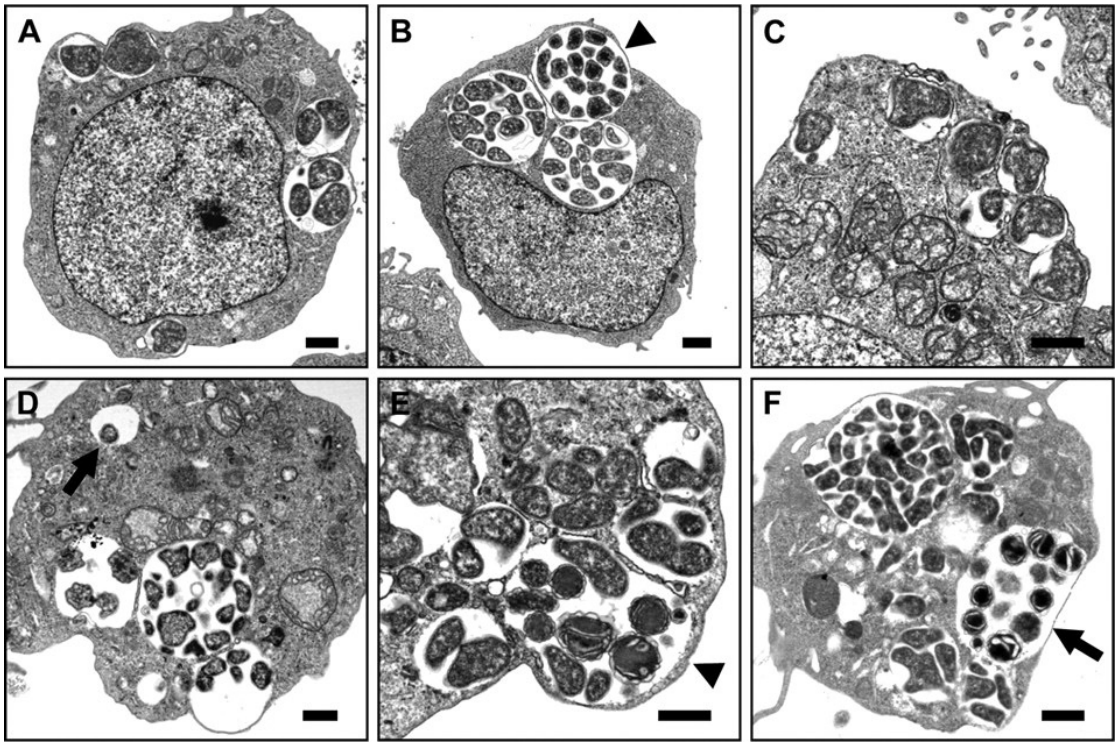


Figure 7. Intracellular development of *A. phagocytophilum* in HL-60 cells. Host cell-free *A. phagocytophilum* organisms were incubated with HL-60 cells. After 40 min, unbound bacteria were removed and host cells were examined at 12 (**A**), 24 (**B**), 36 (**C and D**), 48 (**E**), and 72 h (**F**) 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. Scale bars, 1.0 µm. Representative results of one of two separate experiments are shown.



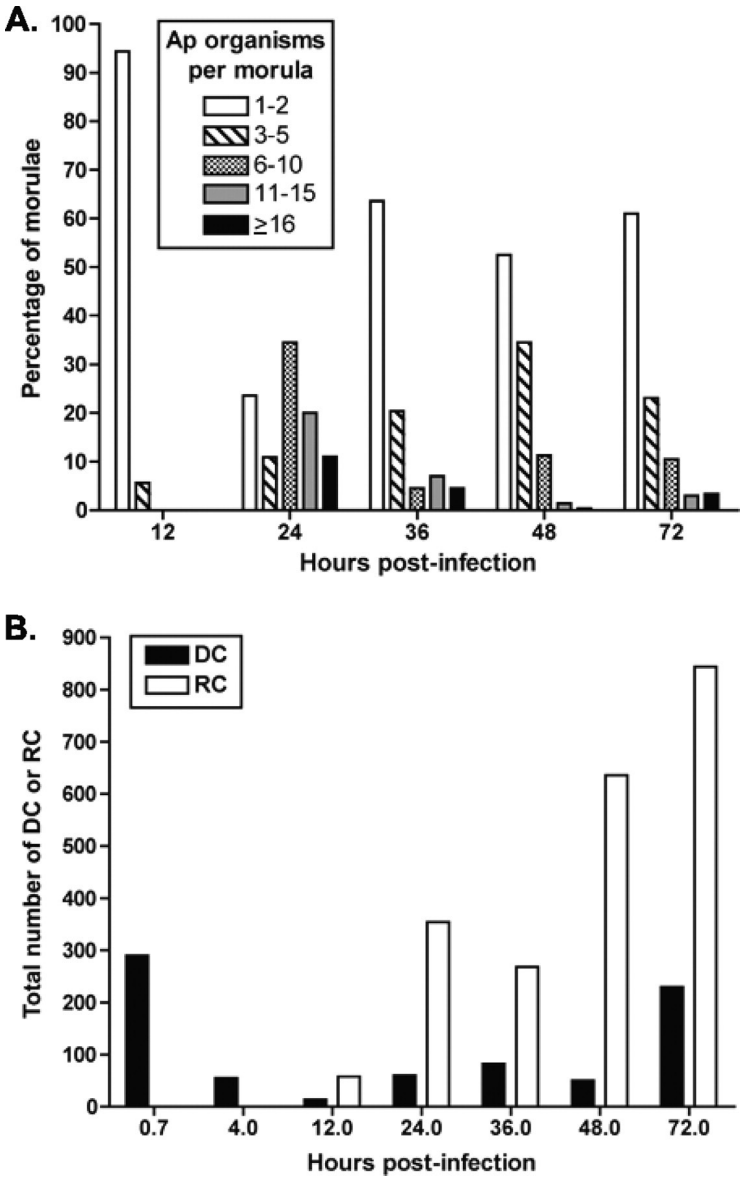
numerous DCs or RCs (**Fig. 7E and F**). Also observed were inclusions that contained bacteria that were transitioning from RCs to DCs (**Fig. 7E**). At no time point was fusion of morulae observed.

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. 8A**). 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. 7C and D and 8A**). 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

Figure 8. Number of *A. phagocytophilum* 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. 6 and 7** (**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.



the time course analysis. It is important to keep in mind that 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. 8B**). Though RCs outnumbered DCs at all postreplication time points, the greatest number of DCs was observed at 72 h.

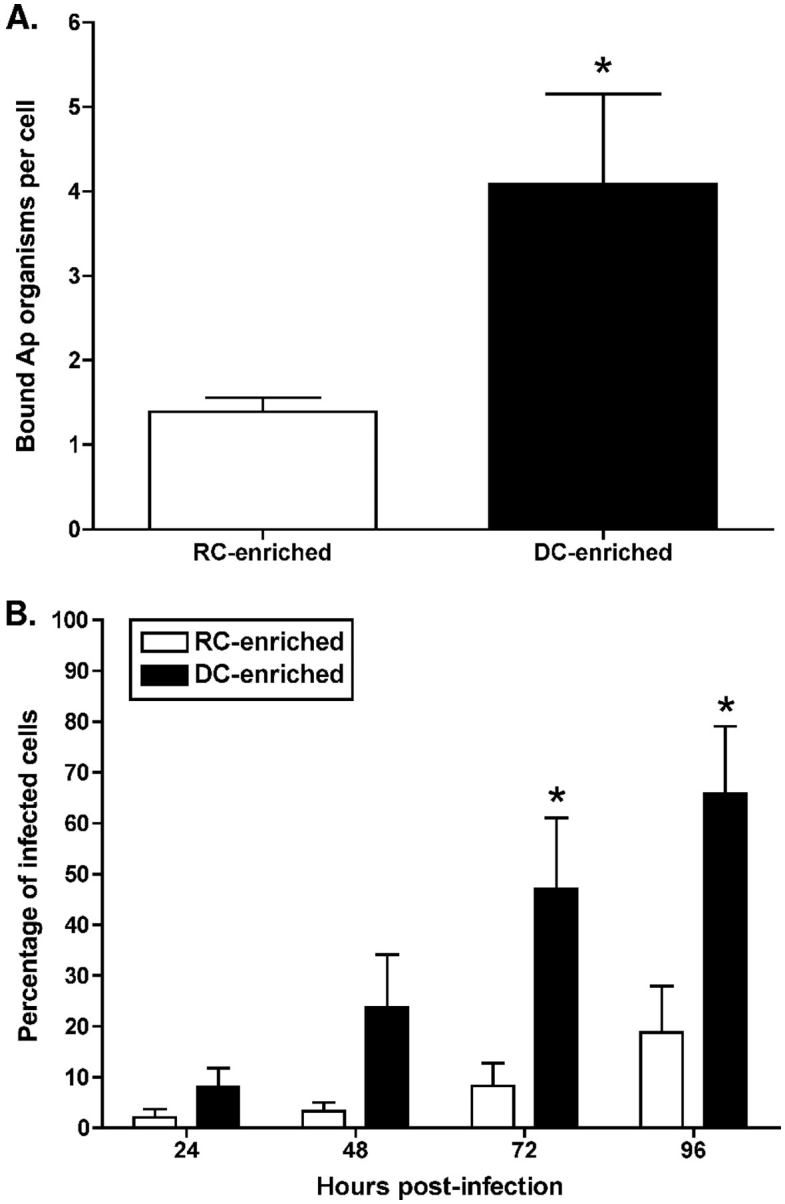
DC-enriched *A. phagocytophilum* organisms bind and infect HL-60 cells more effectively than RC-enriched bacteria.

Our electron microscopy time course studies revealed that DC, but not RC, organisms bound and were internalized into HL-60 cells (**Fig. 6 to 8**). 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% ± 3.4% RCs and 29.9% ± 3.4% DCs (**Table 1**). At 72 h, 53% more DC organisms were present, as host cell-free bacterial populations contained 45.8% ± 14.7% DCs. Accordingly, *A. phagocytophilum* populations recovered from infected HL-60 cells at 24 h and 72 h are denoted 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.

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

Experiment no.	*A. phagocytophilum* organisms isolated at[a]:					
	24 h			72 h		
	Total no. of organisms	% RCs (70.1 ± 3.4)	% DCs (29.9 ± 3.4)	Total no. of organisms	% RCs (54.2 ± 14.7)	% DCs (45.8 ± 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

Figure 9. Binding and infection of RC- and DC-enriched *A. phagocytophilum* 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 (*, $P < 0.05$).



Significantly more DC-enriched organisms bound to HL-60 cells than RC-enriched bacteria (**Fig. 9A**). 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. 9B**).

***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 (58, 74, 144), we next compared the abilities of comparable amounts of DC- and RC-enriched organisms to bind to the surfaces of PSGL-1 CHO cells (100, 192). In addition to PSGL-1, these cells coexpress α1,3-fucosyltransferase VII and core 2 β1,6-*N*-acetylgluocsaminyltransferase, which are required to glycosylate PSGL-1 such that it is decorated with sLe^x, binds to P-selectin, and supports *A. phagocytophilum* binding (29, 74, 100, 194). Flow cytometry analyses confirmed that PSGL-1 CHO cells express PSGL-1 and sLe^x on their cell surfaces (**Fig. 10A 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. 11**). Monoclonal antibody (MAb) KPL1, which interferes with *A. phagocytophilum* binding by blocking access to the PSGL-1 N-terminal peptide

Figure 10. 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.

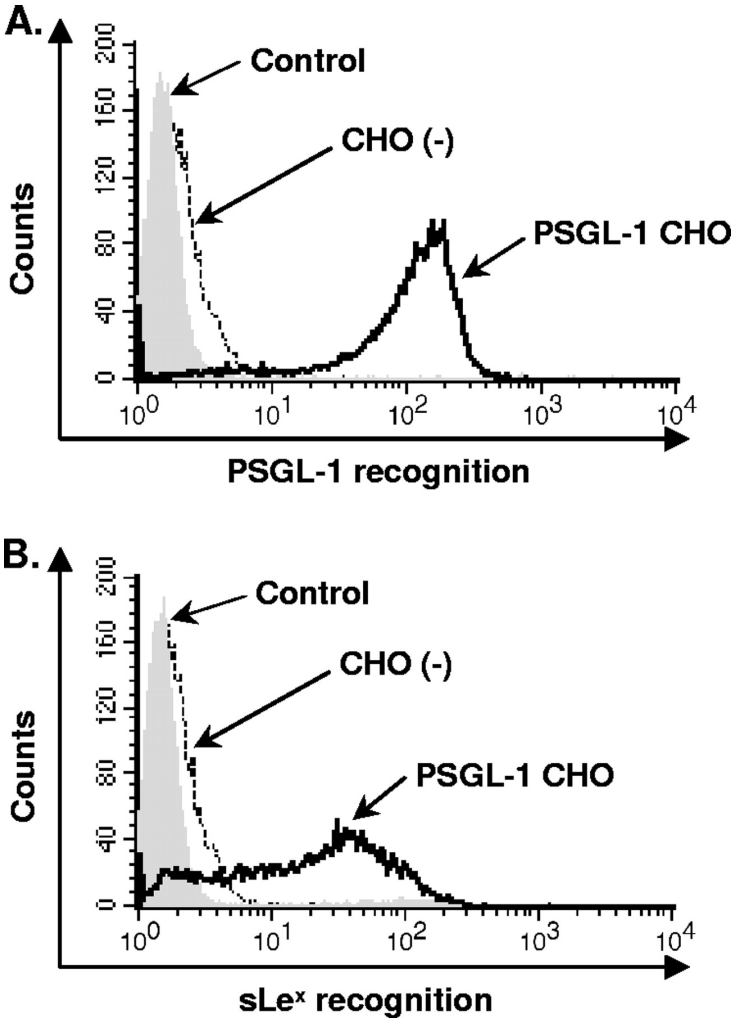
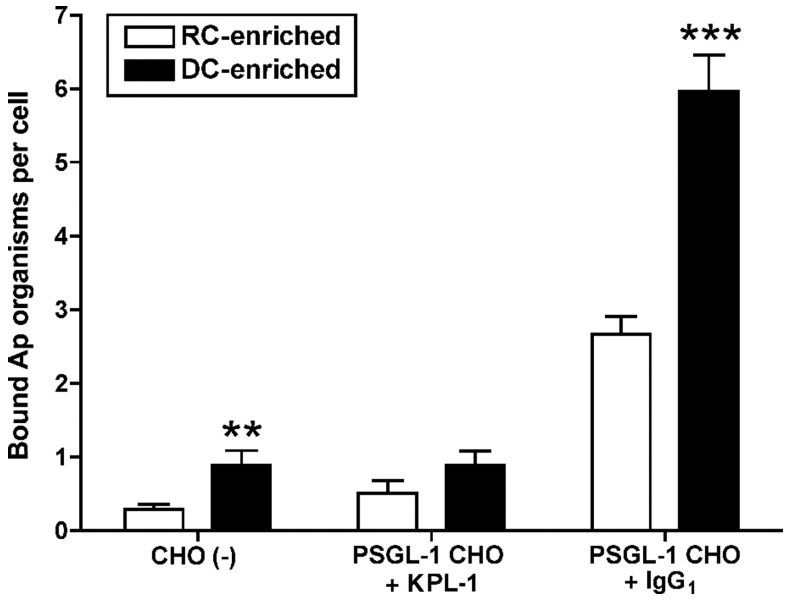


Figure 11. Binding of RC- and DC-enriched *A. phagocytophilum* organisms to PSGL-1 CHO and CHO (–) cells. Comparable amounts of host cell-free *A. phagocytophilum* organisms isolated at 24 h (RC-enriched) and 72 h (DC-enriched) post-synchronous infection were incubated with PSGL-1 CHO cells in the presence 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 (**, $P < 0.01$; ***, $P < 0.001$).

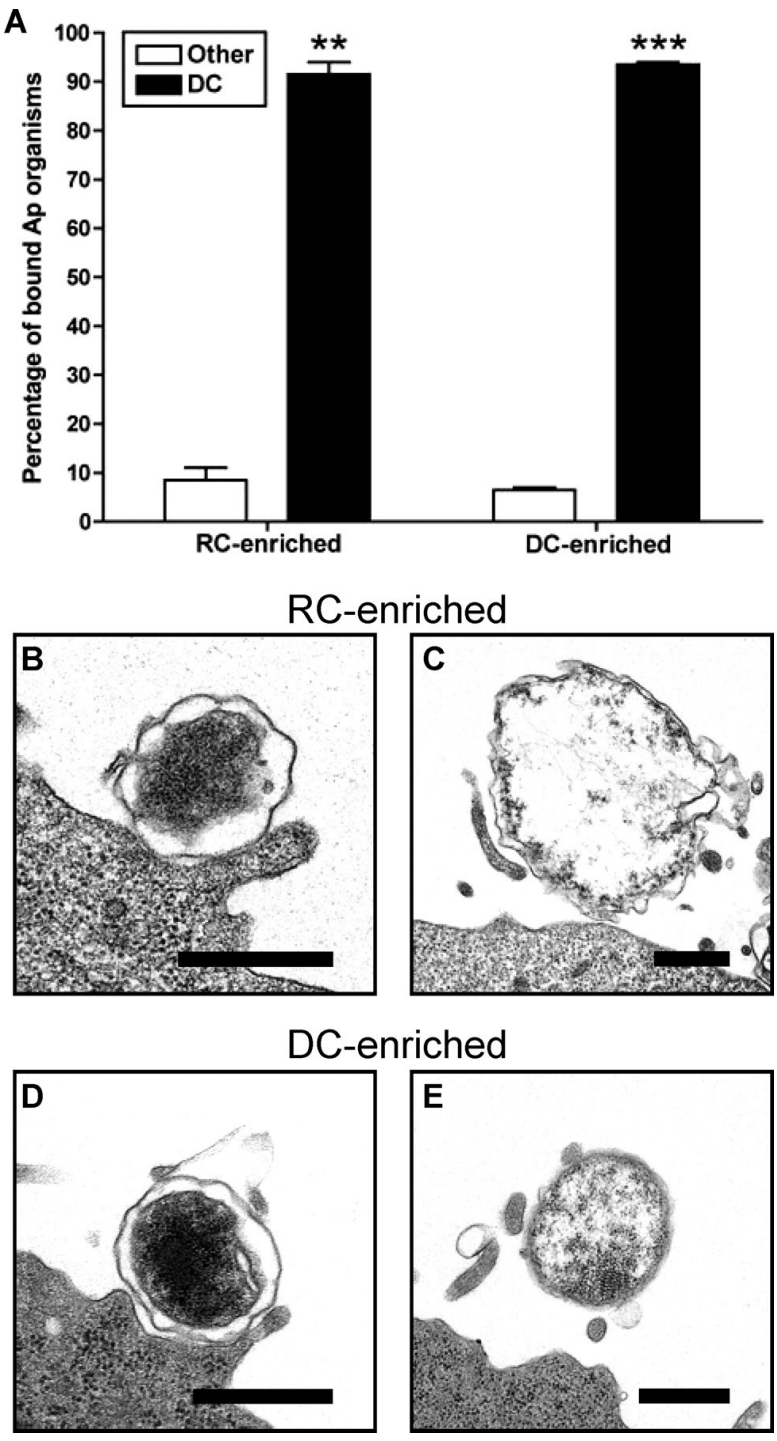


(74, 144, 145, 160), reduced binding by DC- and RC-enriched populations to PSGL-1 CHO cells to the background binding levels observed for CHO (–) cells.

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. 9 and 11**), 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. 11**), 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, ≥91.5% ± 3.5% of the *A. phagocytophilum* organisms that bound to the surfaces of PSGL-1 CHO cells were the DC form (**Fig. 12A, 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. 12C**) 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. 12E**). 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.

Figure 12. ***A. phagocytophilum*** **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. Scale bars, 0.5 μm. 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.



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 (123, 199). 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* (143, 162, 164, 186, 199).

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 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 (23, 28, 83). 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 (81, 101) 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 (26, 135), 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 (123,

199) but contradicts the supposition that the bacterium enters via receptor-mediated endocytosis, not phagocytosis (103, 195). PSGL-1 is a signaling molecule in leukocytes (178). *A. phagocytophilum* engagement of PSGL-1 activates the tyrosine kinase, Syk, a step that is essential for PSGL-1-dependent entry (145, 171, 178). PSGL-1 is present and interacts with Syk in lipid rafts at the myeloid cell surface (2). PSGL-1 association with Syk is mediated through ezrin and moesin (6, 178), which also function as membrane-actin cytoskeleton linkers and play roles in the formation of protrusive membrane structures such as filopodia and membrane ruffles (87). 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 (66), prevents Syk recruitment to interact with PSGL-1 (2) and prevents *A. phagocytophilum* entry (103).

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) (96), 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 (123, 199). Interestingly, the chlamydial developmental cycle within inclusions is asynchronous (3). 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 (76). 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* species (3) 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 (145).

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. 9**). 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 (74) 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.

METHODS

Cultivation of *A. phagocytophilum* and CHO cells. *A. phagocytophilum* strains HZ and NCH-1 were cultured in HL-60 cells (28). 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, α1,3-fucosyltransferase VII, and core 2 β1,6-*N* acetylglucosaminyltransferase (PSGL-1 CHO) were gifts from Rodger McEver (Oklahoma Medical Research Foundation) and were maintained as described previously (100).

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 degrees Celsius (°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(dimethylaminomethyl) 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 (≥90%) HL-60 cells by syringe lysis, followed by differential centrifugation to separate bacteria from host cell debris (27). 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 x 10^6 HL-60 cells in 1 milliliter (ml) of Iscove's modified Dulbecco's Eagle medium (IMDM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (IMDM-10). Host cells were sedimented at 300 x *g* for 5 min, washed twice with phosphate-buffered saline (PBS) to remove unbound bacteria, and incubated at 37°C in 5% carbon dioxide (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 (27).

Measurement of PSGL-1 and sLe^x expression of CHO cells by flow cytometry. CHO cells were removed from adherent flasks with 0.5 millimolar (mM) ethylenediaminetetraacetic acid (EDTA) (Irvine Scientific, Santa Ana, CA). Cells were treated with Fc block (5µg/ml) (BD Pharmingen, San Diego, CA) for 5 min, followed by incubation with CSLEX (binds to sLe_x) (BD Pharmingen) (51), KPL1 (binds to the human PSGL-1 N terminus) (BD Pharmingen) (160), or murine immunoglobulin G_1 (IgG_1) or murine IgM isotype control antibodies (5 µg/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 µg/ml. After 30 min on ice, the cells were washed with PBS, fixed with 1% PFA, and analyzed on a BD Biosciences FACScan 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 KPL-1 or mouse IgG_1 at 10 µg/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 (27).

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 x 10^6 CHO cells in 1 ml of PBS for 30 min at 25°C. The cells were centrifuged at 300 x *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.

CHAPTER 3: In Silico Identification of *A. phagocytophilum* Outer Membrane Proteins

INTRODUCTION

Having established that the *A. phagocytophilum* DC is responsible for binding human sLe^x-modified PSGL-1 and the adhesin(s) are likely surface exposed proteins, the next approach was to predict how many outer membrane proteins (OMPs) may be encoded. In 2006, the complete genome of *A. phagocytophilum* (strain HZ) was sequenced and annotated (45). Sequence analysis identified several important observations about membrane structure. It revealed that, although classified as a gram-negative organism by gram stain, *A. phagocytophilum* has lost all genes for Lipid A synthesis and lacks most of the genes for peptidoglycan synthesis (45, 102). As a means to potentially compensate for the loss of Lipid A, host cell cholesterol is inserted in the membrane (193). Sequence analysis also noted *A. phagocytophilum* does not encode any pili or capsules (45), so all OMPs are most likely afimbrial integral membrane proteins.

To generate a list of putative surface proteins, we sought to analyze the *A. phagocytophilum* genome by computer programs. There are a variety of prediction programs available and some even claim to have a lower error of prediction rate than experimental laboratory approaches (147). To obtain an approximate number of how

many *A. phagocytophilum* OMPs may be encoded, we compared 2 computer prediction program results to those predicted when the genome was sequenced.

RESULTS

TIGR predicted OMPs.

The Institute of Genomic Research (TIGR) sequenced *A. phagocytophilum* (strain HZ) (45), which was annotated and divided into 25 gene role categories. A total of 43 cell envelope proteins were predicted by TIGR and is listed in **Table 2**. Due to the large number of Msp2(P44) paralogs and its known surface localization, it was excluded from all computer analysis.

CELLO and PSORTb predicted OMPs.

The *A. phagocytophilum* genome was analyzed by the subCELlular LOcalization predictor program (CELLO) (196) and PSORTb (197) to predict potential OMPs. CELLO confidently identified 65 OMPs as well as 65 proteins that could localize to the outer membrane and elsewhere. In total, CELLO predicted up to 130 proteins that could potentially function as OMPs (**Table 3**). PSORTb was more conservative in analysis as it only predicted 16 OMPs (**Table 4**).

DISCUSSION

When results of the different computer prediction programs were compared, the data revealed there were discrepancies and similarities between predictions. When

Table 2. TIGR predicted Ap OMPs

	Ap protein	Predicted Cellular Location
1	APH_0094	Outer Membrane
2	APH_0221	Outer Membrane
3	APH_0234	Outer Membrane
4	APH_0338	Outer Membrane
5	APH_0382	Outer Membrane
6	APH_0383	Outer Membrane
7	APH_0385	Outer Membrane
8	APH_0386	Outer Membrane
9	APH_0387	Outer Membrane
10	APH_0449	Outer Membrane
11	APH_0452	Outer Membrane
12	APH_0453	Outer Membrane
13	APH_0454	Outer Membrane
14	APH_0455	Outer Membrane
15	APH_0457	Outer Membrane
16	APH_0510	Outer Membrane
17	APH_0540	Outer Membrane
18	APH_0541	Outer Membrane
19	APH_0594	Outer Membrane
20	APH_0611	Outer Membrane
21	APH_0612	Outer Membrane
22	APH_0625	Outer Membrane
23	APH_0728	Outer Membrane
24	APH_0878	Outer Membrane
25	APH_0919	Outer Membrane
26	APH_0922	Outer Membrane
27	APH_0924	Outer Membrane
28	APH_0949	Outer Membrane
29	APH_0985	Outer Membrane
30	APH_0995	Outer Membrane
31	APH_1017	Outer Membrane
32	APH_1049	Outer Membrane
33	APH_1210	Outer Membrane
34	APH_1219	Outer Membrane
35	APH_1220	Outer Membrane
36	APH_1240	Outer Membrane
37	APH_1250	Outer Membrane
38	APH_1325	Outer Membrane
39	APH_1345	Outer Membrane
40	APH_1358	Outer Membrane
41	APH_1359	Outer Membrane
42	APH_1361	Outer Membrane
43	APH_1387	Outer Membrane

Table 3. CELLO predicted Ap OMPs

	Ap protein	Predicted Cellular Location
1	APH_0015	Outer
2	APH_0018	Outer
3	APH_0023	Outer
4	APH_0035	Outer
5	APH_0043	Outer
6	APH_0050	Outer
7	APH_0051	Outer
8	APH_0068	Outer
9	APH_0081	Outer
10	APH_0110	Outer
11	APH_0117	Outer
12	APH_0221	Outer
13	APH_0328	Outer
14	APH_0376	Outer
15	APH_0392	Outer
16	APH_0404	Outer
17	APH_0405	Outer
18	APH_0406	Outer
19	APH_0469	Outer
20	APH_0506	Outer
21	APH_0537	Outer
22	APH_0582	Outer
23	APH_0597	Outer
24	APH_0614	Outer
25	APH_0634	Outer
26	APH_0683	Outer
27	APH_0705	Outer
28	APH_0707	Outer
29	APH_0708	Outer
30	APH_0716	Outer
31	APH_0723	Outer
32	APH_0755	Outer
33	APH_0805	Outer
34	APH_0807	Outer
35	APH_0808	Outer
36	APH_0811	Outer
37	APH_0838	Outer
38	APH_0839	Outer
39	APH_0849	Outer
40	APH_0861	Outer
41	APH_0874	Outer
42	APH_0905	Outer
43	APH_0906	Outer
44	APH_0976	Outer
45	APH_1013	Outer

46	APH_1014	Outer
47	APH_1037	Outer
48	APH_1050	Outer
49	APH_1069	Outer
50	APH_1117	Outer
51	APH_1127	Outer
52	APH_1153	Outer
53	APH_1170	Outer
54	APH_1210	Outer
55	APH_1219	Outer
56	APH_1220	Outer
57	APH_1240	Outer
58	APH_1310	Outer
59	APH_1348	Outer
60	APH_1358	Outer
61	APH_1359	Outer
62	APH_1361	Outer
63	APH_1378	Outer
64	APH_1404	Outer
65	APH_1406	Outer
66	APH_0112	Outer / Cytoplasmic
67	APH_0331	Outer / Cytoplasmic
68	APH_0377	Outer / Cytoplasmic
69	APH_0385	Outer / Cytoplasmic
70	APH_0390	Outer / Cytoplasmic
71	APH_0420	Outer / Cytoplasmic
72	APH_0421	Outer / Cytoplasmic
73	APH_0465	Outer / Cytoplasmic
74	APH_0503	Outer / Cytoplasmic
75	APH_0504	Outer / Cytoplasmic
76	APH_0625	Outer / Cytoplasmic
77	APH_0673	Outer / Cytoplasmic
78	APH_0761	Outer / Cytoplasmic
79	APH_0832	Outer / Cytoplasmic
80	APH_0847	Outer / Cytoplasmic
81	APH_0852	Outer / Cytoplasmic
82	APH_0897	Outer / Cytoplasmic
83	APH_0928	Outer / Cytoplasmic
84	APH_0933	Outer / Cytoplasmic
85	APH_0997	Outer / Cytoplasmic
86	APH_1148	Outer / Cytoplasmic
87	APH_1158	Outer / Cytoplasmic
88	APH_1233	Outer / Cytoplasmic
89	APH_1270	Outer / Cytoplasmic
90	APH_1303	Outer / Cytoplasmic
91	APH_1320	Outer / Cytoplasmic
92	APH_1322	Outer / Cytoplasmic

93	APH_1325	Outer / Cytoplasmic
94	APH_1346	Outer / Cytoplasmic
95	APH_0314	Outer / Cytoplasmic / Extracellular
96	APH_0514	Outer / Cytoplasmic / Extracellular
97	APH_0709	Outer / Cytoplasmic / Extracellular
98	APH_0829	Outer / Cytoplasmic / Extracellular
99	APH_0876	Outer / Cytoplasmic / Extracellular
100	APH_1120	Outer / Cytoplasmic / Extracellular
101	APH_0473	Outer / Cytoplasmic / Extracellular / Periplasmic
102	APH_0198	Outer / Cytoplasmic / Inner
103	APH_0916	Outer / Cytoplasmic / Inner
104	APH_0944	Outer / Cytoplasmic / Inner
105	APH_1110	Outer / Cytoplasmic / Inner
106	APH_0045	Outer / Cytoplasmic / Periplasmic
107	APH_0104	Outer / Cytoplasmic / Periplasmic
108	APH_0311	Outer / Cytoplasmic / Periplasmic
109	APH_0985	Outer / Cytoplasmic / Periplasmic
110	APH_1049	Outer / Cytoplasmic / Periplasmic
111	APH_1107	Outer / Cytoplasmic / Periplasmic
112	APH_1246	Outer / Cytoplasmic / Periplasmic
113	APH_1412	Outer / Cytoplasmic / Periplasmic
114	APH_0032	Outer / Extracellular
115	APH_0049	Outer / Extracellular
116	APH_0378	Outer / Extracellular
117	APH_0387	Outer / Extracellular
118	APH_0546	Outer / Extracellular
119	APH_0688	Outer / Extracellular
120	APH_0941	Outer / Extracellular
121	APH_0206	Outer / Inner
122	APH_0256	Outer / Inner
123	APH_0386	Outer / Inner
124	APH_0430	Outer / Inner
125	APH_0918	Outer / Inner
126	APH_0740	Outer / Periplasmic
127	APH_1015	Outer / Periplasmic
128	APH_1090	Outer / Periplasmic
129	APH_1150	Outer / Periplasmic
130	APH_1384	Outer / Periplasmic

Outer = Outer Membrane

Table 4. PSORTb predicted Ap OMPs

	Ap protein	Predicted Cellular Location
1	APH_0043	Outer Membrane
2	APH_0068	Outer Membrane
3	APH_0338	Outer Membrane
4	APH_0377	Outer Membrane
5	APH_0404	Outer Membrane
6	APH_0405	Outer Membrane
7	APH_0406	Outer Membrane
8	APH_0506	Outer Membrane
9	APH_0709	Outer Membrane
10	APH_0906	Outer Membrane
11	APH_1087	Outer Membrane
12	APH_1110	Outer Membrane
13	APH_1210	Outer Membrane
14	APH_1240	Outer Membrane
15	APH_1359	Outer Membrane
16	APH_1361	Outer Membrane

comparing the TIGR predicted OMPs to those of CELLO, only 15 proteins were shared. They were APH_0221, 0385, 0386, 0387, 0625, 0985, 1049, 1210, 1219, 1220, 1240, 1325, 1358, 1359 and 1361. Only 5 of the TIGR proteins (APH_0338, 1210, 1240, 1359 and 1361) were identified by PSORTb. A total of 4 proteins (APH_1210, 1240, 1359 and 1361) were identified by all 3 computer programs. All but 2 proteins (APH_0338 and 1087) identified by PSORTb were also identified by CELLO, indicating PSORTb is stricter in its predictions. CELLO and PSORTb, but not TIGR was able to predict the confirmed surface proteins APH_0405 (Asp55) and APH_0404 (Asp62). All programs also predicted the dominant OMP, Msp2(P44) (data not shown).

The validity of the computer programs is partially validated by the Yan Ge and Yasuko Rikihisa's surface biotinylation study of *A. phagocytophilum* (55). They identified 14 proteins, 7 of which were predicted by the computer programs. Three proteins (APH_1210, 1359 and Msp2[P44]) were predicted by all 3 computer programs. The two confirmed OMPs (APH_0405 [Asp55] and 0404 [Asp62]) were predicted by CELLO and PSORTb. The final two proteins (APH_1170 and 1406) were only predicted by CELLO. This is reminiscent of a study of *Helicobacter pylori* in which biotinylation experimentally identified 18 OMPs, and only 2 were predicted as surface proteins (153).

One limitation of prediction programs for our purposes is that it still does not determine whether or not the OMP is specifically expressed on the DC, RC or both developmental forms. As demonstrated in Chapter 2, the adhesin(s) are upregulated or posttranslationally modified on the adherent/infectious DC form. Therefore, our goal is to only pursue OMPs on the DC. A further concern is that other organisms, such as

Chlamydia, that also lack peptidoglycan and undergo biphasic development, stabilize the infectious EB's outer membrane by forming numerous disulfide bonds with two cysteine-rich proteins (47, 67). This results in the environmentally resistant and stable infectious Chlamydial EB that can survive harsh treatments with detergents and even sonication, whereas the fragile non-cross-linked chlamydial replicative RB is destroyed by these treatments (68, 156, 167). The two cysteine-rich proteins responsible for stability are believed to be located in the inner leaflet of the outer membrane and periplasmic space. Their inter- and intra-molecular bonds has made their solubility properties variable, depending on the treatment used for isolation (47), representing the overall complexity and unusualness of the Chlamydial membrane. *A. phagocytophilum* likely uses a similar strategy for stability as the infectious DC form can remain structurally intact even after several minutes of sonication. This property of the infectious *A. phagocytophilum* DC is discussed in further detail in Chapter 5. Although unknown at this time, if *A. phagocytophilum* also were to stabilize the DC by disulfide bonds or other unknown mechanisms, it could potentially stabilize proteins in the outer membrane that do not possess typical membrane features. If this were true, computer prediction programs may not identify such proteins as they typically classify proteins based on structural features.

Although computer predictions can serve as a valuable resource, their use in predicting OMPs of *A. phagocytophilum* may be limiting. The inconsistency of prediction between programs, the uniqueness of the membrane (lack of Lipid A and peptidoglycan, and the DC's resistance to sonication), and the fact experimentally identified OMPs of

other pathogens (153) were not predicted correctly, suggests some *A. phagocytophilum* OMPs will also need to be verified by experimentation in addition to predictions.

Having the sequenced genome is a crucial step forward in the understanding of any pathogen. Nevertheless, there are even more restrictions when applying this data directly towards the study of *A. phagocytophilum*. One of the biggest limitations of working with *A. phagocytophilum*, as well as any obligate intracellular pathogen is direct genetic manipulation of the organism. At this time, site-specific knockouts of genes are not a possibility. A transposase system has been successfully utilized to insert GFP randomly into the *A. phagocytophilum* chromosome (50), suggesting promise for better genetic tools in the future. Even if genetic manipulations were possible, the small genome of 1.47 Mb suggests that many genes may be essential for obligate intracellular survival. If an essential gene of *A. phagocytophilum* such as an adhesin was disrupted, the mutant would not survive and could not be isolated for validation or characterization as it could not be isolated and grown under any other conditions.

Since direct genetic manipulation of *A. phagocytophilum* is not an option for experimentation, it means that any putative surface protein/adhesin will have to be tested by traditional methods. This will entail expressing a protein in recombinant form, generating antiserum, and testing the antiserum to confirm expression and localization of the protein to the outer membrane. Regardless, computer predictions are still valuable data, therefore other members of the Carlyon lab have compared these lists to other experimental data (55, 128) and are working on expressing some of the predicted proteins of interest. Because it is impractical to express great numbers of potential membrane

proteins in recombinant form, as these are still just predictions, the focus of my dissertation project was to test other approaches to directly identify OMPs as potential adhesins of *A. phagocytophilum*.

METHODS

TIGR predicted cell envelope genes of unknown function. *A. phagocytophilum* (strain HZ) genome (45) is divided into 25 gene role categories (http://cmr.jcvi.org/cgi-bin/CMR/shared/GetNumAndPercentGenesInARole.cgi). The cell envelope category consists of 163 (11.9% of the genome) proteins. All 43 proteins (excluding the Msp2[P44] paralogs) of unknown function in the sub role of "other" were selected for analysis.

CELLO and PSORTb outer membrane protein computer prediction programs. The entire Ap genome in the protein FASTA format (http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome&cmd=Retrieve&dopt=Protein+Table&list_uids=19215 Display: protein FASTA) was copied and pasted into CELLO program v2.5: (http://cello.life.nctu.edu.tw/) (196). Gram-negative organisms and protein sequences parameters were selected and submitted. All potential OMPs were listed. CELLO is a multi-class support vector machine classification system. Support vector machines are learning-based classifiers trained to classify proteins as belonging to a specific localization site. CELLO uses a single module, based on multiple feature vectors that analyze *n*-peptide compositions to predict the subcellular localization for Gram-negative bacteria. CELLO analyzes 4 types of sequence coding schemes; the amino acid composition, the di-

peptide composition, the partitioned amino acid composition and the sequence composition based on the physico-chemical properties of amino acids. Scores from these classifications determine the proteins final location.

The program PSORTb v3.0 (http://www.psort.org/) (197) was used to analyze the Ap genome. PSORTb Version 3.0 has the Ap (strain HZ) genome computed (http://db.psort.org/browse/genome?id=7470). All predicted outer membrane proteins were listed. The confirmed and the well documented outer membrane Msp2(P44) protein was excluded from all results. PSORTb comprises six modules to examine protein sequence. The modules are listed as follows: (1) SCL-BLAST & SCL-BLASTe, searches proteins against the database of proteins of known subcellular localization. (2) Support Vector Machines, are machine learning-based classifiers trained to classify a protein as belonging or not belonging to the set of proteins at a specific localization site. (3) Motif & Profile Analysis relies on the observation that a proteins function is closely linked to its localization. (4) Outer Membrane Motif Analysis uses motifs generated from data mining applied to a set of 425 beta-barrel proteins to classify a query protein as outer membrane or non-outer membrane. (5) ModHMM identifies transmembrane alpha helices, which in turn identifies proteins spanning the cytoplasmic membrane. (6) Signal Peptide directs a protein for export past the cytoplasmic membrane, and can be used to differentiate cytoplasmic and non-cytoplasmic proteins. The proteins final location is derived from a combination of each module.

CHAPTER 4: Crosslinking and Affinity Purification Approaches to Capture *A. phagocytophilum* PSGL-1 Targeting Adhesins

INTRODUCTION

As a more direct means to identify *A. phagocytophilum* surface proteins as PSGL-1 adhesins, we next chose to test several affinity capture approaches. *A. phagocytophilum* recognition of the sLe^x-modified PSGL-1 N-terminus is a very specific interaction, which could serve as a means to potentially purify the respective adhesin(s). Affinity purification allows for the enrichment of a variety of molecules, such as receptor-adhesin, enzyme-substrate and antibody-antigen interactions from complex mixtures (9, 177). Affinity based approaches using overlays and pull-downs have identified rickettsial adhesins (111, 146) and a heparin binding outer membrane protein of *Chlamydia* (162). A complementary approach that has also been successful in identifying molecular interactions is chemical crosslinking (120, 172). The advantage of crosslinking is it allows for noncovalent or transient interactions to be captured, purified and then identified. This type of approach successfully identified an adhesin of *Helicobacter pylori* that bound a fucosylated histo-blood group antigen (8, 86).

To identify adhesins by crosslinking and affinity purification we used commercially available recombinant human PSGL-1 (**rhPSGL-1**). rhPSGL-1 is a

chimeric protein consisting of the extracellular domain of human PSGL-1 fused to the Fc region of human IgG_1. rhPSGL-1 contains all three receptor determinants essential for *A. phagocytophilum* binding as modeled in **Figure 3B** of Chapter 1. An advantage of using rhPSGL-1 is the presence of the Fc receptor. The Fc receptor will allow us to purify rhPSGL-1 with protein A/G beads, as the beads contain high affinity Fc-binding domains. Using the above approaches and reagents we set out to capture *A. phagocytophilum* sLe^x-modified PSGL-1 targeting adhesin(s).

RESULTS

A. phagocytophilum binds immobilized but not soluble rhPSGL-1.

To assess under which conditions *A. phagocytophilum* binds PSGL-1, we tested bacterial adherence to rhPSGL-1 in soluble form, as well as to rhPSGL-1 immobilized to beads. Even though only DC organisms bind PSGL-1 as demonstrated in Chapter 2, at the onset of this study there were no available techniques to separate RC and DC organisms from each other. Therefore, we had to isolate and use total bacterial populations consisting of both RC and DC organisms for experiments.

We first tested the bacterium's ability to bind soluble rhPSGL-1 by mixing the two together, after which unbound rhPSGL-1 was washed away. The bacteria were then solubilized, and the soluble fraction was mixed with Protein A/G beads to recover any rhPSGL-1 that had bound to the organisms. The initial supernatant, washes and Protein A/G beads were analyzed by western blot for rhPSGL-1 (**Fig. 13**). The greatest abundance of rhPSGL-1 was detected in the initial supernatant. No rhPSGL-1 was detected in the

Figure 13. ***A. phagocytophilum*** **does not bind soluble rhPSGL-1.** Three µg of rhPSGL-1 was mixed with *A. phagocytophilum* RC and DC organisms in PBS for 3 h. Organisms were pelleted and washed to remove unbound rhPSGL-1. The initial supernatant removed from the bacterial pellet and the washes were checked for rhPSGL-1. The bacterial pellet was lysed in RIPA buffer and the soluble fraction was mixed with Protein A/G beads overnight to bind rhPSGL-1. Protein A/G beads were recovered, boiled and the eluted sample was resolved by SDS-PAGE and examined by western blot for recovery of rhPSGL-1. Any recovered rhPSGL-1 would indicate that *A. phagocytophilum* adhesin(s) can bind its receptor in soluble form. Representative results of one of two separate experiments are shown.

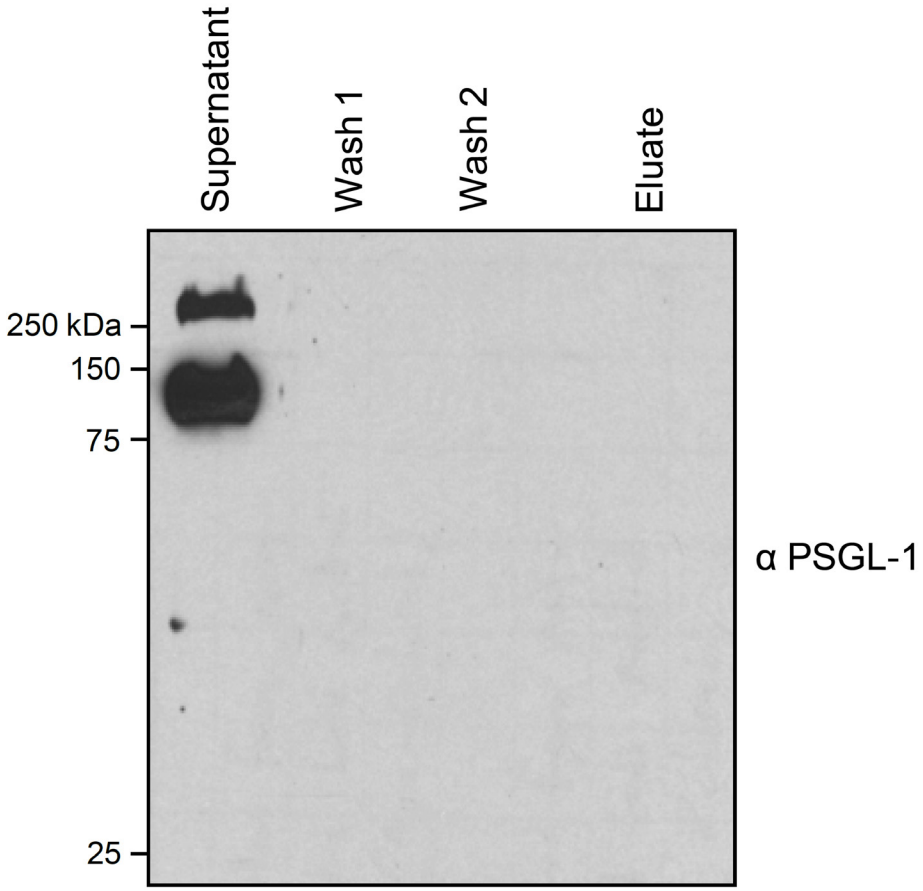
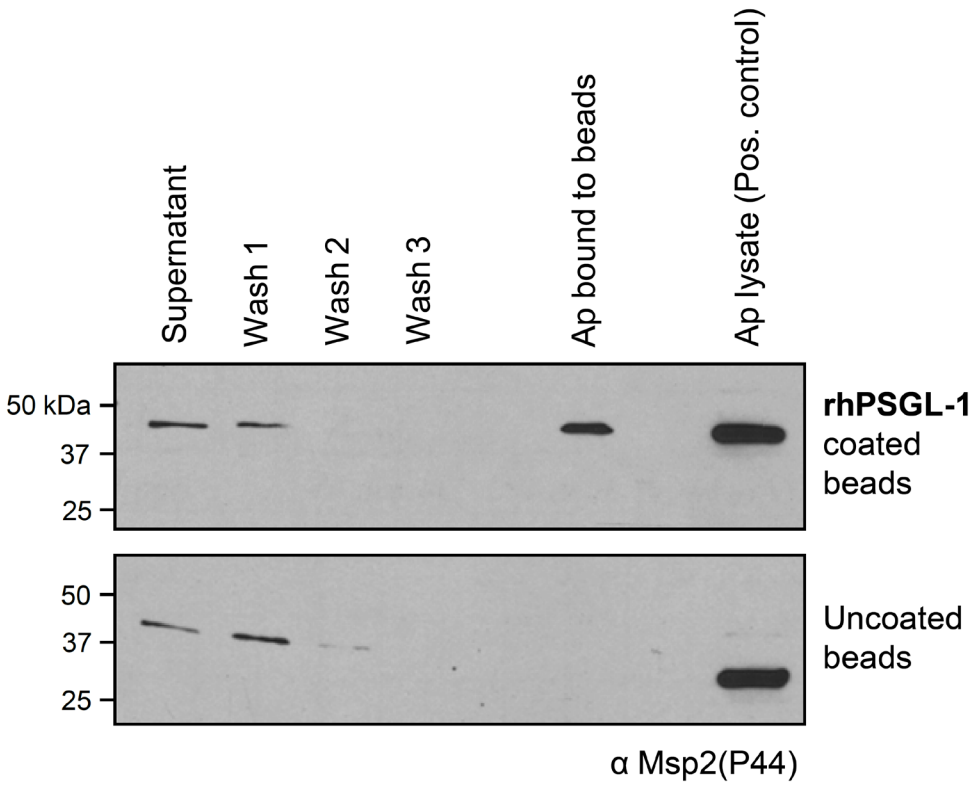


Figure 14. ***A. phagocytophilum*** **binds to rhPSGL-1 immobilized on beads.** Protein A/G beads were coated with (rhPSGL-1 coated) or without (uncoated) rhPSGL-1 and blocked with 5% BSA/PBS. *A. phagocytophilum* RC and DC organisms were mixed in 1% BSA/PBS with rhPSGL-1 coated and uncoated beads for 1 h. Beads were pelleted and washed to remove unbound organisms, then lysed in RIPA buffer to solubilize any remaining bound organisms. Samples were resolved by SDS-PAGE and examined by western blot for Msp2(P44) to determine if organisms bound to the beads. The initial supernatant, washes and beads were checked for organisms. *A. phagocytophilum* lysate served as a positive (Pos.) control for Msp2(P44) detection. Representative results of one of two separate experiments are shown.



washes nor was any recovered by the Protein A/G beads, suggesting that *A. phagocytophilum* adhesin(s) cannot bind soluble rhPSGL-1. Under reducing conditions rhPSGL-1 typically migrates around 125 kDa, but occasionally multimers around 250 kDa can still be detected (**Fig 13**).

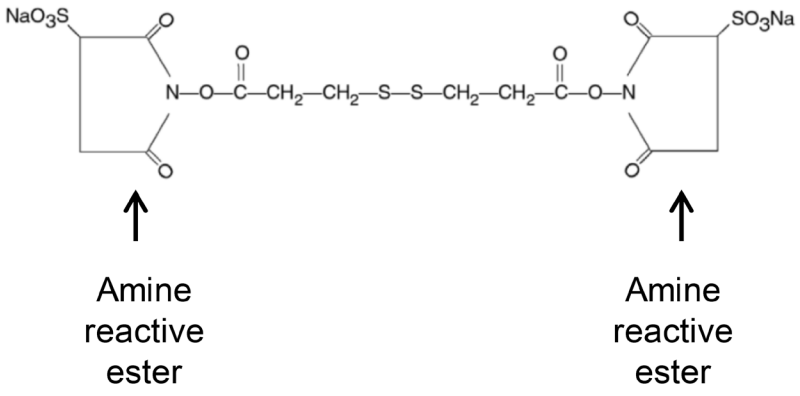
We next tested the organism's ability to bind rhPSGL-1 immobilized on Protein A/G beads. Protein A/G beads were mixed with rhPSGL-1, resulting in Protein A/G binding to the IgG_1 Fc receptor fused to rhPSGL-1, thus coating the beads with rhPSGL-1. Beads coated with (rhPSGL-1 coated) and without (uncoated) rhPSGL-1 were blocked in BSA and then mixed with *A. phagocytophilum*. After 1 h, the supernatant was removed and the beads were washed and analyzed by western blot for organisms bound to the beads (**Fig. 14**). *A. phagocytophilum* could be detected specifically binding to the beads coated with rhPSGL-1, whereas organisms did not bind to beads that lacked rhPSGL-1 (uncoated).

Crosslinking approach to capture *A. phagocytophilum* PSGL-1 adhesins.

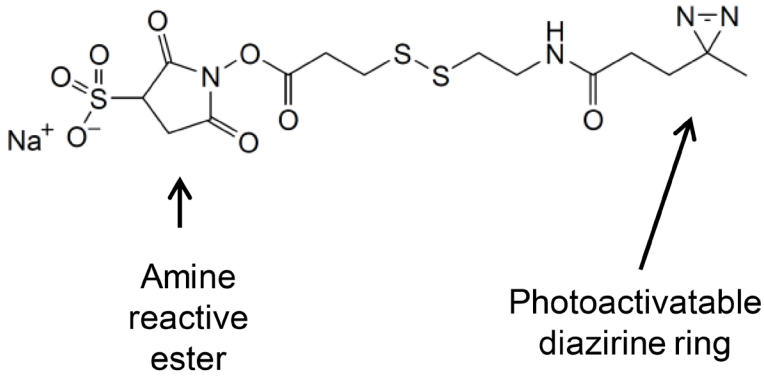
Having determined that *A. phagocytophilum* organisms bind rhPSGL-1 immobilized to Protein A/G beads, we next sought to use this binding event in adjunct with chemical crosslinking. The principle of the approach is to allow organisms to bind rhPSGL-1, at which time a crosslinker is added to form a covalent bond between rhPSGL-1 and bacterial adhesin(s). The advantage of this approach is binding occurs in a native state. Two commercially available crosslinkers, Dithiobis(sulfosuccinimidylpropionate) (DTSSP) and Sulfo-NHS-SS-Diazirine (Sulfo-SDAD) were used in this study (**Fig. 15**).

Figure 15. Crosslinkers utilized during study. Two commercially available crosslinkers were used in this study. (**A**) DTSSP is a homobifunctional NHS-ester primary amine-reactive crosslinker with a spacer arm length of 12 angstroms (Å). (**B**) Sulfo-SDAD is a heterobifunctional crosslinker. The NHS-ester reacts with primary amines, whereas the diazirine ring when exposed to UV light creates a reactive carbene intermediate to form a covalent bond with any amino acid side chain or peptide backbone within the spacer arm distance of 13.6 Å. Both crosslinkers are water soluble and membrane impermeable. Each crosslinker contains a disulfide bond, allowing for separation of any crosslinked proteins for downstream analysis.

A.



B.

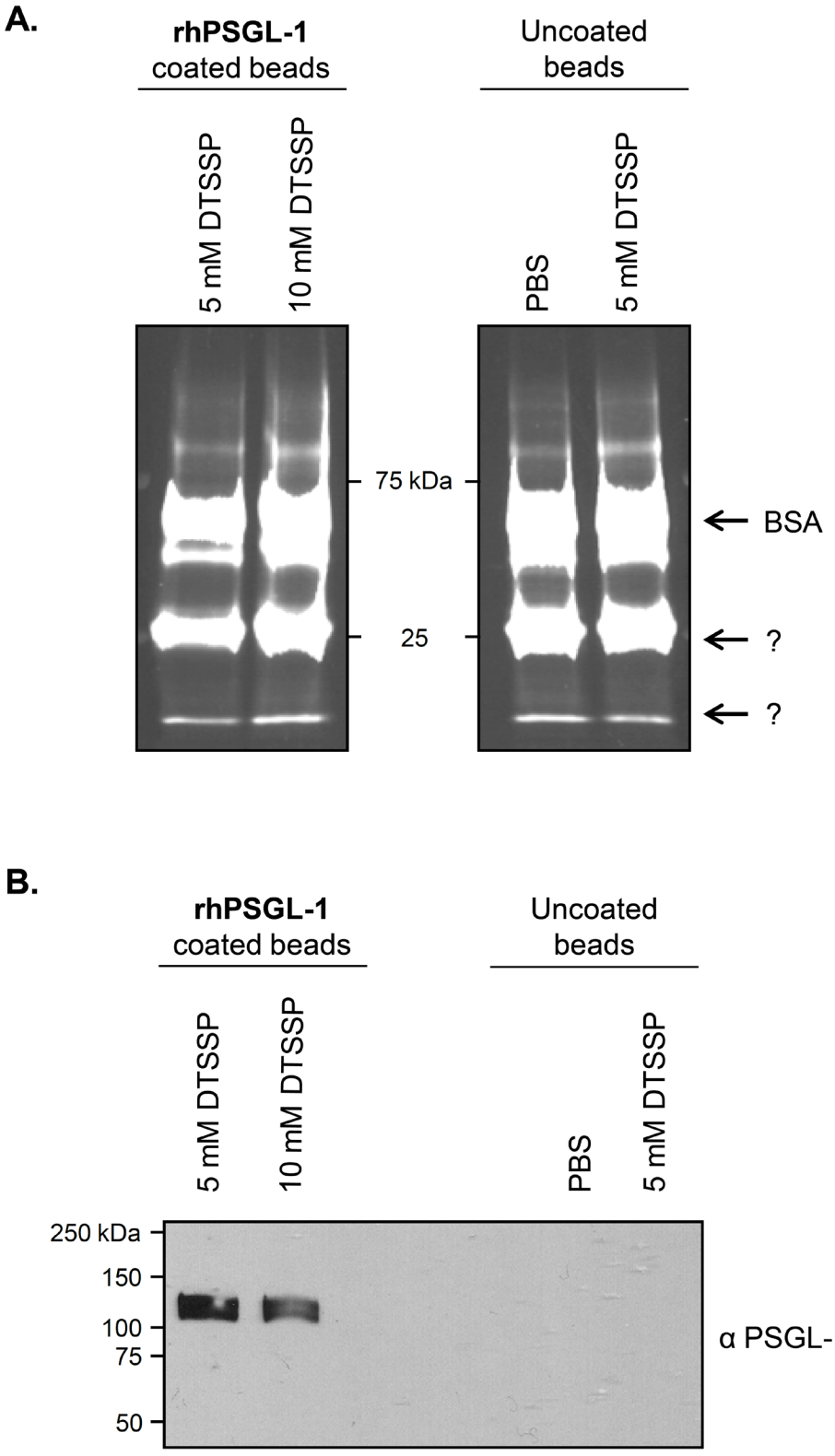


Both crosslinkers contain n-hydroxysuccinimide-ester (NHS-ester) that react with amines, which are typically found in high abundance in proteins due to the amino acid lysine (172).

To identify adhesin(s) by crosslinking, we mixed *A. phagocytophilum* organisms with rhPSGL-1 coated or uncoated beads to allow for bacterial binding, followed by the addition of 5 or 10 mM DTSSP crosslinker to covalently crosslink *A. phagocytophilum* adhesin(s) to rhPSGL-1. Bacteria were solubilized, leaving the crosslinkers' covalent bond between any adhesin(s) and rhPSGL-1 intact. Beads with rhPSGL-1 still bound along with any crosslinked adhesin(s) were recovered. The recovered beads were analyzed by two methods to determine if adhesin(s) were captured with crosslinking (**Fig. 16**).

We first boiled the recovered beads under reducing (with BME) conditions to break the disulfide bond of the crosslinker to release any adhesin(s) from rhPSGL-1. Samples were resolved by SDS-PAGE gel and stained with Sypro Ruby protein stain (**Fig. 16A**) which has a sensitivity of 1 nanogram (ng), to detect any recovered adhesin(s). All samples including the negative control of uncoated beads, to which *A. phagocytophilum* cannot bind and which no crosslinker was incubated, looked identical. We determined that the BSA that is required to block the beads and achieve specificity of *A. phagocytophilum* binding to rhPSGL-1, is boiled off the beads resulting in very high background staining. The prominent band of 66 kDa, the known size of BSA, as well as several other BSA breakdown products are detected. Because of the high background caused by BSA, we cannot exclude the possibility it could be masking recovered adhesin(s) of low abundance. As an alternative, we boiled the recovered beads under nonreducing conditions (No BME) to elute rhPSGL-1 off the beads, keeping the crosslinker intact between rhPSGL-1 and any

Figure 16. *A. phagocytophilum* adhesins are not crosslinked to rhPSGL-1 by DTSSP. Protein A/G beads were coated (rhPSGL-1 coated) with or without (uncoated) rhPSGL-1 and blocked with 5% BSA/PBS. *A. phagocytophilum* RC and DC organisms were mixed in 1% BSA/PBS with rhPSGL-1 coated or uncoated beads for 1 h. Beads were washed to remove unbound organisms followed by the addition of 5 or 10 mM DTSSP crosslinker to covalently crosslink *A. phagocytophilum* adhesin(s) bound to or within proximity of rhPSGL-1. Samples were lysed in RIPA buffer to solubilize the bacteria, which does not disturb the covalent bonds between any adhesin(s) crosslinked to rhPSGL-1. Beads were then washed and recovered (rhPSGL-1 remains bound to beads along with any crosslinked adhesins). Samples were analyzed by Sypro Ruby protein stain (**A**) and western blot (**B**). (**A**) Recovered beads were boiled under reducing (with BME) conditions to elute any recovered *A. phagocytophilum* adhesin(s) crosslinked to rhPSGL-1 and resolved by SDS-PAGE gel. Gel was stained with Sypro Ruby protein stain and examined for recovered proteins. Uncoated beads serve as the negative control. Representative results of one of two separate experiments are shown. (**B**) Recovered beads were boiled under non-reducing (No BME) conditions to elute rhPSGL-1 from beads along with any attached adhesin(s). Samples were analyzed by western blot to detect any shifts in rhPSGL-1, which would indicate crosslinking had occurred. Uncoated beads serve as the negative control. Representative results of one of two separate experiments are shown.



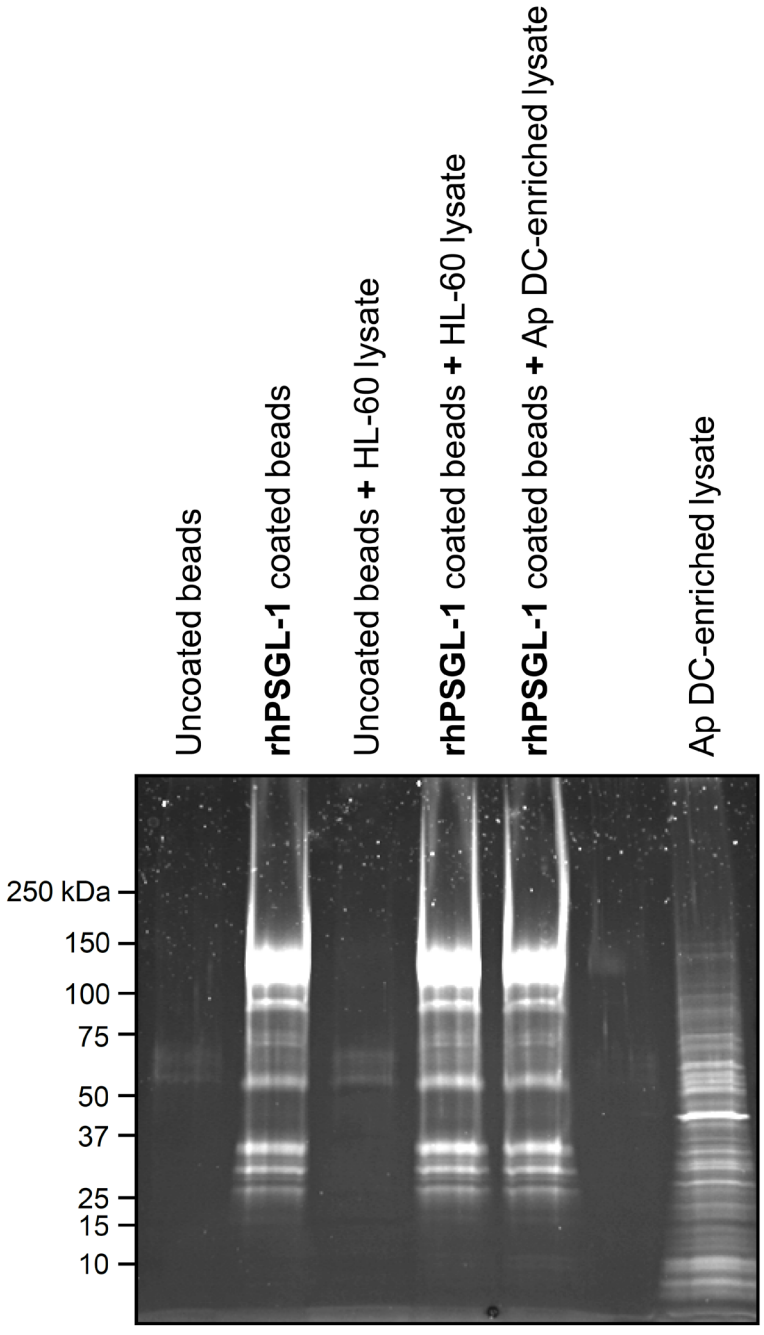
adhesin(s). Samples were resolved by SDS-PAGE gel and analyzed by western blot (**Fig. 16B**) to look for shifts in rhPSGL-1, which would indicate crosslinking of rhPSGL-1 to an adhesin(s) occurred. No shifts in rhPSGL-1 were detected. We also tested by western blot and determined that not even the dominant (Msp2[P44]) surface protein of *A. phagocytophilum*, was recovered with DTSSP crosslinking (data not shown).

A variety of alternatives conditions were taken to try and troubleshoot these approaches (data not shown). These included blocking rhPSGL-1 coated beads with dry milk, and testing bacterial binding in the presence of tween-20, neither of which were successful. One disadvantage of using the DTSSP crosslinker is its dependence on free amines for crosslinking. Because we do not know the surface characteristics of *A. phagocytophilum*, we cannot exclude the possibility there are only a few accessible amines. Therefore we tested the Sulfo-SDAD crosslinker (**Fig. 15B**) which can react with primary amines and any amino acid side chain or peptide backbone. We allowed organisms to bind rhPSGL-1 coated beads, then added Sulfo-SDAD post binding. Alternatively, we labeled organisms with the amine reactive end of Sulfo-SDAD, allowed the organisms to bind rhPSGL-1 coated beads, then activated the diazirine ring. Sulfo-SDAD was also unsuccessful (data not shown).

Immunoprecipitation approach to capture *A. phagocytophilum* PSGL-1 adhesins.

Since crosslinking was unsuccessful in capturing adhesin(s), we next tested immunoprecipitation (IP) as an alternative approach to identify *A. phagocytophilum* adhesin(s). We had found that we can enrich for the adherent DC organisms by

Figure 17. Immunoprecipitation was unsuccessful in recovering *A. phagocytophilum* adhesins. Protein A/G beads were coated (rhPSGL-1 coated) with or without (uncoated) rhPSGL-1. *A. phagocytophilum* DC-enriched organisms or HL-60 controls were lysed in non-denaturing lysis buffer and incubated with beads overnight. Beads were washed to remove unbound proteins and then boiled under reducing conditions to elute any adhesin(s) bound to rhPSGL-1. Samples were resolved by SDS-PAGE gel and stained with Sypro Ruby to detect recovered proteins. The *A. phagocytophilum* DC-enriched lysate mixed with rhPSGL-1 coated beads was resolved in the far right lane. Representative results of one of five separate experiments are shown.



sonication of infected cells, which destroy the fragile RC and leave the resistant DC intact (79). DC-enriched organisms were recovered and lysed in non-denaturing lysis buffer. The soluble fraction was then mixed overnight with rhPSGL-1 coated beads. Beads were recovered, washed to remove unbound proteins and boiled to elute any captured adhesin(s). Samples were resolved by SDS-PAGE gel and stained with Sypro Ruby (**Fig. 17**). Because we did not use intact organisms in this experiment it was not necessary to block the beads with BSA, so we did encounter the high background issues as with the crosslinking approach. We did not detect any recovered proteins with this approach. A prominent band of 125 kDa (known size of rhPSGL-1) is detected, as well as several other rhPSGL-1 breakdown products. The non-denaturing lysis buffer is sufficient in lysing DC-enriched organisms as the prominent surface protein Msp2(P44) is detected in the lysate control. This approach was also tested with *A. phagocytophilum* DC lysates generated with denaturing lysis buffer, but was also unsuccessful.

DISCUSSION

Our data was able to demonstrate that *A. phagocytophilum* organisms only adhere to PSGL-1 immobilized on a surface. It is not surprising that *A. phagocytophilum* adhered to rhPSGL-1 coated beads, as these beads fundamentally mimic HL-60 cells, to which *A. phagocytophilum* readily binds (173). *A. phagocytophilum* can also bind PSGL-1 glycopeptides immobilized on beads (194), and others in the Carlyon lab determined that *A. phagocytophilum* binds rhPSGL-1 coated to the well of an enzyme-linked immunosorbent assay (ELISA) plate (unpublished data). Although we used both RC and

DC organisms in many of these binding experiments, we are confident that only DC organisms bind to rhPSGL-1.

Even though we were unsuccessful in identifying adhesins by crosslinking, it is an ideal approach as it allows organisms to bind their receptor in a natural state. Other than the accessibility of binding sites on proteins for the crosslinker, they have no bias or selective conditions in which to crosslink proteins. As long as two proteins with free amines are in close enough proximity, they should become crosslinked. Both DTSSP and Sulfo-SDAD were tested on *A. phagocytophilum* organisms and shifts in Msp2(P44) were detectable by western blot (data not shown). This indicates the crosslinkers were properly functioning and that Msp2(P44) has accessible amines. The extreme N-termini of rhPSGL-1 is in close proximity to the region where *A. phagocytophilum* organisms bind, which should have been ideal for crosslinking. Crosslinkers should have been sufficient to recover both soluble and insoluble proteins. The αPSGL-1 Ab (KPL-1) is extremely sensitive and can detect as little as 0.05 μg of protein (unpublished data). Even if adhesin(s) were recovered in low abundance, shifts in rhPSGL-1 should have been detectable (**Fig. 16B**).

There are several reasons why crosslinking may have been unsuccessful. First, when *A. phagocytophilum* organisms bind PSGL-1, it may do so in a manner where the whole organism sterically hinders accessibility of the crosslinker from gaining access to the receptor-adhesin interaction site. Second, free amines have to occur within the distance of the spacer arm of the crosslinker for crosslinking to occur. If the crosslinker is too short, and there are not enough accessible amines within proximity of each other,

crosslinking will not occur. This should have been less of a problem with the crosslinker Sulfo-SDAD, as it binds amines at one end and any amino acid at the other end. Nevertheless, use of the crosslinker post binding and labeling organisms pre binding to rhPSGL-1 was unsuccessful in recovering adhesin(s). Others in the Carlyon lab also tested labeled rhPSGL-1 with crosslinkers first, allowed organisms to bind, then activated the crosslinker to capture adhesin(s) (unpublished data). This approach was also unsuccessful.

The use of bacterial lysates has also proven to be equally important in identifying adhesins (111, 146). The biggest limitation of this approach is the adhesin(s) have to be soluble, as all insoluble material from lysates is discarded. Given the resistant properties of the *A. phagocytophilum* DC, we cannot exclude the possibility some surface proteins are insoluble and not recovered. However, the facts soluble bacterial lysates did not bind rhPSGL-1 (**Fig. 17**) and intact organisms did not bind soluble rhPSGL-1 (**Fig. 13**) suggest the affinity of the adhesin or receptor interaction in soluble form is too weak to occur. When intact organisms bind immobilized PSGL-1 it is a specific and strong interaction. We believe that multiple adhesins may be involved in binding, but we do not know the affinity of a given adhesin for its respective receptor determinant. A specific adhesin-receptor interaction could be individually weak, but the synergistic effect of all the adhesins working together may support strong binding of intact organisms. If this were true, once the adhesins are isolated from the membrane they may not have a strong enough affinity for their individual receptor determinants to be isolated and identified.

The structural requirements required for binding at this time are also unknown. We cannot exclude the possibility that an adhesin could be a complex of proteins. Surface

proteins within a heterodimer complex of the veterinary pathogen *A. marginale* were found to be an adhesin for erythrocytes (21, 38, 91). If a complex of proteins is required for *A. phagocytophilum* binding, this will be disrupted when generating lysates. We also cannot exclude the importance of protein conformation during binding. When P-selectin binds sialic acid on PSGL-1, a conformational epitope is required (161). It is possible that the structural requirements of the adhesin required for binding sLe^x-modified PSGL-1 is only found on intact organisms and when denatured may result in the loss of adhesin activity. Non-denaturing lysis buffers should increase the chance of recovering proteins in a native state with IPs (22), but did not appear successful when tested with *A. phagocytophilum* (**Fig. 17**).

In conclusion, we determined adherence of *A. phagocytophilum* to sLe^x-modified PSGL-1 must occur on a fixed surface, whether it is HL-60 cells, beads or ELISA plates. The data presented here as well as other unpublished data from the Carlyon lab, demonstrates known methods used in the recovery of other bacterial adhesins were unsuccessful in recovering *A. phagocytophilum* adhesins. *A. phagocytophilum* adhesion to PSGL-1 appears to be a very complex scenario, one that we are determined to solve.

METHODS

Mixing soluble rhPSGL-1 with *A. phagocytophilum*. Ap RC and DC organisms (strain NCH-1) were recovered by syringe lysis as described (27) from 200 ml of culture and mixed in a total of 100 microliters (µl) PBS containing 3 micrograms (µg) rhPSGL-1

(R&D Systems; Minneapolis, MN, Cat. No. 3345-PS) with protease inhibitors (Roche; Indianapolis, IN, Cat. No. 118361153001) for 3 h at 4°C with rotation. Bacteria were pelleted at 10,000 x *g* for 6 min at 4°C and were washed 2 times with 1 ml cold PBS (supernatant from initial spin and washes were saved for western blot analysis). Bacteria were lysed in 1 ml of radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM sodium chloride [NaCl], 1 mM EDTA, 1 mM sodium orthovanadate [Na_3VO_4] and 1mM sodium fluoride[NaF]) with protease inhibitors for 45 min on ice. Insoluble material was removed at 10,000 x *g* for 10 min after which the soluble fraction was mixed with 25 µl of Protein A/G beads (Pierce; Waltham, MA, Cat. No. 20421) overnight (O/N) at 4°C with rotation. Beads were pelleted at 3,000 x *g* at 4°C for 1 min and washed with 1 ml cold PBS 3 times. Forty µl of Laemmli 2X sample buffer (Bio-Rad; Hercules, CA, Cat. No. 161-0737) with 5% BME (Fisher Scientific; Pittsburgh PA, Cat. No. BP176-100) was added to beads and boiled for 5 min at 100°C. Beads were pelleted at 3,000 x *g* for 1 min, supernatant was saved and analyzed for rhPSGL-1. 25 µl of supernatant (wash steps) was mixed with equal volumes of 2X sample buffer with 5% BME and boiled for 5 min at 100°C.

Samples were resolved on 4-15% Tris-HCl gel (Bio-Rad Cat. No. 161-1158) at 115v for 15 min and 130v for 45 min and then transferred to nitrocellulose at 100v for 1 h. The blot was blocked in 5% milk in tris-buffered saline with 5% tween-20 (TBS-t[0.5%]) O/N at 4°C. Primary antibody (Ab) mouse anti-PSGL-1 (KPL-1) (BD Pharmingen; San Diego, CA, Cat. No. 556053) was added at room temperature (RT) for 1 h at 1:2,000 dilution in 1% milk-TBS-t(0.5%). The blot was washed for 5, 5, 15, 5, and 5 min in TBS-

t(0.5%). Secondary Ab anti-mouse IgG horseradish peroxidase (HRP)-conjugated (Cell Signaling; Danvers, MA, Cat. No. 7076) was added at RT for 1 h at 1:30,000 dilution in 1% milk-TBS-t(0.5%). The blot was washed for 5, 5, 15, 5, and 5 min in TBS-t(0.5%). The blot was incubated with SuperSignal West Pico chemiluminescent substrate (Pierce, Cat. No. 34080) for 5 min and exposed to film (Pierce, Cat. No. 34091).

***A. phagocytophilum* binding to Protein A/G beads coated with rhPSGL-1.** 10 µg of rhPSGL-1 was mixed with 25 µl of Protein A/G beads in 1 ml PBS with protease inhibitors O/N. The next day, beads were pelleted at 3,000 x *g* for 30 seconds (sec) and supernatant discarded. 1 ml of 5% bovine serum albumin (BSA)/PBS was added for 1 h at RT with rotation to block beads. Uncoated beads were prepared in same manner without the addition of rhPSGL-1. Ap RC and DC organisms (strain NCH-1) were recovered by syringe lysis as described (27) from 120 ml of culture and split equally with rhPSGL-1 coated and uncoated beads in 1 ml of 1% BSA/PBS for 1 h at RT with rotation. Beads were pelleted at 1,000 x *g* for 1 min at RT and washed 3 times with 1 ml PBS. Supernatant from initial spin and washes were saved and centrifuged at 10,000 x *g* for 6 min to test for Ap. rhPSGL-1 coated, uncoated beads and Ap recovered from supernatant and washes were lysed in 250 µl of RIPA buffer with protease inhibitors for 45 min on ice. Insoluble material and beads were removed by spinning at 10,000 x *g* for 10 min. Twenty-five µl of supernatants were mixed with 25 µl of Laemmli 2X sample buffer with 5% BME and boiled for 5 min at 100°C.

Samples were resolved on 4-15% Tris-HCl gel at 115v for 15 min and 130v for 45 min and transferred to nitrocellulose at 100v for 1 h. Blot was blocked in 5% milk-TBS-t(0.5%) O/N at 4°C. Primary Ab rabbit anti-Ap was added at RT for 1 h at 1:10,000 dilution in 1 % milk-TBS-t(0.5%). The blot was washed for 5, 5, 15, 5, and 5 min in TBS-t(0.5%). Secondary Ab anti-rabbit IgG HRP-conjugated (Cell Signaling, Cat. No. 7074) was added at RT for 1 h at 1:20,000 dilution in 1 % milk-TBS-t(0.5%). The blot was washed for 5, 5, 15, 5, and 5 min in TBS-t(0.5%). The blot was incubated with SuperSignal West Pico chemiluminescent substrate for 5 min and exposed to film.

Crosslinking *A. phagocytophilum* adhesins to rhPSGL-1 with DTSSP. Ap (strain NCH-1) was mixed with rhPSGL-1 coated and uncoated beads as described above. Unbound organisms were washed off and beads were split equally into two samples and resuspended in 1 ml of 5 or 10 mM DTSSP (Pierce, Cat. No. 21578) for rhPSGL-1 coated beads or PBS and 5 mM DTSSP for uncoated beads and incubated for 30 min at RT with rotation. Tris was added to a final concentration of 20 mM for 15 min to quench crosslinker. Beads were pelleted at 1,000 x *g* for 1 min at RT, and washed with 1 ml cold PBS. Beads were lysed in 250 µl RIPA buffer with protease inhibitors for 45 min on ice. Beads (with rhPSGL-1 still bound) were pelleted at 1,000 x *g* for 1 min and washed 4 times in 1 m of cold PBS. 40 µl of Laemmli 2X sample buffer with 10% BME was added and boiled for 5 min at 100°C.

Beads were pelleted at 3,000 x *g* for 1 min, supernatant was saved and analyzed for recovery of Ap adhesin(s). Samples were resolved on 4-15% Tris-HCl gels at 115v for 15

min and 130v for 45 min. Gels were stained with Sypro Ruby (Invitrogen; Carlsbad, CA, Cat. No. S-12000) according to manufactures instructions and imaged with trans UV on ChemiDoc XRS imager (Bio-Rad). In some experiments, samples were eluted from beads under non-reducing conditions (No BME), resolved by SDS-PAGE and transferred to nitrocellulose at 100v for 1 h. Blots were screened with α PSGL-1 (KPL-1 Ab) as described above.

Immunoprecipitation with rhPSGL-1 coated beads using non-denatured *A. phagocytophilum* lysate. rhPSGL-1 coated and uncoated beads were prepared as described above. Ap DC-enriched organisms (strain NCH-1) were recovered from 400 ml of culture by sonication as described (79). Ap was lysed in 1 ml of non-denaturing lysis buffer (1% w/v Triton-X-100, 0.02% w/v sodium azide, 50 mM Tris-HCl pH 7.4, 300 mM NaCl, 5 mM EDTA, 10 mM iodoacetamide, 2 µg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride [PMSF]) at 4°C for 45 min with rotation. Insoluble material was removed by spinning at 10,000 x *g* for 10 min. Supernatants were split equally among samples. Equal numbers of uninfected HL-60 cells were prepared in the same manner as infected cells to serve as negative controls. All samples were mixed with rhPSGL-1 coated or uncoated beads in a total of 1 ml non-denaturing lysis buffer O/N at 4°C with rotation. The next day, beads were pelleted at 1,000 x *g* for 1 min and washed 3 times with 1 ml cold PBS. 40 µl of Laemmli 2X sample buffer with 5% BME was added to the washed beads and boiled for 5 min at 100°C. Beads were pelleted at 3,000 x *g* for 1 min, supernatant was saved and analyzed for recovery of Ap adhesin(s). Samples were

resolved on 4-15% Tris-HCl gels at 115v for 15 min and 130v for 45 min. Gels were stained with Sypro Ruby according to manufactures instructions and imaged with trans UV on ChemiDoc XRS imager (Bio-Rad).

CHAPTER 5: Identification of Putative *A. phagocytophilum* Dense-Cored Cell Outer Membrane Proteins by Density-gradient Centrifugation, Biotinylation and Mass Spectrometry

INTRODUCTION

In view of the fact that we were unable to capture or purify *A. phagocytophilum* adhesins by receptor crosslinking and affinity purification, we next chose to take a broader approach to identify OMPs of the adherent DC. The biphasic development cycle exhibited by *A. phagocytophilum* was first documented to occur and is most well studied in *Chlamydia* species. For some research purposes, it is essential to purify these intracellular bacteria away from their host cell components. Separation of the noninfectious RB and infectious EB developmental forms of *Chlamydia* from each other and removal of host cell debris has been accomplished by the use of Renografin density-gradient centrifugation (99, 156). Separation of different developmental forms and removal of host cell debris with density-gradient centrifugation has also been accomplished for intracellular pathogens within the order *Rickettsiales* as well as for *Coxiella burnetii* (65, 187-190, 198).

Due to the relatively recent discovery of human infections in 1994 (34), standardized techniques in use for other organisms have not yet been fully developed for the study of *A. phagocytophilum*. An example is the inability thus far to separate the *A. phagocytophilum* replicative RC and the adherent DC forms from each other. To date,

Renografin density-gradient centrifugation has only been able to separate both RC and DC organisms mixed together from host cell debris (27). To identify OMPs of the adherent DC form we chose to use the sonication resistant properties of the DC for their selection, followed by Renografin discontinuous density-gradient centrifugation to remove host cell debris. We then used biotinylation to label surface proteins, a proven method previously validated to work with *A. phagocytophilum* and other organisms (25, 55, 88, 138, 141, 153). Biotinylated OMPs were purified and then identified by mass spectrometry (MS).

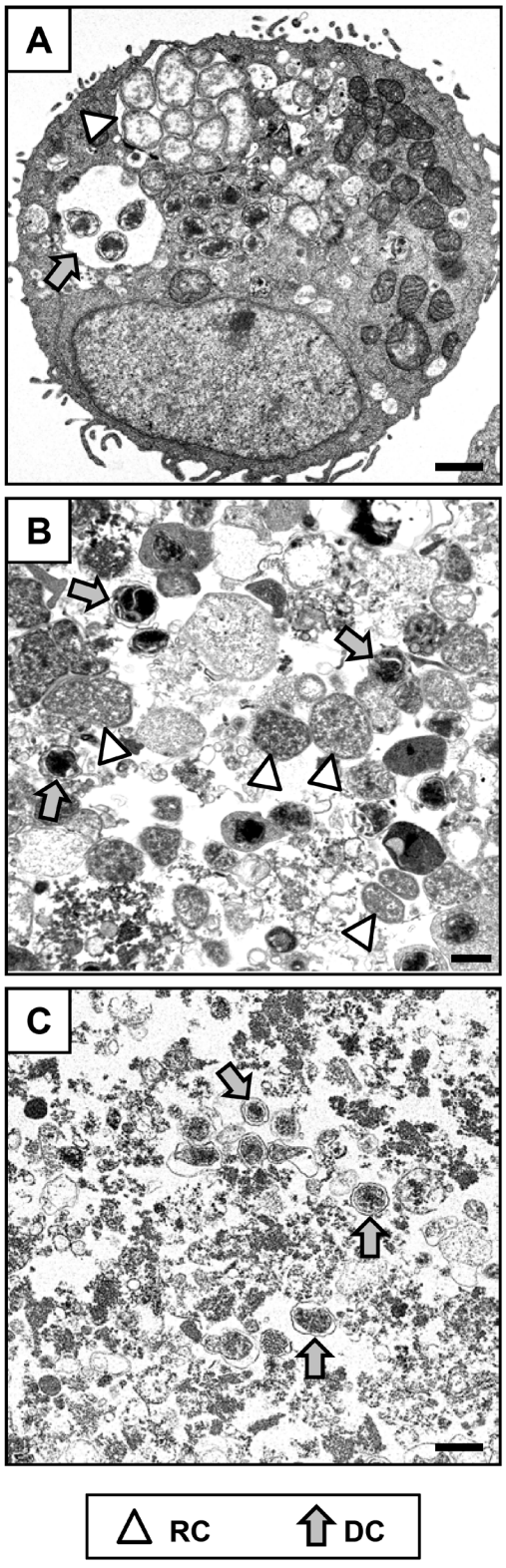
RESULTS

The *A. phagocytophilum* dense-cored cell is resistant to sonication.

The infectious EB of *Chlamydia* is very environmentally resistant and stable due to extensive crosslinking of disulfide bonds within its membrane (67). The EB's stability allows it to survive treatments with both detergents and sonication that destroy the fragile replicative RB (68, 156, 167). To determine if the *A. phagocytophilum* DC is environmentally resistant, we compared the conventional isolation method of syringe lysis to sonication. Samples were monitored for RC and DC organisms by TEM before isolation, after syringe lysis and after sonication.

Organisms for experiments are isolated from cultures of asynchronously infected cells (**Fig. 18A**) containing both RC and DC organisms. The abundance of RC and DC organisms in a given culture before use in experiments cannot be determined at this time, as there are no antibodies or differently expressed markers to distinguish between the two developmental forms. Data from Chapter 2 demonstrates that even from a synchronous

Figure 18. ***A. phagocytophilum*** **organisms at various stages of isolation examined by transmission electron microscopy.** (**A**) Infected HL-60 cells. Infected cells containing both RC and DC organisms are the starting material for all experiments. (**B**) Syringe lysis. Both RC and DC organisms mixed with some host cell debris were recovered by syringe lysis. (**C**) Sonication. Infected cells were sonicated to destroy the host cell and fragile RC organisms, leaving the environmentally resistant DC intact. White arrowheads denote RC and grey arrows denote DC organisms. All scale bars 1 µm.



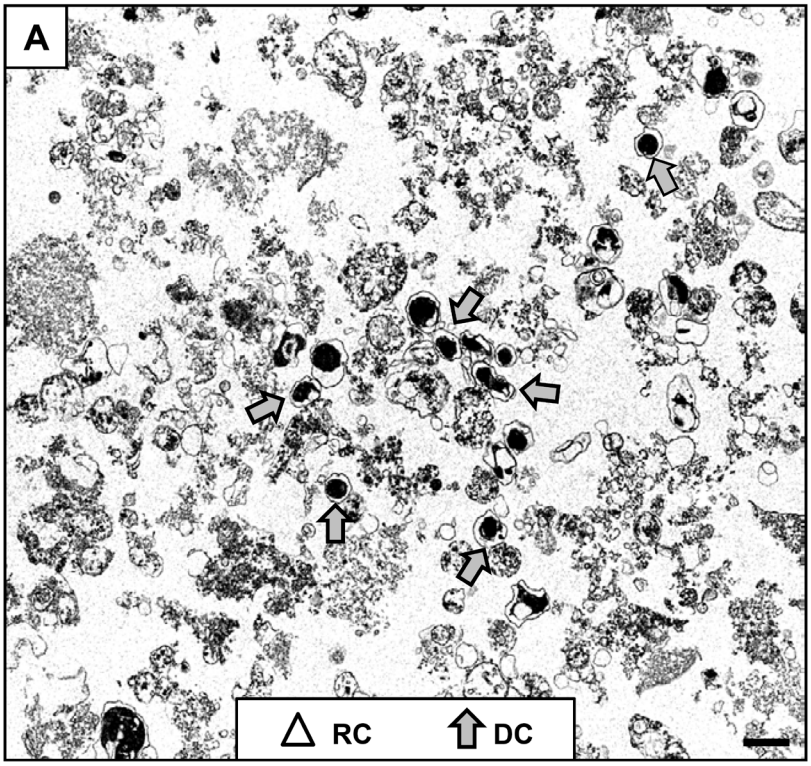
infection, the best time to recover the greatest abundance of organisms is late in infection after the culture has become asynchronous and the cells are heavily infected (**Fig. 8B**). The population of the DC in these asynchronous cultures is approximately 30-55% (**Table 1**). The standard method of isolating *A. phagocytophilum* for experiments is syringe lysis. We observed that this method is best suited for recovery of a mixture of both RC and DC organisms, which is also mixed with host cell debris (**Fig. 18B**). To test whether or not the *A. phagocytophilum* DC is environmentally resistant, former Carlyon lab member Nathan Galloway sonicated infected cells from 32 up to160 sec. The DC was able to remain visually intact even after 160 sec of sonication as observed by TEM (data not shown). We chose to use 64 sec of sonication for experiments as this was the minimum time required for destruction of the host cells and RC organisms (**Fig. 18C**).

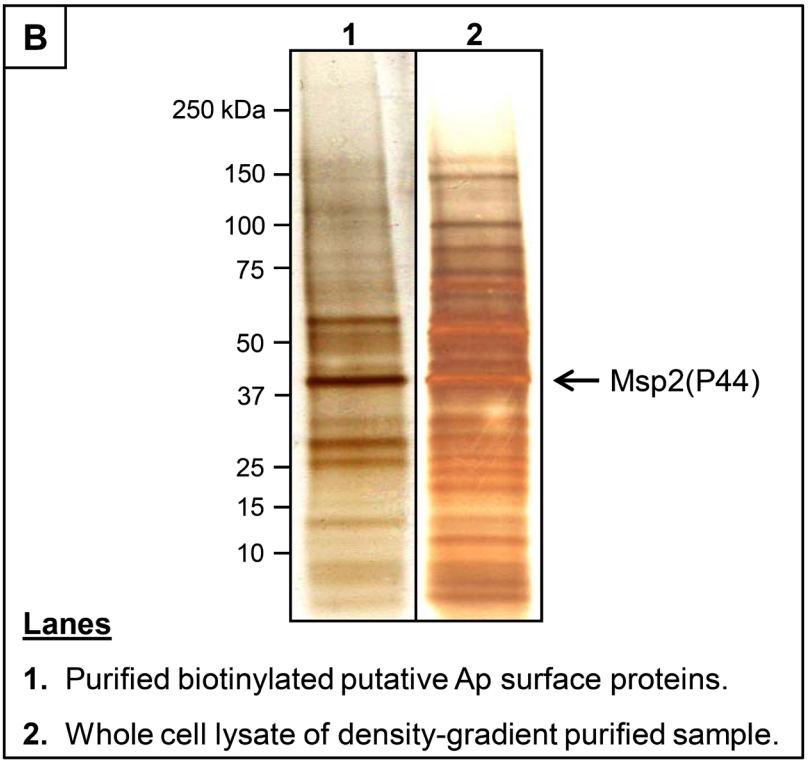
Renografin purification and biotinylation of *A. phagocytophilum* DC OMPs.

Establishing that sonication was an efficient means to destroy RC organisms while leaving the DC form intact, we next used this technique in combination with the published protocol of Renografin discontinuous density-gradient centrifugation (27) to purify the DC organisms and reduce the amount of cellular debris. **Figure 19A** is a representative TEM image of sonicated DC organisms recovered after Renografin purification.

The purified DC organisms were then mixed with an excess of the water soluble, non-membrane permeable Sulfo-NHS-SS-Biotin to label OMPs. Bacteria were solubilized in RIPA buffer, and the soluble fraction was mixed with NeutrAvidin beads to recover

Figure 19. Density-gradient purified DC organisms and recovered biotinylated putative surface proteins. Infected cells were sonicated to enrich for DC organisms, and then purified by Renografin discontinuous density-gradient centrifugation. (**A**) TEM image of DC organisms recovered between the 30% - 42% Renografin interface. Grey arrows denote DC organisms. Scale bar 1 µm. (**B**) Renografin density-gradient purified DC OMPs were labeled with the non-membrane permeable Sulfo-NHS-SS-Biotin. Bacteria were lysed in RIPA buffer and soluble biotinylated proteins were purified with NeutrAvidin Beads. Recovered biotinylated proteins were resolved by SDS-PAGE and examined by silver stain (lane **1**). A whole cell lysate control was resolved in lane **2**. The dominant Ap surface protein Msp2(P44) is indicated. Representative results of one experiment are shown.





biotinylated proteins. Recovered proteins were resolved by SDS-PAGE and examined by silver stain. Selective enrichment of specific proteins can be observed in **lane 1** when compared to the whole cell lysate control of **lane** 2 (**Fig. 19B**). The dominant *A. phagocytophilum* surface protein Msp2(P44) is clearly enriched with biotinylation, as would be expected due to its known surface localization.

Identification of recovered proteins by mass spectrometry.

To identify recovered *A. phagocytophilum* proteins, we chose to use a type of MS referred to as multi-dimensional protein identification technology (MudPIT). MudPIT is suited to identify hundreds of proteins from complex mixtures (108). It allows peptides to be separated by 2D liquid chromatography, thus eliminating the need to resolve samples by gels. From our biotinylation approach, MudPIT identified a total of 55 *A. phagocytophilum* proteins (**Table 5**). However, only 16 of these proteins can be confidently accepted as an authentic result with a 1% chance of error. For proteins to be considered authentic they should have a confidence score of above 300 with several recovered peptides. Proteins below this threshold have a higher probability of being a false positive. MudPIT also confidently identified 153 human proteins (data not shown), indicating that the sample still had considerable host cell debris contamination.

DISCUSSION

Our data demonstrates that the *A. phagocytophilum* DC is environmentally resistant and is capable of surviving mechanical means of disruption, a property that we were able

Table 5. Putative Ap biotinylated OMPs identified by MudPIT

	Ap gene	kDa[a]	Protein description	Score	Peptides	Coverage[b]
1	APH_1221	45.8	P44 18ES OMP	20,608	131	78
2	APH_1235	14.7	hypothetical protein	4,191	33	92
3	APH_0240	58.2	chaperonin GroEL	1,437	69	69
4	APH_0494	51.5	ATP synthase subunit	641	32	59
5	APH_0405	57.5	hypothetical protein (Asp55)	490	27	46
6	APH_1087	26.1	putative lipoprotein	458	10	37
7	APH_1032	42.8	elongation factor Tu	415	19	45
8	APH_1190	18.8	ATP synthase subunit	415	2	14
9	APH_0404	63.6	hypothetical protein (Asp62)	413	21	27
10	APH_0397	32.1	30S ribosomal protein S2	406	12	33
11	APH_0036	22.6	co chaperone GrpE	395	4	33
12	APH_1404	46.9	T4SS protein VirB10	389	8	23
13	APH_0346	69.7	chaperone protein DnaK	381	25	34
14	APH_0248	13.8	hypothetical protein	359	10	58
15	APH_1049	23.3	major surface protein 5	354	4	22
16	APH_1334	54.1	ATP synthase subunit alpha	312	30	35
17	APH_0051	37.3	iron binding protein	253	4	15
18	APH_0853	10.8	hypothetical protein	250	4	63
19	APH_0625	34.7	immunogenic protein	229	6	29
20	APH_1050	37.6	putative ABC transporter	221	3	17
21	APH_1246	52.4	glutamine synthetase type I	216	9	10
22	APH_1232	45.6	citrate synthase I	214	5	20
23	APH_0600	61.5	thiamine biosynthesis - ThiC	203	4	11
24	APH_0059	39.2	phenylalanyl tRNA synthetase	197	7	14
25	APH_0555	51.8	cysteinyl tRNA synthetase	193	5	19
26	APH_0985	19.8	putative lipoprotein	185	1	14
27	APH_0794	27.1	hypothetical protein	184	2	8
28	APH_0740	131.1	ankyrin	183	11	7
29	APH_1258	32.7	fructose bisphosphate aldolase	182	5	9
30	APH_0723	27.2	hypothetical protein	182	1	3
31	APH_1025	14.1	50S ribosomal protein L7 L12	181	2	24
32	APH_1292	42.0	cell division protein FtsZ	181	3	13
33	APH_1210	85.7	OMP85 family OMP	174	7	8
34	APH_0130	20.6	triphosphate deaminase	172	1	5
35	APH_0283	29.8	50S ribosomal protein L2	170	3	8

36	APH_0893	71.1	heat shock protein 90	168	6	13
37	APH_1412	16.0	hypothetical protein	168	1	7
38	APH_1217	15.6	nucleoside diphosphate kinase	167	1	10
39	APH_0111	26.3	uridylate kinase	164	2	13
40	APH_1151	21.4	hypothetical protein	162	1	5
41	APH_0608	67.4	ppiC parvulin rotamase protein	161	10	13
42	APH_1359	31.6	major OMP 1A	158	2	6
43	APH_1084	29.8	cytochrome c oxidase subunit II	155	3	13
44	APH_0422	35.7	acetylglutamate kinase	152	2	7
45	APH_0792	37.6	hypothetical protein	149	1	3
46	APH_1219	33.4	Omp 1X	145	1	5
47	APH_0971	49.4	putative trigger factor	141	3	13
48	APH_0038	59.4	CTP synthetase	140	2	6
49	APH_0669	114.5	proline dehydrogenase	139	4	7
50	APH_0450	86.7	ATP dependent Clp protease	138	2	2
51	APH_0386	58.6	HGE 14 protein	135	1	2
52	APH_0231	54.6	leucyl aminopeptidase	129	3	11
53	APH_0678	63.4	chaperone protein HscA	127	1	5
54	APH_0393	49.0	dihydrolipoamide dehydrogenase	127	1	2
55	APH_0874	115.4	hypothetical protein	123	5	3

Proteins above the dashed line are 99% authentic with only a 1% false-discovery rate

[a] Size of identified protein

[b] Percentage of protein coverage

to use in its isolation. The focus of this study was to identify surface proteins of the *A. phagocytophilum* DC, using improved purification approaches. We observed several limitations when surface proteins of *A. phagocytophilum* were previously identified (55). First, both RC and DC organisms were used for OMP identification. For our purposes, we only used the adherent DC organism in this study, as our goal is to identify OMPs as putative adhesins. Second, some of previously identified proteins may not have been surface exposed if the biotin reagent gained access through functional porins (25) to label inner membrane proteins. Although we did not control for this issue, it is unlikely to be a problem with DC organisms. Porins function as a transport conduits through which various molecules such as nutrients can pass (130). *A. phagocytophilum* has porins (80), but they are most likely only active on the RC during intracellular growth and replication. The sonication-resistant feature of the DC further demonstrates that its function is to survive harsh extracellular environments during dissemination. It would be disadvantageous for the organisms to have functional porins outside the cell in a potentially hostile environment. Indeed, the *Coxiella* small cell-variant (SCV) and *Chlamydia* EBs either down regulate proteins that function as porins or heavily crosslink the surface, thereby ceasing porin function on their respective infectious forms (17, 180). A third limitation of the previous study was that only a select few bands from a 1D SDS-PAGE gel were chosen for analysis. Our use of MudPIT, which does not require proteins to be resolved on a gel, eliminates any bias by detecting all recovered biotinylated proteins.

Previously, only 14 putative *A. phagocytophilum* surface proteins were identified with biotinylation (55), of which only 3 have been confirmed as surface localized. Our

biotinylation approach of the DC recovered 55 proteins. However, only 16 proteins could be confidently identified as an authentic result. Of the proteins that we confidently identified, 3 were known surface proteins (Msp2[P44], Asp55 [APH_0405] and Asp62 [APH_0404]). Of all 55 proteins we potentially identified, 9 of these were identified in the previous study. They were Msp2(p44), APH_0036, 0240, 0346, 0404, 0405, 0608, 1210 and 1359. All but 3 of these proteins (APH_0608, 1210 and 1359) were confidently identified in our analysis. We also confidently identified Msp5 (APH_1049), a well conserved protein amongst *Anaplasma* species (163).

Even though our study corroborates the previous biotinylation study, some of the recovered proteins are not ones that would be expected to be surface exposed, while others appear to be missing. A total of 3 chaperones GroEL (APH_0240), GrpE (APH_0036) and DnaK (APH_0346) were confidently identified. Chaperones assist in protein folding in the cytoplasm (77), and would not be expected as surface exposed proteins. The same is true of elongation factor Tu (APH_1032), which is associated with the ribosome (131). As odd as it may be for these proteins to be surface exposed, some evidence not only suggests they are on the surface in some instances, but also act as adhesins. Elongation factor Tu of *Lactobacillus johnsonii* has been demonstrated to mediate attachment to human intestinal cells and mucins (63). Whereas GroEL of both *Lactobacillus johnsonii* and *Plesiomonas shigelloides* has also demonstrated adhesin function (19, 175). It may be predicted that because the DCs chromatin is compacted, the organism is likely to be metabolically inactive and thus would not require chaperones. Nonetheless, characterization of the proteome of the infectious Chlamydial EB has found many proteins associated with

metabolism, replication, and protein folding (159, 179). These proteins are likely prepackaged in the infectious form so the organisms can quickly use them upon entry into a host cell for replication and intracellular survival. Two other confidently identified proteins, APH_1235 and APH_0248 were of interest due to their small sizes of 14.7 and 13.8 kDa, respectively. It is interesting to speculate how such small proteins are integrated into the membrane. Proteins such as these would not be predicted as OMPs, as their small size would prevent them from containing enough structural features to allow them to be classified as traditional membrane proteins. One group of proteins that would have been expected to be recovered are the *A. phagocytophilum* T4SS components (133). DCs should express the T4SS, as AnkA is delivered into host cells upon bacterial binding. Of all the T4SS components, only VirB10 was detected. VirB10 is predicted to localize within the inner membrane, yet all the outer membrane protein components of the T4SS (32), VirB2, VirB3, VirB5, VirB7 and VirB9 were not detected.

The unusual ability of *A. phagocytophilum* to bind human PSGL-1 suggest that the adhesin(s) are unusual as well. Even though chaperones GroEL, GrpE , DnaK as well as elongation factor Tu may be unusual if they localize to the surface of *A. phagocytophilum*, it does not yet directly implicate them as adhesin(s). Some of these proteins as well as predicted OMPs APH_1210 (OMP85 family) and APH_1359 (OMP 1A) have very high protein sequence homologies to other bacterial species (data not shown). This raises the question of how could these proteins. if adhesin(s), achieve specificity of binding to PSGL-1 for *A. phagocytophilum* if also expressed on the outer membrane of other pathogens. We presume that we are looking for unique proteins. This would include

hypothetical proteins, as these proteins are predicted to existence, but for which there is no experimental evidence that they are expressed in vivo. They can also have little homology to other proteins of known function. The *A. phagocytophilum* genome contains 540 hypothetical proteins, comprising 38.3% of the genome.

Overall, our approach of purification and biotinylation of the DC was successful in recovering known and potentially new OMPs. However, there are several limitations of this experimental approach that will need to be addressed further for us to be confident that we are truly identifying DC specific OMPs. One concern is the volume of contaminating cellular debris. We confidently identified 153 human proteins (derived from HL-60 cells), but only 16 *A. phagocytophilum* proteins. Debris contamination was visually observed by TEM as well (**Fig. 19A**). An excess of debris could interfere with efficient labeling of OMPs on the DC, preventing recovery of low abundant proteins. The published Renografin density-gradient centrifugation protocol has only been tested on recovering organisms from syringe lysis (27) and the purity of the organisms was never confirmed by TEM. We did not take into consideration that sonication generates very fine debris particles that could not be readily removed. We have since tried different sonication times and added more gradient layers, but were unsuccessful in eliminating debris from comigrating with *A. phagocytophilum* DC organisms. A greater concern is whether or not this debris could contain destroyed RC organisms. If debris from RC organisms comigrates with the DC, then we cannot be confident that the proteins we identified are truly DC-specific or even surface proteins. It is possible that some identified proteins could be derived from the RC cytoplasm. Indeed, one of the confidently identified

proteins was 30S ribosomal protein S2 (APH_0397). Also, many of the proteins we identified, albeit of low confidence, are associated with metabolism. Generally, these proteins would not be predicted to be surface exposed. Another concern is we also have to question how efficient RIPA buffer extracts surface proteins from the membrane given the resistant nature of the *A. phagocytophilum* DC. Although RIPA buffer is commonly used for lysing *A. phagocytophilum* and most certainly can solubilize surface proteins, the efficiency of extraction is unknown. Msp2(P44) was easily recovered and confidently detected, yet predicted OMPs APH_1210 (OMP85) and APH_1359 (OMP1A) were also identified, but below a confident level. The low level of OMP85 and OMP1A may simply be due to low levels of expression on the DC or due to inefficient extraction from the outer membrane.

Many of the proteins identified in this study are likely surface proteins as the results corroborate well with a previous biotinylation study of *A. phagocytophilum* (55). Although the above concerns will have to be addressed in future experiments, proteins identified from this study are currently being investigated by other members of the Carlyon lab. The remainder of my dissertation was to focus on improving gradient purification as a means to isolate pure DC organisms.

METHODS

***A. phagocytophilum* organisms at various stages of isolation examined by transmission electron microscopy**. Infected HL-60 cells were pelleted at 300 x *g* for 5 min, supernatant

was discarded and cell pellet was processed for TEM. All samples were processed for TEM as previously described (173). Ap RC and DC organisms (strain NCH-1) were recovered by syringe lysis as follows. Heavily infected HL-60 cells with high bacterial loads were pelleted at 5,000 x *g* for 15 min at 4°C. Cells were resuspended in a total of 9 ml cold PBS with protease inhibitors (Roche; Indianapolis, IN, Cat. No. 11836153001) for every 250 x 10^6 cells. Cells were lysed by 5 passages through a 27½ gauge needle (BD; Franklin Lakes, NJ, Cat. Nos. 305196, 305109) in a 50 ml tube. The sample was distributed at a ratio of 100 x 10^6 cells in 45 ml PBS and centrifuged at 1,200 x *g* for 10 minutes at 4°C to pellet large debris. The supernatant was transferred to new tube and centrifuged at 4,500 x *g* for 15 minutes at 4°C to pellet Ap. The bacterial pellet was processed for TEM. DC organisms were recovered by sonication as follows. Heavily infected HL-60 cells with high bacterial loads were pelleted at 5,000 x *g* for 15 min at 4°C. Cells were resuspended in 6 ml cold PBS with protease inhibitors for every 250 x 10^6 cells, and subjected to eight 8-sec bursts on ice interspersed with 8-sec rest periods using a Misonix S4000 ultrasonic processor (Farmingdale, NY) on an amplitude setting of 30. The 6 ml sample was brought up to 50 ml with PBS and centrifuged at 750 x *g* for 5 minutes at 4°C to pellet large debris. Supernatant was transferred to a new tube and centrifuged at 1,000 x *g* for 10 minutes at 4°C to remove more debris. Supernatant was transferred to a new tube and centrifuged at 4,500 x *g* for 10 minutes at 4°C to pellet Ap. The bacterial pellet was processed for TEM.

Renografin purification of *A. phagocytophilum* DC organisms. DC organisms were recovered by sonication and differential centrifugation as described above. Ap pellet from 500 x 10^6 cells was resuspended in 10 ml PBS with 0.2% glucose and treated with 50 µg/ml of DNAse I (Roche, Cat. No. 10104159001) for 45 min at 37°C. The sample was then layered on top of 30% - 42% discontinuous Renografin gradient, centrifuged, and organisms were recovered from 30% - 42 % interface as previously described (27).

Biotinylation and purification of *A. phagocytophilum* DC surface proteins. DC organisms recovered from the Renografin gradient were washed 2 times in 1 ml cold PBS at 5,000 x *g* for 5 minutes. For biotinylation, Ap recovered from up to 1 x 10^9 cells was mixed in 1 ml PBS with 10 mM water soluble non-membrane permeable Sulfo-NHS-SS-Biotin (Pierce; Waltham, MA, Cat. No. 21331) for 30 min at RT with rotation. Organisms were pelleted at 5,000 x *g* for 5 min and the supernatant was discarded. The Ap pellet was thoroughly resuspended in 1 ml of 50 mM Tris (pH 8.0) to quench the biotinylation reagent. Ap pellet was washed two more times with 500 µl cold PBS. The Ap pellet was lysed in 1 ml RIPA solution with protease inhibitors for 1 h on ice. Every 20 min, the sample was subjected to sonication on an amplitude setting of 30 for four 8-sec bursts on ice interspersed with 8-sec rest periods. Insoluble material was removed by centrifugation for 10 min at 10,000 x *g* at 4°C. The supernatant was saved and transferred to ice.

The soluble fraction of the biotinylated sample was mixed with High Capacity NeutrAvidin Agarose Beads (Pierce, Cat. No. 29202) in 10 ml RIPA buffer with protease inhibitors O/N at 4°C with rotation. The sample was centrifuged at 1,000 x *g* for 1 min,

supernatant was removed (saved to verify purification of biotinylated proteins) and beads were resuspended in 10 ml cold PBS. The sample was centrifuged again at 1,000 x *g* for 1 min and the supernatant was discarded. The beads were resuspended in 8 ml cold PBS. The sample was thoroughly mixed and 800 µl aliquots of slurry were removed from the tube and placed in one spin column (Pierce, Cat. No. 69725) a total of 10 times. Columns were centrifuged at 1,000 x *g* for 1 min at 4°C. The flow through was discarded. Five hundred µl of cold PBS was added to the column, mixed and centrifuged again for a total of 3 washes. A cap was placed on the bottom of spin column tube. Five hundred µl of 0.25% Na deoxycholate solution with 150 mM dithiothreitol (DTT) was added to the column. The tube lid was closed and the sample was mixed for 2 h at RT with rotation. The cap from the bottom of the tube was removed and proteins were eluted into a new clean tube by spinning at 1,000 x *g* for 2 minutes. All recovered/eluted samples were pooled into one tube and placed on ice. The sample concentration was determined. Sample was stored at 4°C to prevent proteins from falling out of solution.

MS identification of *A. phagocytophilum* DC surface proteins. Two hundred µg purified biotinylated sample was concentrated with ultra-0.5ml centrifugal filter units (Fisher Scientific; Pittsburgh, PA, Cat. No. UFC501024). Four hundred µl of sample was added as many times as needed to the centrifugal filter and centrifuged at 10,000 x *g* at RT to reduce the retentate to <50 µl. Upon reduction, 400 µl of 50 mM ammonium bicarbonate (ABC) buffer with 0.05% Na deoxycholate was added and the sample was centrifuged at 10,000 x *g* at RT to reduce the retentate. The wash was repeated a second

time and centrifuged to near dryness. Filters were inverted and placed into new microfuge tubes, which were centrifuged at 3,000 x *g* at RT for 10 min to collect retentate. The eluted volume was measured. The eluted volume was subtracted from 25 µl. A 90:10 (50 mM ABC / Acetonitrile [ACN]) buffer of the subtracted volume was added to the top of the filter and incubated for 15 min. The sample was eluted into the previously collected sample to yield a total volume of 25 µl. One µl of fresh 500 mM DTT made in 50 mM ABC was added to sample and incubated for 30 min at 90°C. The sample was then cooled to room temperature. Trypsin (Promega; Madison, WI, Cat. No. V5280) (100 ng/µl) was added to a final 1:100 enzyme:protein ratio (20 µl trypsin for 200 µg of protein). If a precipitate formed, then 0.5 µl ammonium hydroxide was added to the sample. The sample was vortexed briefly and placed at 37°C O/N. Samples were analyzed by MudPIT. Recovered peptide spectra data was search against the *A. phagocytophilum* (strain HZ) and human genome. All identified *A. phagocytophilum* proteins were reported.

For protein identification to be considered an authentic result, we determined that the protein needed to have a minimal score of 300 with multiple recovered peptides. This would allow for identified proteins to be considered as 99% authentic with only a 1% chance of error. The score of the identified protein is the sum of several values, which includes the number of peptides recovered and percent coverage of proteins. The number of peptides identified is derived from the total number of different ion measurements. These ion measurements depend on how a protein is digested and will include peptides that can contain multiple overlapping sequences from the same region of a protein. The proteins in Table 5 are ranked by score, which is generally related to the abundance of a

given protein. The more protein present in a sample, the more peptides should be recovered and the better score the protein will receive.

CHAPTER 6: Density-gradient Purification of the *A. phagocytophilum* Reticulate Cell and Dense-Cored Cell Forms

INTRODUCTION

Our ability to confidently identify OMPs as adhesin(s) is dependent on isolation of the adherent *A. phagocytophilum* DC. In the previous chapter, we were able to demonstrate that sonication combined with discontinuous density-gradient centrifugation was successful in recovering DC organisms, but we could not be confident that contamination from destroyed RC organisms did not occur. The large abundance of host cell debris contamination generated from sonication was also an issue, as it could interfere with biotinylation and MS identification of *A. phagocytophilum* proteins. Therefore, individual separation of undamaged RC and DC organisms will be required to ensure we identify and pursue only DC specific surface proteins.

Separation of the different biphasic developmental forms has been achieved with gradient-centrifugation for *Chlamydia* species and *Coxiella burnetii* (36, 156). Isolation and characterization of pure developmental forms has allowed great insight into their structures and functions. Because separation of RC and DC organisms has not been established for *A. phagocytophilum*, we set out to devise new methods to individually separate the two forms by means of discontinuous density-gradient centrifugation.

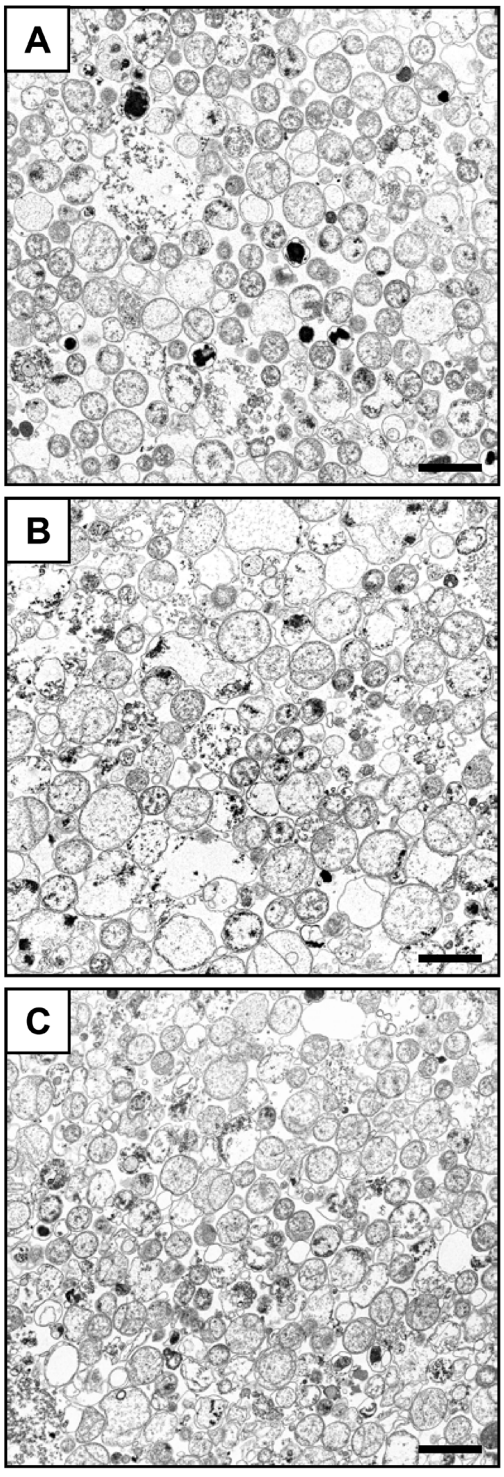
RESULTS

Isolation and purification of *A. phagocytophilum* RC organisms by dounce homogenization and OptiPrep density-gradient centrifugation.

In Chapter 5, we discovered that the destroyed RCs and host cell debris generated from sonication was a problem when selecting for DC organisms. As an alternative, we chose to test dounce homogenization as a method for RC and DC isolation, as this is a gentler manner of cell lysis. This method combined with density-gradient centrifugation has been successful in isolating mitochondria, lysosomes and other organelles from various cells and tissue (60-62), as well as for isolation of *Neorickettsia risticii* (104). In an effort to lyse infected cells while keeping the *A. phagocytophilum* RC and DC undamaged, and reduce host cell debris contamination, we tested the efficiency of dounce homogenization.

Infected HL-60 cells were first placed in a hypo-osmotic solution to make them more susceptible to lysis from swelling. Cells were lysed by dounce homogenization after which they were returned to an iso-osmotic state to prevent damage to the organisms and host cell vesicles. Large cellular debris was removed by differential centrifugation, after which the recovered organisms were centrifuged through an OptiPrep discontinuous density-layered gradient. We used OptiPrep in our gradients because the Renografin solution we used previously in Chapter 5 was no longer commercially available. After centrifugation, we analyzed several interfaces of the gradient by TEM and found that the 25% - 30% interface was the only layer that contained pure bacteria (**Fig. 20**). The 25% - 30% interface was enriched for RC organisms with minimal host cell debris contamination.

Figure 20. ***A. phagocytophilum*** **RC organisms recovered by dounce homogenization and density-gradient centrifugation.** Infected host cells were dounce homogenized and Ap organisms were purified by OptiPrep discontinuous density-gradient centrifugation. (**A-C**) TEM images of organisms recovered between the 25% - 30% OptiPrep interface. Results from 3 separate experiments are shown. Scale bar 2 μm.



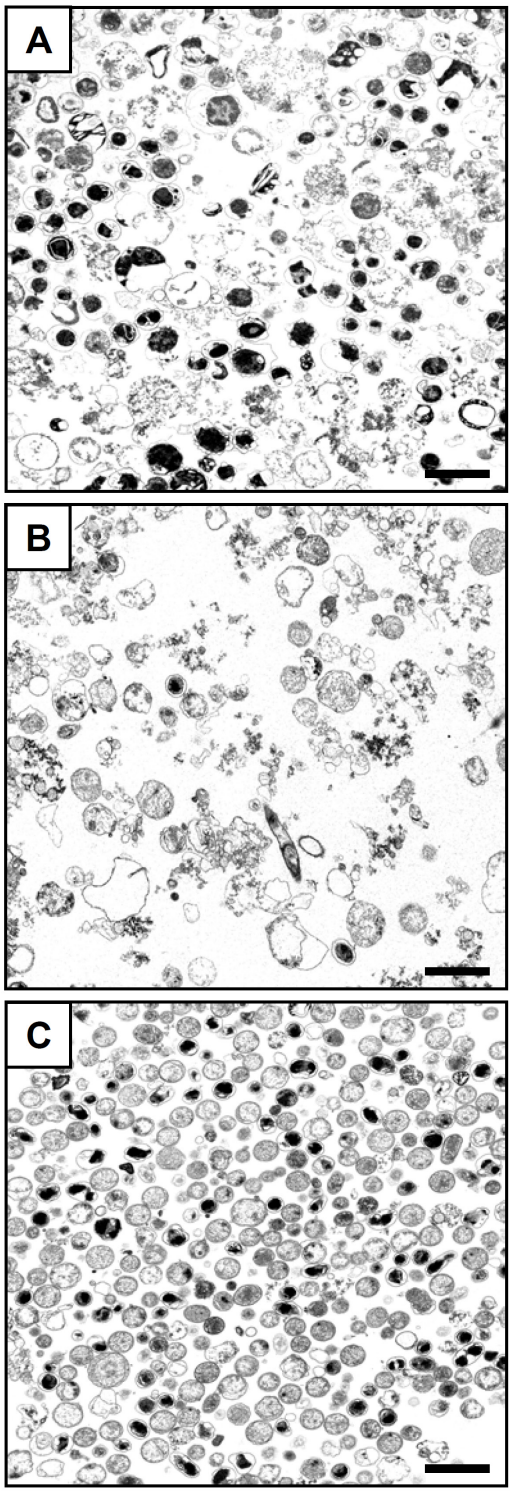
None of the other analyzed layers contained any abundance or purity of DC organisms (data not shown).

Attempts to isolate and purify *A. phagocytophilum* DC organisms by syringe lysis and OptiPrep density-gradient centrifugation.

Our TEM results revealed that dounce homogenization only recovered an RC-enriched bacterial population. Therefore, we had to try alternative approaches of lysing infected cells to maximize recovery of DC organisms. We previously determined that syringe lysis is best suited for recovery of a mixture of both RC and DC organisms as seen in **Figure 18B** of Chapter 5.

To recover DC organisms, we repeatedly passed infected HL-60 cells through a 18½ and 27½ gauge needles to destroy the host cell and release intact RC and DC *A. phagocytophilum* organisms. Large cellular debris was removed by differential centrifugation, after which the recovered organisms were centrifuged through an OptiPrep discontinuous density-layered gradient. After centrifugation, we analyzed several interfaces of the density-gradient by TEM and found that the 20% - 25% interface contained the greatest abundance of bacteria (**Fig. 21**). The first time we tested this approach, we recovered a fairly pure population of DC organisms with little to no RCs (**Fig. 21A**). However, repeated attempts to recover this DC-enriched population were unsuccessful, as only a mixed population of RC and DC organisms were recovered (**Fig. 21B and C**).

Figure 21. Attempts to purify *A. phagocytophilum* DC organisms by syringe lysis and density-gradient centrifugation. Infected host cells were syringe lysed and organisms were purified by OptiPrep discontinuous density-gradient centrifugation. (**A-C**) TEM images of organisms recovered between the 20% - 25% OptiPrep interface. Results from 3 separate experiments are shown. Scale bar 2 µm.



DISCUSSION

These approaches were designed to isolate organisms from HL-60 cells while keeping the RC undamaged, as to prevent destroyed RC debris from comigrating and contaminating recovered DC organisms. We observed that dounce homogenization and density-gradient purification was suited for recovery of intact RC organisms and greatly reduced cellular debris contamination compared to that observed when using sonication in Chapter 5 (**Fig. 19**). Using this approach we obtained an RC-enriched bacterial population (**Fig. 20)**, but unfortunately did not recover any significant population of DCs. We determined that the lack of recovered DC organisms was a result of how the cells were lysed. We know dounce homogenization releases *A. phagocytophilum* organisms from their inclusions, as indicated by the recovered RC-enriched population. But we also know the host cells are gently lysed, which keeps many large membrane and cellular structures intact, including the host cell nucleus. We think that as the *A. phagocytophilum* inclusions are broken open and the bacteria are released, the adherent DC organisms most likely binds to PSGL-1 on intact membrane cellular debris. This large cellular debris is removed by differential centrifugation before it is ever added to the OptiPrep discontinuous density-gradient, thus potentially preventing recovery of DC organisms. Indeed, others have noted that intracellular organisms adhere persistently to host cell material (190, 198). We also observed that the recovered RC organisms migrate at the 25% - 30% interface. We ascertained that as the infected HL-60 cells are treated with the hypo-osmotic solution to aid in their lysis, the density of RC organisms' increases, which "shifts" the majority of the RC population to the 25% - 30% interface. This in fact results in a more consistently

recovered RC-enriched population with minimal cellular debris contamination (**Fig. 20**). A few DC organisms can be observed within the RC-enriched population, but their presence is minimal.

In spite of the fact that purification of the *A. phagocytophilum* RC is of great benefit, our intent was to purify DC organisms. Therefore, we next used syringe lysis to isolate organisms from infected cells, which we know recovers both intact RC and DC organisms, as previously demonstrated in Chapter 2 (**Table 1**) and Chapter 5 (**Fig. 18B**). Syringe lysis is more destructive to HL-60 cells and efficiently destroys host cell membrane structures, which ensures recovery of DC organisms. Syringe lysis will result in more cellular debris contamination, but is a necessity to ensure DC recovery. The first time we tested syringe lysis with OptiPrep discontinuous density-layered gradients, we recovered a DC-enriched bacterial population with little to no RC organisms at the 20% - 25% interface (**Fig. 21A**). Unfortunately, repeated attempts following the same protocol resulted in recovery of a mixed RC and DC bacterial population (**Fig. 21B and C**). There could be several reasons for these discrepancies between experiments. First, the population of DC organisms within the cultures during the first experiment may have simply been much greater. Second, it could be a consequence of the biphasic developmental cycle exhibited by *A. phagocytophilum*. The constant transitional continuum between RC and DC forms may prevent them from having a consistent density to allow for individual separation. We have observed by TEM that DC organisms stopped at the 20%, 25%, and 30% layers of the OptiPrep density gradients (data not shown) thereby validating that the density of the organisms can vary. Although the majority of the

DCs migrate to the 20% - 25% interface, the DC that migrates elsewhere is too few to pursue. This variation in density contributes to an overall loss in recovery of DC organisms.

An assortment of attempts to remove the RC organisms from the DC were taken. These included, but were not limited to changing the speed of density-gradient centrifugation, as well as adding more or less density gradient layers, which were all unsuccessful. We learned during dounce homogenization that the hypo-osmotic solution used to aid in cell lysis "shifted" the RCs to the 25% - 30% interface. Since the DC was resistant to sonication, we thought they may also be resistant to osmotic changes as well. We tried treating the mixed bacterial population recovered by syringe lysis at the 20% - 25% interface with the hypo-osmotic solution, then centrifuged the sample through a second OptiPrep discontinuous density-gradient to determine if we could "shift" the RC organisms at the 20% - 25% interface to the 25% - 30% interface, while leaving the DC at the 20% - 25% interface. Although many RC organisms were "shifted" to the 25% - 30% interface, some DC organisms "shifted" as well. This resulted in fewer DC at the 20% - 25% interface, which was still was contaminated with a few RCs and a fair amount of cellular debris. Overall, the approaches we took to remove RC organisms and cellular debris were unsuccessful in obtaining sufficient quantities of pure DC organisms.

METHODS

OptiPrep density-gradient purification of *A. phagocytophilum* RC organisms by dounce homogenization. Infected HL-60 cells were pelleted at 500 x *g* for 10 min at 4°C.

The supernatant was discarded and cell pellets were resuspended at a ratio of 250 x 10^6 cells in 50 ml cold PBS and centrifuged at 500 x *g* for 8 min at 4°C to wash the cells. The supernatant was discarded and cells were resuspended at a ratio of 100 x 10^6 cells in 2 ml of hypo-osmotic solution (10 mM hepes, 10 mM potassium chloride [KCl], 1.5 mM magnesium chloride [$MgCl_2$]) and placed on ice for 10 min. Up to 7 ml at a time was placed in a dounce homogenizer (Kimble and Chase; Vineland, NJ, Part No. 885303-0007) and dounced with a tight fitting pestle (Kimble and Chase, Part No. 885302-0007) 20 times. Immediately after douncing, 1/3 of the total volume of a cold 1 M Sucrose solution (10 mM hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 1 M sucrose) was added to the dounced cells to return the solution to an iso-osmotic state of 0.25 M sucrose. Dounced cells were resuspended at a ratio of 100 x 10^6 cells in 45 ml of cold 0.25 M sucrose solution (10 mM hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 0.25 M sucrose) and centrifuged at 1,000 x *g* for 10 minutes at 4°C to pellet large cellular debris. Supernatant was transferred to a new 50 ml tube and Ap organisms were pelleted at 4,500 x *g* for 15 minutes at 4°C. The supernatant was discarded and the Ap pellet was resuspended at ratio of 500 x 10^6 cells in 6 ml cold 0.25 M sucrose solution. DNAse I (Roche; Indianapolis, IN, Cat. No. 10104159001) was added to a final concentration of 250 µg/ml and incubated at 37°C for 30 min. The sample was added onto a discontinuous density-layered OptiPrep (Sigma-Aldrich; St. Louis, MO, Cat. No. 1556-250) gradient. OptiPrep solution was diluted with the 0.25 M sucrose solution. The OptiPrep discontinuous density-gradient was layered bottom to top with the following volumes and percentages of OptiPrep solutions; 6 ml of 30%, 5 ml of 25%, 5 ml of 20%, 3.5 ml of 17.5%, 3.5 ml of 15% and 3.5 ml of 12.5%. Solutions were layered in

35 ml ultracentrifuge tubes (Beckman Coulter; Brea, CA, Part No. 344058). Samples were centrifuged at 87,000 x *g* in an ultracentrifuge swinging bucket rotor for 1 hour and 15 minutes at 4°C. After centrifugation, the bands at the 25% - 30% interface were recovered and mixed with an equal volume of sucrose-phosphate-glutamate (SPG) buffer (0.25 M Sucrose, 7 mM KH_2PO_4, 3.7 mMK_2HPO_4, 5 mM L-glutamine [pH 7]). The sample was centrifuged at 15,000 x *g* for 10 min at 4°C. The supernatant was discarded and Ap pellet was processed for TEM as previously described (173).

OptiPrep density-gradient purification of *A. phagocytophilum* RC and DC organisms by syringe lysis. Infected HL-60 cells were pelleted at 5,000 x *g* for 15 min at 4°C. The supernatant was discarded and the cell pellet was resuspended in a total of 9 ml cold PBS with protease inhibitors (Roche; Indianapolis, IN, Cat. No. 11836153001) for every 250 x 10^6 cells. Cells were lysed by 5 passages through 18½ and 27½ gauge needles (BD; Franklin Lakes, NJ, Cat. Nos. 305196, 305109) in a 50 ml tube. The sample was distributed at a ratio of 100 x 10^6 cells in 45 ml PBS, and centrifuged at 1,200 x *g* for 10 minutes at 4°C to pellet large debris. The supernatant was transferred to new tube and centrifuged at 4,500 x *g* for 15 minutes at 4°C to pellet Ap. The supernatant was discarded and the Ap pellet was resuspended at ratio of 500 x 10^6 cells in 6 ml cold 0.25 M sucrose solution (10 mM hepes, 10 mM KCl, 1.5 mM $MgCl_2$ and 0.25 M sucrose). DNAse I (Roche, Cat. No. 10104159001) was added to a final concentration of 250 µg/ml and incubated at 37°C for 30 min. The sample was processed as described above except that organisms were recovered from the 20% - 25% interface.

CHAPTER 7: Characterization of Putative *A. phagocytophilum* Dense-Cored Cell Outer Membrane Proteins

INTRODUCTION

Even though the main focus of Chapters 5 and 6 were to improve upon recovery of DC organisms, several proteins identified in Chapter 5 were a priority to study while we continued to test new gradient procedures. In Chapter 5, we used biotinylation to identify surface proteins of *A. phagocytophilum* DC organisms. Using this approach, we identified known OMPs of *A. phagocytophilum* as well as OMPs identified for other *Anaplasma* species. We rationalized that since we identified known OMPs, some of the newly identified proteins were most likely newly discovered OMPs. Three hypothetical proteins recovered by biotinylation and identified by MS (**Table 5**) were of interest. These proteins were APH_1235, APH_0248 and APH_0853. These proteins have little to no homology with other bacterial species. Since we presume that we are looking for unique proteins as adhesins to human PSGL-1, these proteins were of great interest to us and we set out to further characterize these proteins as putative DC cell OMPs.

RESULTS

APH_1235 and APH_0248.

Two proteins of interest recovered by MS in significant enough quantities to designate them as authentic proteins were APH_1235 and APH_0248. APH_1235 was the second most abundant protein identified by MS (**Table 5**) after the dominant surface protein of *A. phagocytophilum*, Msp2(P44). Both APH_1235 and APH_0248 are small proteins. APH_1235 is 14.7 kDa, while APH_0248 is 13.8 kDa. We initiated our characterization of APH_1235 and APH_0248 by expressing them as GST fusion proteins in *E. coli* and using the recombinant proteins to raise mouse polyclonal antisera. We next screened *A. phagocytophilum* synchronously infected HL-60 cells with anti-APH_1235 and anti-APH_0248 in conjunction with an Ab against Msp2(P44), which is a constitutively expressed OMP. The synchronously infected cells were screened at 18 h when the organisms should be a highly pure RC population, and 24 h, which is when DC organisms first begin to appear (**Fig. 22**). At 18 h and 24 h, 0% and 21% of inclusions, respectively, expressed APH_1235. At 18 h and 24 h, 97% and 100% of inclusions, respectively, expressed APH_0248. This suggests that APH_1235 is a DC-specific protein, and APH_0248 is expressed on RC and DC organisms.

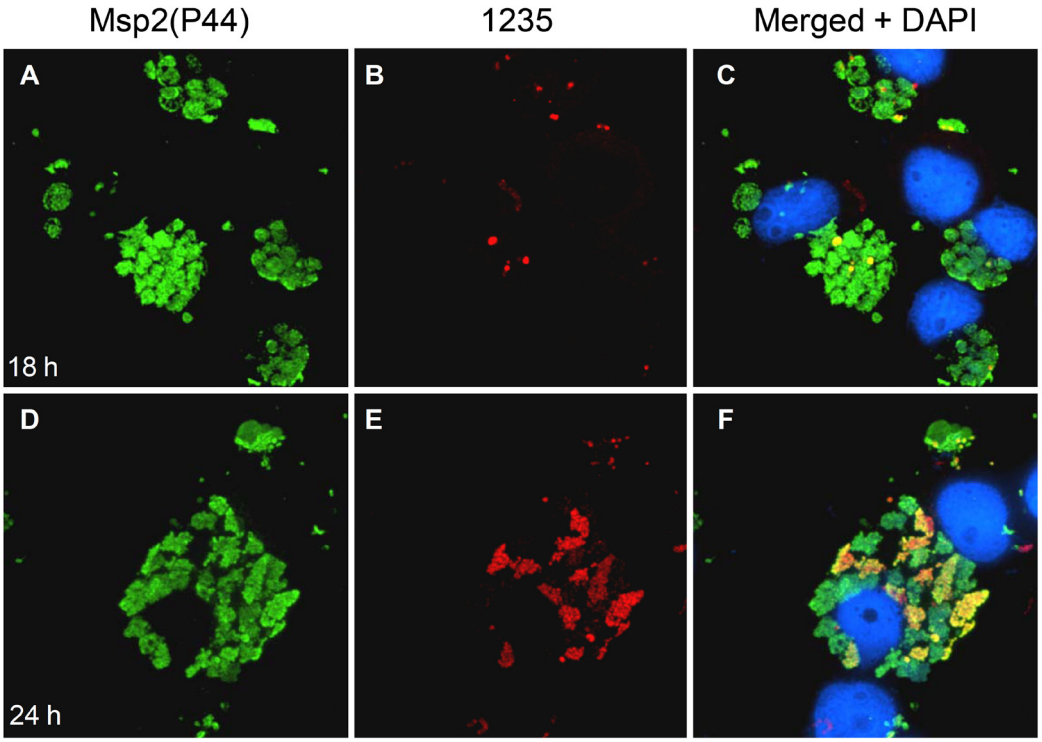
APH_0853

One very unique protein we identified, although it is just below the threshold of authenticity as determined by MS, was hypothetical protein APH_0853. When searched against The National Center for Biotechnology Information (NCBI) protein database using

the basic local alignment search tool (BLAST), this 10.8 kDa protein did not have homology to any single protein (**Fig. 23A**). Because of the uniqueness of APH_0853, we decided to compare the DNA sequence of our *A. phagocytophilum* strain to *A. phagocytophilum*-Variant 1 (ApVar-1). ApVar-1 is a strain of *A. phagocytophilum* that does not infect mice or human cell lines (114, 115). The reservoir in nature is believed to be White-tailed deer, although goats are also susceptible to infection (113). The ApVar-1 strain is cultivated in tick cell lines for laboratory studies (112).

Although this strain cannot infect human cell lines, we do not yet know if it can bind human PSGL-1. Nonetheless, we compared the sizes of PCR products from 5 full-length putative OMP genes identified by biotinylation (**Table 5**). The only gene that had an obvious size difference was *aph_0853* (**Fig 23B**). We next sequenced *aph_0853* from ApVar-1 (genome sequence not yet available) and compared its DNA sequence and translated protein sequence to those of the NCH-1 strain, which is infectious for humans. We found that the *aph_0853* gene of ApVar-1 had several point mutations as well as large and small insertions (**Fig 24A**). If this protein were expressed, it would be nonfunctional as the mutations in the DNA sequence produce a premature stop codon at amino acid 17, which would result in a 1.9 kDa protein. This data demonstrates that APH_0853 is expressed in an *A. phagocytophilum* strain that infects humans, but is severely truncated in a stain that cannot infect human cells.

Figure 22. APH_1235 and APH_0248 expression during intracellular infection. HL-60 cells were synchronously infected with *A. phagocytophilum*. At 18 h (**A-C** and **G-I**) and 24 h **(D-F and J-L**) post-infection, cells were fixed with methanol and viewed by immunofluorescence microscopy for immunoreactivity with antibodies against Msp2(P44); used to denote bacteria in **A, D, G and J**), anti-APH_1235 (**B and E**) and anti-APH_0248 (H and K). Merged fluorescent images with DAPI (stains host cell nuclei) are presented in **C, F, I and L**. Representative images from 1 experiment are shown.



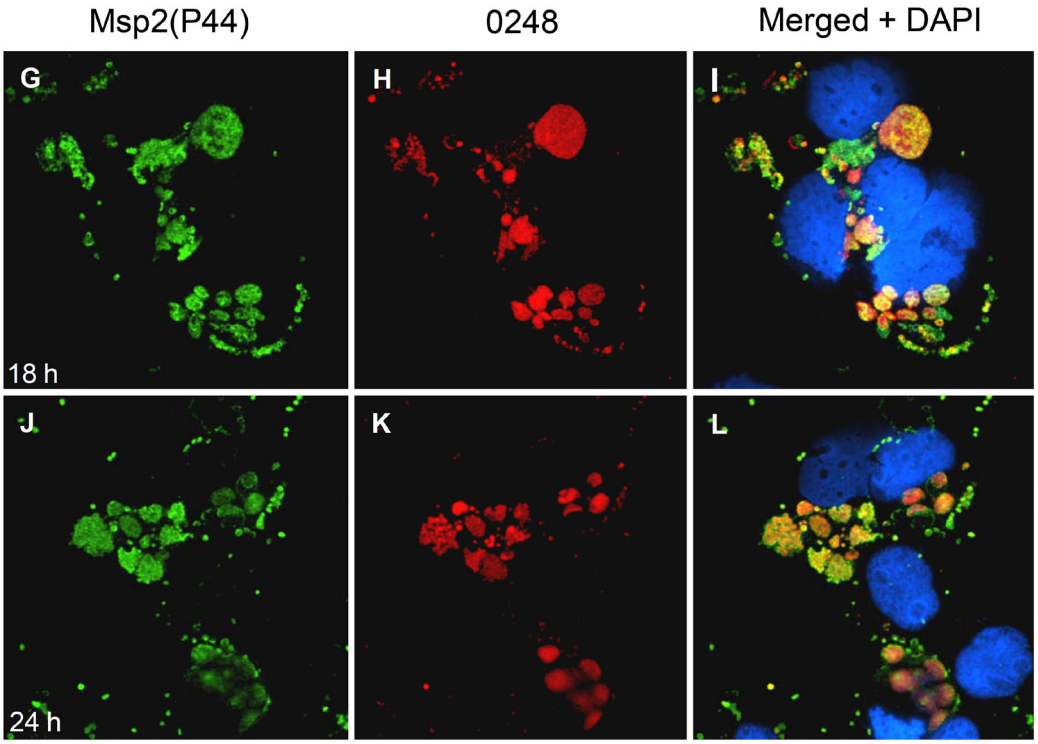
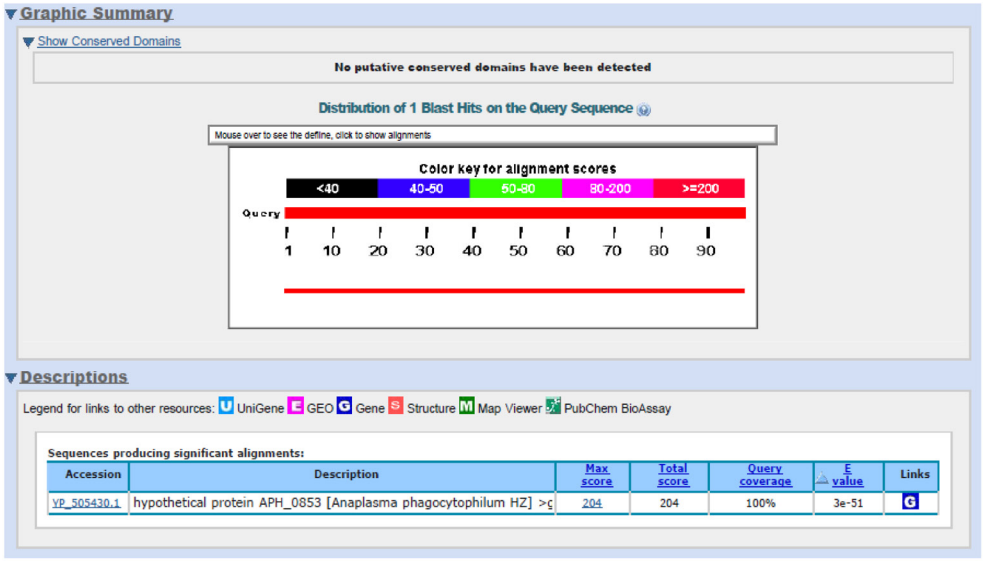


Figure 23. APH_0853 BLAST search and PCR results. (**A**) Result of APH_0853 protein BLAST search of the NCBI database. (**B**) PCR products of full length genes were compared between a human infectious *A. phagocytophilum* (Ap) strain and the non human strain (ApVar-1).

A.



B.

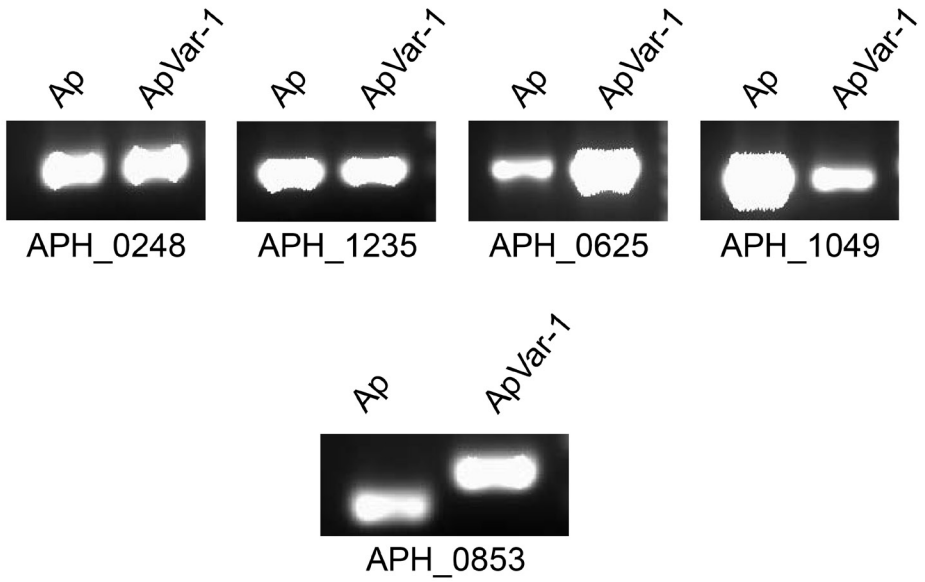


Figure 24. DNA and protein sequences of APH_0853. (**A**) Alignment of DNA sequences of full length *aph_0853* of the human infectious *A. phagocytophilum* (Ap) strain and the non human strain (ApVar-1). (**B**) Alignment of amino acid sequence of the two strains. Red asterisks indicate homology and blue bold text indicate differences between the two strains.

A.

```
                  10         20         30         40         50         60
                   |          |          |          |          |          |
Ap          ATGTTGATAGATGATCATGCAGCTGATTTTTTTT-GCAAGATTATCTGTACGTTTAAGGT
ApVar-1     ATGTTGATAGATGATCATGCAGCTGATTTTTTTTTGCAAGATTATCTGTA-GTTTAAGGT
            ********************************** *************** *********
                  70         80         90        100        110        120
                   |          |          |          |          |          |
Ap          AGCAAAGAGATTTAGTAGTGTAGCAGAATATGT--------------------------
ApVar-1     AGCAAAGAGATTTAATAGTGCAGCAGAATATGTGCTGATATCACTCTGTTACGGACAACT
            ************** ***** ************

                 130        140        150        160        170        180
                   |          |          |          |          |          |
Ap          ------------------------CTTGCTGCAGATAAACACAAATCTTTACCCAGGAGC
ApVar-1     AACTCAGTACATAAGAGTGCTATGCTCGCTGCAGATAAACACAAATCTTTACCCAGGAGC
                                    ** *******************************

                 190        200        210        220        230        240
                   |          |          |          |          |          |
Ap          GCGCTCCTCTAGGAAAGGCATGGCTCTTTCTGAGATCAACCGCATGTCCACGCAATTTTA
ApVar-1     GCGCTCCTCTAGGAAAGGCATGGCTCTTTCTGAGATCAACCGCATGTCCATGCAATTTTA
            ************************************************* *********

                 250        260        270        280        290        300
                   |          |          |          |          |          |
Ap          TGCTGTTCAGCCAACATCC---CGAGATTGTGGAATAAAAAAATGCCAACACAGTAGTAG
ApVar-1     TGCTGTT-AGCCAATATTTATATAAGACTGCGGAATAAAAAA-TGCCAATACAGTAGCAA
            ******* ****** **       *** ** *********** ****** ******* *

                 310        320        330        340
                   |          |          |          |
Ap          TTTTGTTTTTTATGATTTGGCACGTATGTCCGGTCCTGGTGCAACCTGA
ApVar-1     TTTTGTTTTTTATGATTTGATACGTATGTCCGGTCCTGGTGCAACCTGA
            ******************  ****************************
```

B.

```
Ap          MLIDDHAADFFCKIICTFKVAKRFSSVAEYVLLQINTNLYPGARSSRKGMALSEINRMST
ApVar-1     MLIDDHAADFFLQDYL(STOP)--------------------------------------
            ***********

Ap          QFYAVQPTSRDCGIKKCQHSSSFVFYDLARMSGPGAT(Stop)
ApVar-1     -----------------------------------
```

DISCUSSION

Our data demonstrates that we have identified APH_1235 as the first DC-specific protein of *A. phagocytophilum*. Although further characterization will be required to prove that the protein is in fact an OMP, its identification as the second most abundant protein after Msp2(P44) in our biotinylation study (**Table 5**) of DC organisms strongly suggests that APH_1235 is a DC-specific protein. This is further reinforced by the lack of detection of APH_1235 expression at 18 h (**Fig. 22B**), a time point at which the *A. phagocytophilum* population should exclusively consist of RC organisms. However, at 24 h, a time point concurrent with the appearance of DC organisms, APH_1235 expression is detectable (**Fig. 22E**). When APH_0248 expression was monitored, it was detected at both 18 and 24 h, suggesting that this protein is expressed on both RC and DC organisms. Even though MS identified APH_0248 from a DC-enriched population, it is not surprising that some proteins that are on DC organisms will also be present on RCs.

Attempts to raise antiserum to APH_0853 were unsuccessful and are currently being retried. Although we have not yet determined when this protein is expressed during development, we have determined that it is exclusively unique to *A. phagocytophilum*. A BLAST search demonstrated that APH_0853 does not have homology to a single other protein in the NCBI database (**Fig. 23A**). Also, APH_0853 is non-functional in the non-human strain ApVar-1 due to a premature stop codon. APH_0853 is unique to the human infectious strain of *A. phagocytophilum* and its recovery from DC-enriched organisms makes this protein a priority to pursue as a potential DC-specific OMP that could function as an adhesin.

The manner by which these proteins are associated with the *A. phagocytophilum* outer membrane is unknown. None of these proteins were predicted as OMPs by any of the programs used in Chapter 3 to analyze the *A. phagocytophilum* genome. The program CELLO, which predicted 130 potential OMPs (**Table 3**), classifies these 3 small proteins as cytoplasmic (data not shown). None of the proteins contain signal sequences that would conceivably direct them to the outer membrane when analyzed by the signalP program. Computer analyses to look for potential transmembrane helices were inconsistent, as programs predicted 0, 1 or 2 helices for each protein (data not shown). Lastly, since we demonstrate that APH_1235 and APH_0248 are expressed during infection, these proteins should no longer be classified as hypothetical proteins but instead be designated as uncharacterized proteins.

METHODS

Recombinant protein expression and generation of antisera. Full length proteins of interest were expressed as GST fusion proteins according to manufacturer's instructions using the pENTR directional TOPO cloning kits pENTR/TEV/D-TOPO (Invitrogen; Carlsbad, CA, Cat. No. K2525-20) and *E. coli* expression system with gateway technology pDEST-15 (Invitrogen, Cat. No. 11802-014). Insoluble proteins were solubilized in Sarkosyl as previously described (168). SnakeSkin dialysis tubing (Pierce; Waltham, MA) was used to remove detergents and place proteins into a PBS solution. Purified proteins

were submitted for production of mouse polyclonal antiserum. Anti-GST-APH_0248 and Anti-GST-APH_1235 is referred to as anti-APH_0248 and anti-APH_1235, respectively.

APH_1235 and APH_0248 infection time course and immunofluorescence microscopy. *A. phagocytophilum* organisms were recovered from 10 times the number of infected HL-60 cells by syringe lysis as described (27) and added to naïve HL-60 cells to initiate a synchronous infection. For bacterial adherence, *A. phagocytophilum* organisms were incubated at a ratio of $2.0x10^6$ HL-60 cells in 1 ml of IMDM-10 (Invitrogen) for 1 hour. Host cells were pelleted at 300 x *g* for 5 min, washed twice with PBS to remove unbound bacteria, and incubated in IMDM-10 at 37°C in 5% CO_2. At 18 h and 24 h, 35,000 cells per sample were removed and centrifuged onto glass slides using a Shandon Cytospin 4 cytocentrifuge (Thermo Scientific, Waltham, MA) as described previously (27), followed by fixation in methanol for 4 min and stored at -20°C.

For immunofluorescence microscopy slides were blocked with 5% BSA/PBS for 1 hour. Mouse anti-APH_1235 and anti-APH_0248 sera were added for 45 min at dilutions of 1:100 and 1:200, respectively, in 1% BSA/PBS. The antisera were removed by aspiration and then rabbit anti-MPB-Msp2(P44) was added for 30 min at a 1:500 dilution in 1% BSA/PBS. The slides were washed 3 times in TBS-t (0.5%) for 2 min each, followed by Alexa Fluor 594-conjugated goat anti-mouse IgG (Invitrogen) for 30 min at a 1:1,000 dilution in 1% BSA/PBS. The antisera were removed by aspiration and then Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen) was added for 30 min at a 1:500 dilution in 1% BSA/PBS. The slides were washed 3 times in TBS-t (0.5%) for 2

min each, followed by mounting with Prolong Antifade Gold with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen). The cells were examined for inclusions using an Olympus BX51 microscope. A minimum of 100 inclusions was scored per time point.

DNA sequencing and alignments. Primers were designed to amplify 5 full-length genes of interest. The primers were as follows: APH_0248 (F) 5'-CACCATGATACCATTAGC TCCTTGGAAGAGC-3' and (R) 5'-TTAGCTTTCTTTAGGAGTATTGGCACCGTAA-3', APH_0625 (F) 5'-CACCATGACCGGAGTGTACTATTCCATTGGTG-3' and (R) 5'-CTAGCCTGTTATTAGACCAAAGTCTCTATAAAACTTTTCTGC-3', APH_0853 (F) 5'-CACCATGTTGATAGATGATCATGCAGCTGATTTTT-3' and (R) 5'-TCAGGTTG CACCAGGACC-3', APH_1049 (F) 5'-CACCTATGTTAACAAAAAGGGAATATTCAG TGACATGGG-3' and (R) CTAAGAATTAAGGTACTTATTAACGAAATCAAAAAGT CTACCTACT-3', and APH_1235 (F) 5'-CACCATGAAAGGAAAGTCAGATTCTGAAA TACGTACG-3' and (R) 5'-CTAACCTTGGGTCGATGCCACAATTTTC-3'. The underlined nucleotides correspond to a Gateway-compatible sequence. Genes were amplified from the NCH-1 strain, which is infectious for humans, and the non-human strain ApVar-1. PCR size products were compared by agarose gel electrophoresis. PCR products for *aph_0853* from ApVar-1 were cloned into the pENTR/TEV/D-TOPO cloning vector (Invitrogen, Cat. No. K2525-20). The insert sequences were sequenced and verified as correct.

Sequence alignments of APH_0853 were aligned using the http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalwan.html program for

protein and http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalwan.html for DNA.

CHAPTER 8: Conclusions

The results of this dissertation highlight the unusual and complex features of *A. phagocytophilum*. We characterized many important features about adhesion to PSGL-1 and potentially identified the first DC-specific *A. phagocytophilum* OMP. Previous work largely suggests that the *A. phagocytophilum* adhesins are surface exposed proteins. Our work was able to further demonstrate that the *A. phagocytophilum* DC is environmentally resistant, and responsible for binding human PSGL-1. We have established the DC's role as the adherent/infectious form of *A. phagocytophilum*, whereas the RC is fragile, does not bind human PSGL-1 and is the replicative form. This is in agreement with analogous forms of other obligate intracellular bacteria that undergo biphasic development.

Our attempts to capture adhesins by receptor crosslinking and affinity purification met with little success. We did learn that *A. phagocytophilum* must adhere to immobilized PSGL-1. The negative results from crosslinking, immunoprecipitation, and data from other lab members demonstrate that adherence to PSGL-1 is more complex than first realized. All of the approaches we tested have successfully identified adhesins of other pathogens. The inability to recover *A. phagocytophilum* adhesin(s) either suggests that there is a very weak affinity between the adhesin and receptor or that a multifaceted protein complex is required for adhesion to bind PSGL-1, thus preventing its direct

recovery by the methods employed in this study. Although we used computer predictions to identify outer membrane proteins in this study, they mainly serve as a good reference for comparison to experimental data. It is likely that some of these predictions are accurate, but the focus of my dissertation was to specifically identify proteins by experimental means. This would allow time to be spent focusing on proteins that were actually proven to be OMPs.

Our first attempt to identify OMPs by sonication, Renografin density-gradient purification and biotinylation was successful. We identified 3 known surface proteins as well as those previously identified by biotinylation. It is likely that many of the proteins we identified in our biotinylation approach are DC-specific. However, some of the identified proteins may have come from RC organisms. Consequently, we had to devise and test new gradient procedures using new and untested approaches.

Our expectation of using discontinuous density-gradient centrifugation to purify DC organisms was partially successful. The best results we obtained thus far are recovery of mixed populations of RC and DC organisms from syringe lysis. These organisms are contaminated with less cellular debris after gradient-centrifugation than if they were isolated by syringe lysis alone. Although not ideal, they can still serve as a template in biotinylation studies to identify surface proteins. This would identify surface proteins on both developmental forms, but will still provide a list of proteins on which to focus our attention. Although we may try to improve these gradient procedures, the continual transition between RC and DC organisms may prevent us from ever recovering a pure DC bacterial population.

Future directions

If we cannot isolate pure DC organisms by discontinuous density-gradient centrifugation, then there is an alternative approach that we will try. We can use the adherent property of the DC as a means to isolate it from RC organisms and cellular debris. We know that *A. phagocytophilum* organisms will bind to rhPSGL-1 immobilized to ELISA plates. We could isolate organisms by syringe lysis, and incubate them over a plate coated with rhPSGL-1. Only adherent DC organisms will bind to rhPSGL-1, thereby allowing for RC organisms and cellular debris to be washed away. DC organisms can then be scrapped off the plate and pelleted. Although theoretically possible, the yield of recovered DC organisms may be minimal and, if so, would require large-scale application.

Identification of putative OMPs in Chapter 5 and characterization of APH_1235 and APH_0248 in Chapter 7 demonstrates the strength of biotinylation to identify new proteins. If additional work confirms APH_1235 and APH_0248 as OMPs, it will reinforce the argument that some OMPs of *A. phagocytophilum* will have to be discovered by experimental methods and cannot be predicted. As mentioned above, use of mixed RC and DC organisms recovered from OptiPrep discontinuous density-gradient centrifugation still serves as a valuable resource for biotinylation studies to identify OMPs. We do not know how many proteins are differently expressed between the RC and DC. All we know for certain is that the DC upregulates or optimally modifies proteins allowing them to functions as adhesins. If a list of OMPs is generated from both RC and DC organisms, there are ways that we could narrow down on which proteins to focus our attention. Once antiserum is generated against a protein of interest, we can screen a lysate of the RC-

enriched bacteria and compare it to a lysate of both RC and DC organisms. Presumably, any surface protein that functions as an adhesin should be detected in the lysate of mixed organisms but not in the RC-enriched lysate. A similar approach would be to monitor genes of interest by real-time PCR over the course of a synchronous infection. Genes expressed late in development, concurrent with the appearance of DC organisms would be a priority to study.

Another potential approach that we could try is to reduce the list of OMPs by comparing the DNA sequence of our NCH-1 and other strains that infect human cells to that of ApVar-1. If and only if the ApVar-1 does not bind human PSGL-1, it would imply that one or more proteins are missing or mutated on the ApVar-1 surface, thereby resulting in the loss of adhesion to human cells. A direct comparison between ApVar-1 and our human strains may allow for identification of putative adhesins. A prime example is protein APH_0853.

Purification of the *A. phagocytophilum* RC will be invaluable for future experiments. Even though our focus was to identify OMPs using these purified organisms, we have the added bonus of using them to obtain the RC proteome. This will provide great knowledge into which proteins are involved in replication, metabolism, and potentially identify virulence proteins required for intracellular survival. If DC organisms can be purified in the future, characterization of their proteome will potentially identify prepackaged effector proteins and other proteins that are crucial during the early stages of binding and infection. Purification of the *A. phagocytophilum* RC is a vital step forward in this direction. Characterization of the two developmental forms will allow for

identification of RC and DC specific markers. This was indeed the case for the purified developmental forms of *Coxiella burnetii* (36). Two highly upregulated proteins were found to be associated with the infectious SCV (71, 72), and have since been identified as DNA-binding proteins that are only present in the compacted chromatin of the infectious SCV. Presumably *A. phagocytophilum* has proteins of similar function. We have since tested improved urea-based lysis buffers, which greatly enhances solubilization of purified samples compared to RIPA. Complete solubilization of samples will be critical to recover all the proteins associated with each developmental form. APH_1235 is likely an OMP; and preliminary data suggests that it is DC specific. If APH_1235 is confirmed as DC-specific, it will serve as the first maker to distinguish between the two *A. phagocytophilum* developmental forms. This will allow us to rapidly distinguish between forms, thus eliminating the need to confirm experimental results by TEM.

A long term goal of the Carlyon lab is to identify OMPs as adhesins to the confirmed human receptor PSGL-1. Though it may take some time to identify and specifically prove an OMP as an adhesin to human PSGL-1, any OMPs we identify in the process will be of great value. At this time very little is known about how *A. phagocytophilum* is transmitted from a tick and infects a host. I believe that DC organisms are transferred in some fashion from the tick to the host during a blood meal. Ticks feed on a variety of mammals and I do not believe *A. phagocytophilum* has the ability to know the host it will next infect. This implies the *A. phagocytophilum* DC must have the adhesins on its surface ready to bind many diverse receptors. As mentioned earlier, we have observed by TEM the infectious DC form of *A. phagocytophilum* isolated from HL-

60 cells binding to mouse mast cells (unpublished data). Because the DC is presumably metabolically inactive, it would not have the ability to upregulate proteins specific to the host into which it was just transmitted. This strongly implicates that the adhesin(s) for multiple cells types are likely expressed on the DC surface.

I believe that *A. phagocytophilum* has a select number of adhesins that cooperatively act in various combinations that allow the organisms to bind to a variety of different receptors, resulting in infection of various cell types within mammalian and tick hosts. Therefore, if an identified surface protein does not function as an adhesin to human PSGL-1, it may be equally important in infection of another mammal. Nonetheless, identification of other proteins as adhesins of human PSGL-1 will have great value and application to other organisms. Fucosylation of human, mouse and tick cells lines is critical for *A. phagocytophilum* infection (29, 137, 194). If the bacterial adhesin responsible for binding fucose on human PSGL-1 is identified, it will have broad implications for protection against *A. phagocytophilum* infection of more than humans alone. Further identification and characterization of *A. phagocytophilum* surface exposed proteins will greatly enhance our understanding of mammalian infection by this potentially fatal tick-transmitted pathogen.

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Introduction (Major surface protein 2 (Msp2[P44]): The Dominant surface protein):

Infection and Immunity, 2008, Vol. 76, No. 5, p. 2090-2098, doi:10.1128/IAI.01594-07. Reproduced/amended with permission from American Society for Microbiology. License Number 2536641121018. *Anaplasma phagocytophilum* MSP2(P44)-18 Predominates and Is Modified into Multiple Isoforms in Human Myeloid Cells. Portion: Excerpt.

Chapter 1 (Figure 1):

Reprinted from Trends in Parasitiology, Vol. 26, No. 4, Nicholson, W.L., K.E. Allen, J.H. McQuiston, E.B. Breitschwerdt and S.E. Little, The increasing recognition of rickettsial pathogens in dogs and people, Page No. 205-212 , Copyright 2010, with permission from Elsevier. License Number 2562631155562. Portion: Figure.

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Chapter 2:

Infection and Immunity, 2009, Vol. 77, No. 9, p. 4018-4027, doi:10.1128/IAI.00527-09. Reproduced/amended with permission from American Society for Microbiology. License Number 2536640663742. *Anaplasma phagocytophilum* Dense-Cored Organisms Mediate Cellular Adherence through Recognition of Human P-Selectin Glycoprotein Ligand. Portion: Full article

VITA

Matthew James Troese was born in Pennsylvania. He was raised in Mechanicsburg, Pennsylvania and graduated from Cumberland Valley High School in 2001. In May 2005 he received his Bachelor of Science in Biology from Shippensburg University in Shippensburg, Pennsylvania. In the fall of 2005 he began his graduate studies at the University of Kentucky and joined the lab of Dr. Jason A. Carlyon in May 2005 in the Department of Microbiology, Immunology and Molecular Genetics. In the fall of 2007 the Carlyon Lab moved to Virginia Commonwealth University, Medical College of Virginia, Department of Microbiology and Immunology, where he received his Ph.D. in Microbiology.

HONORS/AWARDS

Mid-Atlantic Microbial Pathogenesis Meeting, Travel Grant recipient	2009
The American Society For Rickettsiology (ASR), Travel Grant recipient	2007
Support from NIH, National Institute for Allergy and Infectious Diseases Training Program in Microbial Pathogenesis (PHS grant T32 AI49795)	8/1/06-7/31/07
The National Dean's List	2006-2007
Chancellor's List	2005-2006

PUBLICATIONS (In reverse chronological order):

1. B. Huang, **M.J. Troese**, D. Howe, S. Ye, J.T. Sims, R.A. Heinzen, D.L. Borjesson and J.A. Carlyon. 2010. *Anaplasma phagocytophilum* APH_0032 is expressed late during infection and localizes to the pathogen-occupied vacuolar membrane. Microbial Pathogenesis 49(5): 273-284.

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4. **Troese, M.J.** †, M. Sakar†, N.L. Galloway, R.J. Thomas, S.A. Kearns, D.V. Reneer, T. Yang, and J.A. Carylon. 2009. Differential expression and glycosylation of *Anaplasma phagocytophilum* major surface protein 2 paralogs during cultivation in sialyl lewis x-deficient host cells. Infection and Immunity 77(5): 1746-1756.

5. Reneer, D.V.†, **M.J. Troese**†, B. Huang, S.A. Kearns and J.A. Carlyon. 2008. *Anaplasma phagocytophilum* PSGL-1-independent infection does not require Syk and leads to less efficient AnkA delivery. Cellular Microbiology 10(9): 1827-1838.

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8. D'Orazio, S.E., **M.J. Troese**, and M.N. Starnbach. 2006. Cytosolic localization of *Listeria monocytogenes* triggers an early IFN-gamma response by CD8+ T cells that correlates with innate resistance to infection. Journal of Immunology 177: 7146-7154.

† = Contributed equally to this work.

ABSTRACTS **(In reverse chronological order):**

1. **M.J. Troese*** and J.A. Carlyon. Purification of *A. phagocytophilum* Dense-cored cell (DC) and Reticulate cell (RC) populations by density-gradient centrifugation. 24th Meeting of the American Society for Rickettsiology. Stevenson, Washington, USA. July 31 - August 3, 2010. (Poster)

2. **M.J. Troese*** and J.A. Carlyon. *Anaplasma phagocytophilum* dense-cored organisms mediate cellular adherence through recognition of human P-selectin glycoprotein ligand-1. American Society for Microbiology; Virginia branch 2009 annual meeting. Virginia Commonwealth University, Richmond, VA, USA. November 13-14, 2009. (Oral)

3. **M.J. Troese*** and J.A. Carlyon. Expression and characterization of *A. phagocytophilum* Asp55 and Asp62 throughout the course of infection in human myeloid cells. 23rd Meeting of the American Society for Rickettsiology. Hilton Head Island, South Carolina, USA. August 15-18, 2009. (Poster)

4. B. Huang, P. Clark, **M.J. Troese**, J.A. Carlyon***.** *Anaplasma phagocytophilum* proteins APH_0032 and APH_1387 associate with the pathogen-occupied vacuolar membrane and are phosphorylated. 23rd Meeting of the American Society for Rickettsiology. Hilton Head Island, South Carolina, USA. August 15-18, 2009. (Poster)

5. B. Huang*, P. Clark, **M.J. Troese**, and J.A. Carlyon. Two bacterial proteins - P100 and P130 - are phosphorylated and modify the *Anaplasma phagocytophilum* inclusion membrane. 109th General Meeting; American Society for Microbiology. Philadelphia, Pennsylvania, USA. May 17-21, 2009. (Poster)

6. **M.J. Troese*** and J.A. Carlyon. Characterization of *Anaplasma phagocytophilum* binding, entry, and intracellular development in promyelocytic host cells and identification of putative adhesins mediating cellular attachment. Mid-Atlantic Microbial Pathogenesis Meeting. Wintergreen Resort, Wintergreen, VA, USA. February 8-10, 2009. (Poster)

7. B. Huang*, **M.J. Troese**, P. Clark, and J.A. Carlyon. Two bacterial proteins – P100 and P130 – are phosphorylated and modify the *Anaplasma phagocytophilum* inclusion membrane. Mid-Atlantic Microbial Pathogenesis Meeting. Wintergreen Resort, Wintergreen, VA, USA. February 8-10, 2009. (Poster)

8. **M.J. Troese*** and J.A. Carlyon. Characterization of *Anaplasma phagocytophilum* binding, entry, and intracellular development in promyeolocytic host cells. The 25th Annual Daniel T. Watts Research Poster Symposium. Virginia Commonwealth University, USA. October 28-30, 2008. (Poster)

9. **M.J. Troese*** and J.A. Carlyon. Characterization of *Anaplasma phagocytophilum* binding, entry, and intracellular development in promyeolocytic host cells. 108th General Meeting; American Society for Microbiology. Boston, Massachusetts, USA. June 1-5, 2008. (Poster)

10. J.A. Carlyon*, and **M.J. Troese**. Assessment of differential cellular adherence, invasion, and receptor recognition by *Anaplasma phagocytophilum* dense-cored and reticulate cells. 5th International Conference on Rickettsiae and Rickettsial Diseases. Marseille, France. May 18-20, 2008. (Oral)

11. J.A. Carlyon*, D.V. Reneer, **M.J. Troese**, B. Huang, and S.A. Kearns. *A. phagocytophilum* sLe^x/ PSGL-1-independent infection does not require Syk and leads to less-efficient AnkA delivery. 5th International Conference on Rickettsiae and Rickettsial Diseases. Marseille, France. May 18-20, 2008. (Poster)

12. **M.J. Troese***, D.V. Reneer, and J.A. Carlyon. Two high molecular weight *Anaplasma phagocytophilum* proteins localize to the bacterial inclusion membrane and are differentially expressed throughout the course of infection. 21st Meeting of the American Society for Rickettsiology. Colorado Springs, Colorado, USA. September 8-11, 2007. (Poster)

13. **M.J. Troese**, M. Sarkar, S.A. Kearns, D.V. Reneer, and J.A. Carlyon*. *Anaplasma phagocytophilum* MSP2 (P44)-18 predominates and is modified into multiple isoforms in human myeloid cells. 21st Meeting of the American Society for Rickettsiology. Colorado Springs, Colorado, USA. September 8-11, 2007. (Poster)

14. **M.J. Troese**, M. Sarkar, S.A. Kearns, D.V. Reneer, and J.A. Carlyon*. Differential expression of *Anaplasma phagocytophilum* MSP2 (P44) paralogs is induced upon cultivation in sialyl lewis x-deficient HL-60 Cells. 21st Meeting of the American Society for Rickettsiology. Colorado Springs, Colorado, USA. September 8-11, 2007. (Poster)

15. T. Bykowski*, M.E. Woodman, A.E. Cooley, L.H. Burns, **M.J. Troese**, R. Wallich, P. Kraiczy, and B. Stevenson. *Borrelia burgdorferi* complement regulator-acquiring surface proteins: Expression during the Lyme disease spirochete's mammal-tick infection cycle. IX International Jena Symposium on Tick-borne Diseases. Jena, Germany. March 15-17, 2007. (Oral)

16. **M.J. Troese*,** H. Miwa, T. Hering, and D.M. Snow. Neuronal growth cones can detect subtle differences in chondroitin sulfate proteoglycan structure. Neuroscience Day, Bluegrass Chapter – Society for Neuroscience. Lexington KY, USA. March 13, 2006. (Poster)

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