CLINICAL MICROBIOLOGY REVIEWS, July 2011, p. 469–489 Vol. 24, No. 3
0893-8512/11/$12.00 doi:10.1128/CMR.00064-10
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Mechanisms of Obligatory Intracellular Infection with *Anaplasma phagocytophilum*

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INTRODUCTION 469
Anaplasma phagocytophilum 469
Classification 469
Morphology 470
Natural reservoir 470
Strain variations 470
Human Granulocytic Anaplasmosis and Diseases in Domestic Animals 472
HGA 472
Diseases in domestic animals 472
GENOMIC FEATURES AND GENE REGULATION OF *A. PHAGOCYTOPHILUM* 473
Genomic Features 473
Major Surface Antigen P44 474
Type IV Secretion System 475
Two-Component System and Transcriptional Regulation 475
REPLICATION OF *A. PHAGOCYTOPHILUM* IN EUKARYOTIC CELLS 475
Intracellular Developmental Cycle 475
Host Cell Receptor, Site of Entry, and Internalization Signal 476
Anaplasma Inclusion Biogenesis 477
Contribution of host cells 477
Modification of the inclusion membrane by anaplasma proteins 477
SUBVERSION OF NEUTROPHIL ANTIMICROBIAL DEFENSES 477
Downregulation of Reactive Oxygen Species Generation 478
Inhibition of Host Cell Apoptosis 480
Subversion of Autophagy 480
Other Host Cell Signals Activated and Required for *A. phagocytophilum* Infection 481
Tyrosine kinase activation 481
ERK1/2 activation 481
PATHOGENESIS AND IMMUNE RESPONSES 481
Generation of Chemokines/Cytokines 481
Roles of IFN-γ and IL-8 482
TICK FACTORS REQUIRED FOR TRANSMISSION 483
CONCLUSIONS 484
ACKNOWLEDGMENTS 484
REFERENCES 484

INTRODUCTION

In the early 1990s, an unknown febrile tick-borne illness, now called human granulocytic anaplasmosis (HGA) (57), accompanied with granulocytic inclusions of bacteria closely related to the veterinary pathogens *Ehrlichia phagocytophilum* and *E. equi*, all of which are now called *Anaplasma phagocytophilum*, was discovered in Minnesota and Wisconsin (14, 48). This seminal discovery led to the successful isolation and cultivation of *A. phagocytophilum* (80) and an understanding of clinical, immunological, and pathological characteristics of HGA and the disease distribution, tick vector species, and wild animal reservoirs. Alongside these discoveries, progresses have been made toward an understanding of the mechanisms by which this pathogen can invade and proliferate inside neutrophils, primary host defensive cells, to cause disease. Genome sequences of *A. phagocytophilum* (61) and the vector tick *Ixodes scapularis* and the development of new methods and approaches have been expanding our ability to investigate this extraordinary pathogen and to determine bacterial and host factors critical for its invasion, survival, persistence, and transmission from infected ticks to mammalian host cells.

Anaplasma phagocytophilum

Classification. *A. phagocytophilum* includes the newly discovered human pathogen whose original name was the human granulocytic ehrlichiosis (HGE) agent (14, 48) and previously known ruminant (*Cytoecetes phagocytophila* and *Ehrlichia phagocytophila*) and equine (*Ehrlichia equi*) pathogens (57). *A. phagocytophilum* belongs to the family *Anaplas-*

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mataceae, in the order *Rickettsiales* and the class *Alphaproteobacteria* (57). The family *Anaplasmataceae* includes five well-known genera, *Ehrlichia*, *Anaplasma*, *Neorickettsia*, *Aegyptianella*, and *Wolbachia*, and two less-well-studied genera, "*Candidatus* Neoehrlichia" and "*Candidatus* Xenohaliotis." All of these genera infect specific invertebrate hosts (ticks, insects, trematodes, nematodes, or mollusks) that are abundant in nature. Unlike *Neorickettsia* and *Wolbachia* spp., which can be transmitted through generations of invertebrate hosts, *Anaplasma* and *Ehrlichia* cannot effectively pass from adult ticks to offspring (transovarial passage) (139, 206). All genera except *Wolbachia* and "*Candidatus* Xenohaliotis" are known to infect vertebrates (mammals or birds). Vertebrate infection can be acute or chronic and may result in fatality. The bacteria infect specific host cell types within vertebrates, usually cells of hematopoietic origin, such as neutrophils, monocytes/macrophages, platelets, red blood cells, or endothelial cells. Characteristics of members of the family *Anaplasmataceae* are summarized in Table 1. *A. phagocytophilum* is one of four species belonging to the genus *Anaplasma*, which have different host cell specificities. For *A. phagocytophilum*, primary host cells are granulocytes, and endothelial cells are also infected (48, 80, 92, 142, 197, 235). The phylogenetic relationship of *A. phagocytophilum* to other members of the genus *Anaplasma* and members of the family *Anaplasmataceae* is shown in Fig. 1.

Morphology. *A. phagocytophilum* is a small Gram-negative pleomorphic coccus enveloped by two membranes, as are other members of the family *Anaplasmataceae*. The bacterial size is generally 0.4 to 1.3 μm, but the bacteria can be as large as 2 μm. The outer membrane of the bacterium is often ruffled, which creates an irregular periplasmic space, and there is no capsule layer. Fine DNA strands and ribosomes are distinctly seen within the bacteria (185, 199, 235). Unlike members of the family *Rickettsiaceae*, which escape from phagosomes and replicate directly within the cytoplasm of eukaryotes, members of the family *Anaplasmataceae* replicate in membrane-bound vacuoles (referred to as inclusions or parasitophorous vacuoles) within the cytoplasm of eukaryotic host cells. The bacteria may be tightly packed inside inclusions in part due to a loss of peptidoglycan and lipopolysaccharide (LPS) (130). The loss permits the bacteria to squeeze within a limited intravacuolar space while maintaining the plasticity of the infected granulocytes that is required for capillary circulation.

Gram staining is not suitable to visualize intracellular bacteria because of a lack of contrast against the host cytoplasm. Romanowsky staining is generally used, usually with a quick method such as Diff-Quik. This approach stains the bacteria purple, which allows the visualization of characteristic mulberry-like bacterial clumps called morulae. (The term "morula" is derived from the Latin term "morus," which means mulberry.) Morulae are usually 1.5 to 2.5 μm in diameter but can be as large as 6 μm (185).

Natural reservoir. *A. phagocytophilum* DNA has been detected in several species of *Ixodes* ticks (*I. scapularis*, *I. pacificus*, *I. spinipalpis*, *I. ricinus*, *I. persulcatus*, and *I. ovatus*) in the United States, Europe, and Asia (37, 38a, 170, 171, 193, 216). Naturally infected ticks were shown to transmit *A. phagocytophilum* to naïve mammals (63, 192, 207). Once ticks acquire the bacterium from infected mammals through a blood meal, the bacterium is maintained from the larva or nymph stage to adult stages of metamorphosis and is transmitted to mammals during the next blood meal (169, 216, 250). Since there is no evidence of transovarial (from adult ticks to eggs) transmission, larvae do not transmit the bacterium to mammals, but infected nymphs and adult ticks do. The mammalian reservoir for *A. phagocytophilum* infection within the United States includes white-footed mice (*Peromyscus leucopus*), raccoons (*Procyon lotor*), gray squirrels (*Sciurus carolinensis*), gray foxes (*Urocyon cinereoargenteus*), and redwood chipmunks (69, 127, 165, 216). A variety of other wild animals are also implicated as reservoirs (68; reviewed recently in references 208 and 218). Although *Ixodes* ticks often feed on white-tailed deer, the deer are infected with the Ap-Variant 1 strain of *A. phagocytophilum*, rather than with the human strain, in the United States (146). Diverse *A. phagocytophilum* strains are also found in animals and ticks in Europe, Japan, and Russia (109, 111, 151, 160, 170, 203, 238, 239), where HGA has been rarely reported. These findings imply that the zoonosis potential of *A. phagocytophilum* depends not only on the transmissibility, habitats, and population density of ticks and infected mammals (90) but also on the genetic variations of *A. phagocytophilum*. A primary natural tick-mammalian transmission cycle of *A. phagocytophilum* interlacing with bacterial strain diversity and host susceptibility is depicted in Fig. 2. The transovarial transmission of *A. phagocytophilum* variants occurs in *Dermacentor albipictus* (19). Thus, it is possible that there are atypical systems such as *D. albipictus* feeding into the normal *Ixodes* infection cycle.

Strain variations. All *A. phagocytophilum* isolates appear to have serological cross-reactivity. In mammals, the most common constitutively produced antigens are 42- to 49-kDa proteins, which are expressed on the bacterial outer membrane (11, 56, 105, 111a, 239, 249, 251). The proteins are encoded by the *p44* (also called *msp2*) gene family. Serological tests are generally group specific and cannot be used to distinguish individual strains. *A. phagocytophilum* strains show a minor degree of variation in the nucleotide sequence in 16S rRNA and *groESL*. The *p44*, *p44ESup1* (*omp-1N*), *msp2* (different from *p44*), and *ankA* genes contain major strain variation (23, 44, 61, 67, 111, 134, 135, 150, 153, 203, 224, 239). These and potentially other genes may allow more detailed comparisons among strains. For example, diverse *ankA* sequences are found in naturally infected ticks and wild deer, including those that have not been detected in humans or domestic animals (203, 224).

Several studies reported that *A. phagocytophilum* strains differ in host infectivity. For example, strain Ap-Variant 1 from *I. scapularis* ticks infects goat and deer but not mice (146, 148, 149). A Californian strain infectious to equines was not infectious to ruminants (205). *A. phagocytophilum* strains from wild rodents were reported to differ in horse infectivity (68). Although several human, ruminant, and equine strains are cultivated in ISE6 and/or IDE8 or other tick cell lines (80, 147, 159, 234), so far, only human isolates have been directly cultured by using HL-60 cells, a human promyelocytic leukemia cell line. Recently, new strains of *A. phagocytophilum* from Chinese sheep and wild rodents were cultured in HL-60 cells after initial passage in BALB/c mice (246). The bacterial factors that determine virulence, host mammal species specificity, and cultivability are not known. Arthropod-borne diseases, including *A. phagocytophilum* infection, are on the rise, probably because

TABLE 1. *Anaplasmataceae*, hosts, and diseases[a]

Genus	Species	Disease(s)	Host(s)	Host cells	Vector(s)	Distribution	Old name(s)
Ehrlichia	***E. canis***	Canine ehrlichiosis	Canids, human	Monocytes/macrophages	*Rhipicephalus sanguineus*	Global	
	E. chaffeensis	Human monocytic ehrlichiosis	Human, deer, dog	Monocytes/macrophages	*Amblyomma americanum*	United States, South America, Asia	
	E. ewingii	Canine granulocytic ehrlichiosis, human ewingii ehrlichiosis	Dog, deer, human	Granulocytes	*Amblyomma americanum*	United States	
	E. muris	Splenomegaly	Rodents, human	Monocytes/macrophages	*Hemaphysalis* spp., *Ixodes* spp.	Japan, Russia, United States	
	E. ruminantium	Heartwater	Ruminants, human	Endothelial cells, granulocytes	*Amblyomma* spp.	Africa, Caribbean	*Cowdria ruminantium*
Anaplasma	***A. phagocytophilum***	Human granulocytic anaplasmosis, tick-borne fever, equine ehrlichiosis	Human, horse, ruminants, rodents, dog, cat, deer	Granulocytes, endothelial cells	*Ixodes scapularis*, *Ixodes pacificus*, *Ixodes ricinus*	United States, Europe, Asia	*Ehrlichia* (*Cytoecetes*) *phagocytophila*, human granulocytic ehrlichiosis agent, *E. equi*
	A. marginale	Bovine anaplasmosis	Bovine	Erythrocytes	Various tick species	Global	
	A. bovis	Fever, lymphoadenopathy	Bovine, deer, rabbit	Monocytes	Various tick species	United States, Africa, Japan	*Ehrlichia bovis*
	A. platys	Canine cyclic thrombocytopenia	Dog	Platelets	*Rhipicephalus sanguineus*?	Global	*Ehrlichia platys*
Aegyptianella	*A. pallorum*	Anemia	Birds	Erythrocytes	*Argas* (*Persicargus*) *persicus tick*	Global	
Neorickettsia	***N. risticii***	Potomac horse fever, equine monocytic ehrlichiosis	Horse	Monocytes/macrophages, intestinal epithelial cells, mast cells	*Acanthatrium oregonense* trematode	North and South America	*Ehrlichia risticii*
	N. sennetsu	Sennetsu fever, glandular fever	Human	Monocytes/macrophages	Unknown trematode	Japan, Southeast Asia	*Ehrlichia sennetsu*, *Rickettsia sennetsu*
	N. helminthoeca	Salmon poisoning disease	Canids	Monocytes/macrophages	*Nanophyetus salmincola* trematode	North and South America	
Wolbachia	*W. pipientis*	Feminization, parthenogenesis, male killing, cytoplasmic incompatibility (arthropods); river blindness and elephantitis (mammalian hosts of infected nematodes)	Arthropods, nematodes	Syncytial lateral cord cells, ovary	Not applicable	Global	
"*Candidatus* Neoehrlichia"	"*Ca.* Neoehrlichia mikurensis"	Human neoehrlichiosis	Rodents, human	Endothelial cells	*Ixodes ovatus*, *Ixodes ricinus*	Japan, China, Europe	
	"*Ca.* Neoehrlichia lotoris"	Unknown	Raccoon	Unknown	Unknown	United States	
"*Candidatus* Xenohaliotis"	"*Ca.* Xenohaliotis *californiensis*"	Withering syndrome	Abalone	Gastrointestinal epithelial cells	Unknown	United States	

[a] Well-established human pathogens are shaded. Names of bacteria which have been stably cultured in mammalian host cells are shown in boldface type. Several tick-borne species have been cultured in tick cell lines.

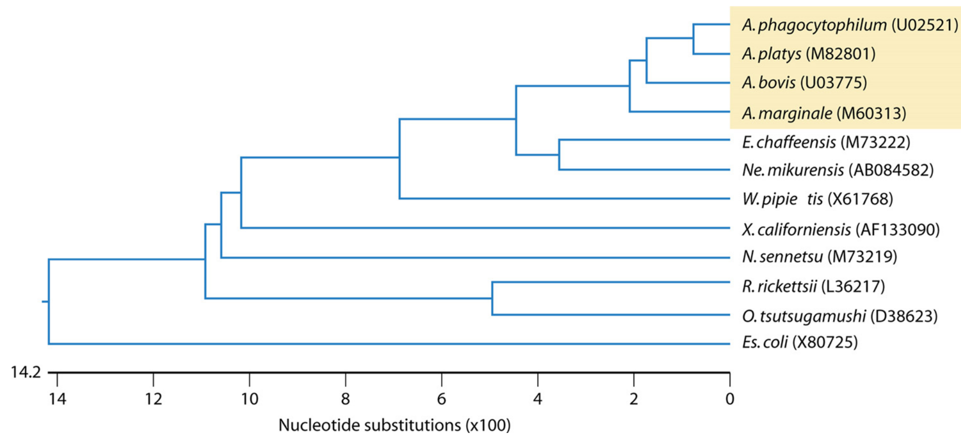

FIG. 1. Phylogram of members of the genus *Anaplasma* in the family *Anaplasmataceae*. The genus *Anaplasma* is highlighted in gray. Phylogenetic trees were constructed based on 16S rRNA sequence alignment by the Clustal W method using the MegAlign program from the Lasergene package. GenBank accession numbers are shown in parentheses.

of changes in human activities and climate (27, 77, 84). The results of molecular studies on *A. phagocytophilum* variants will shed light on the evolution, population dynamics, and ecology of naturally occurring *A. phagocytophilum* strains and will help identify risks for outbreaks of zoonosis and important veterinary diseases.

Human Granulocytic Anaplasmosis and Diseases in Domestic Animals

HGA. HGA is characterized by fever; chills; headache; myalgia; hematological abnormalities, including leucopenia and thrombocytopenia; and increased serum aminotransferase liver enzyme activity, which suggests mild to moderate liver injury (3, 15, 58, 59). Patients generally respond to doxycycline, the treatment of choice. Although fluoroquinolones are active *in vitro* against *A. phagocytophilum*, it causes a relapse of infection in severe combined immunodeficiency mice and HGA patients and is not recommended (237). The case-fatality rate is 0.7% in the Midwestern United States, and the risk of fatality is greater when therapy initiation is delayed, when patients are elderly, and when patients have complicating opportunistic infections and/or antecedent medical conditions, such as diabetes mellitus or immunocompromise (13, 59). In a case series, illness associated with HGA in the Northeastern United States was more mild than that originally described in the Midwestern United States (3), suggesting strain-dependent virulence. The median age of patients with HGA is 50 to 60 years, higher than that associated with other tick-borne diseases (for example, the median age of patients with Lyme disease is around 40 years).

The number of documented cases of HGA has increased every year since 1999, the year when the disease became notifiable in the United States (176), and 1,161 cases of *A. phagocytophilum* infection were reported in 2009 (47). Numbers of HGA cases and *A. phagocytophilum* sero- or DNA-positive clinical cases have also been increasing in Europe and Asia (7, 17, 123, 170a, 175, 182, 200, 247). Minnesota, Wisconsin, New York, and Massachusetts remain states with the highest incidence of HGA (47). In areas of Wisconsin and New York where the disease is endemic, seroepidemiological data suggest that many infections go unrecognized, and it was estimated that 15% and 36% of the populations, respectively, have been infected (2, 16). Results from PCR assays and serological tests suggest that HGA is a major cause of unexplained fever during the tick season in Wisconsin (26). Most cases of HGA occur in the same states that have high incidences of Lyme disease and human babesiosis; this is because *Ixodes* ticks also transmit *Borrelia burgdorferi*, the causative agent of Lyme disease, and *Babesia* species, the causative agent of babesiosis. Simultaneous infection with *A. phagocytophilum* and *B. burgdorferi* in humans has been reported (4, 17, 123, 162). Therefore, the choice of antimicrobial agent needs to take into account whether a patient has a combined infection. For example, amoxicillin can be used to treat early-stage Lyme disease, although it is not effective as a treatment for HGA.

Although the primary mode of transmission of *A. phagocytophilum* to humans is from an infected tick, the transmission of the bacteria can occur perinatally (97) and nosocomially (247). *A. phagocytophilum* in infected human blood specimens is viable at 4°C for up to 18 days (108), and transmission has occurred through blood transfusion (46) and contact with the blood of infected mammals (18).

Diseases in domestic animals. Clinical signs of granulocytic anaplasmosis in domestic animals (horse, dog, and ruminants) include fever accompanied by leucopenia and thrombocytopenia. Opportunistic infections may also occur (reviewed in references 43, 196, 233). Other signs of disease in animals include depression and anorexia in horses and dogs and limb edema and ataxia in horses. *A. phagocytophilum* strains that were previously known as *Cytoecetes phagocytophila* or *Ehrlichia phagocytophila* cause tick-borne fever in ruminants, mainly in Western Europe (66, 82, 102). Strains that were previously known as *Ehrlichia equi* or the HGE agent cause granulocytic ehrlichiosis in equines (also known as equine granulocytic anaplasmosis) (7, 30, 86, 112, 142, 205) and in canines (also known as canine granulocytic anaplasmosis, distinct from *Ehrlichia ewingii* infection) (7, 85, 183, 188; reviewed in reference 43). Equine and canine granulocytic ehrlichioses have been found

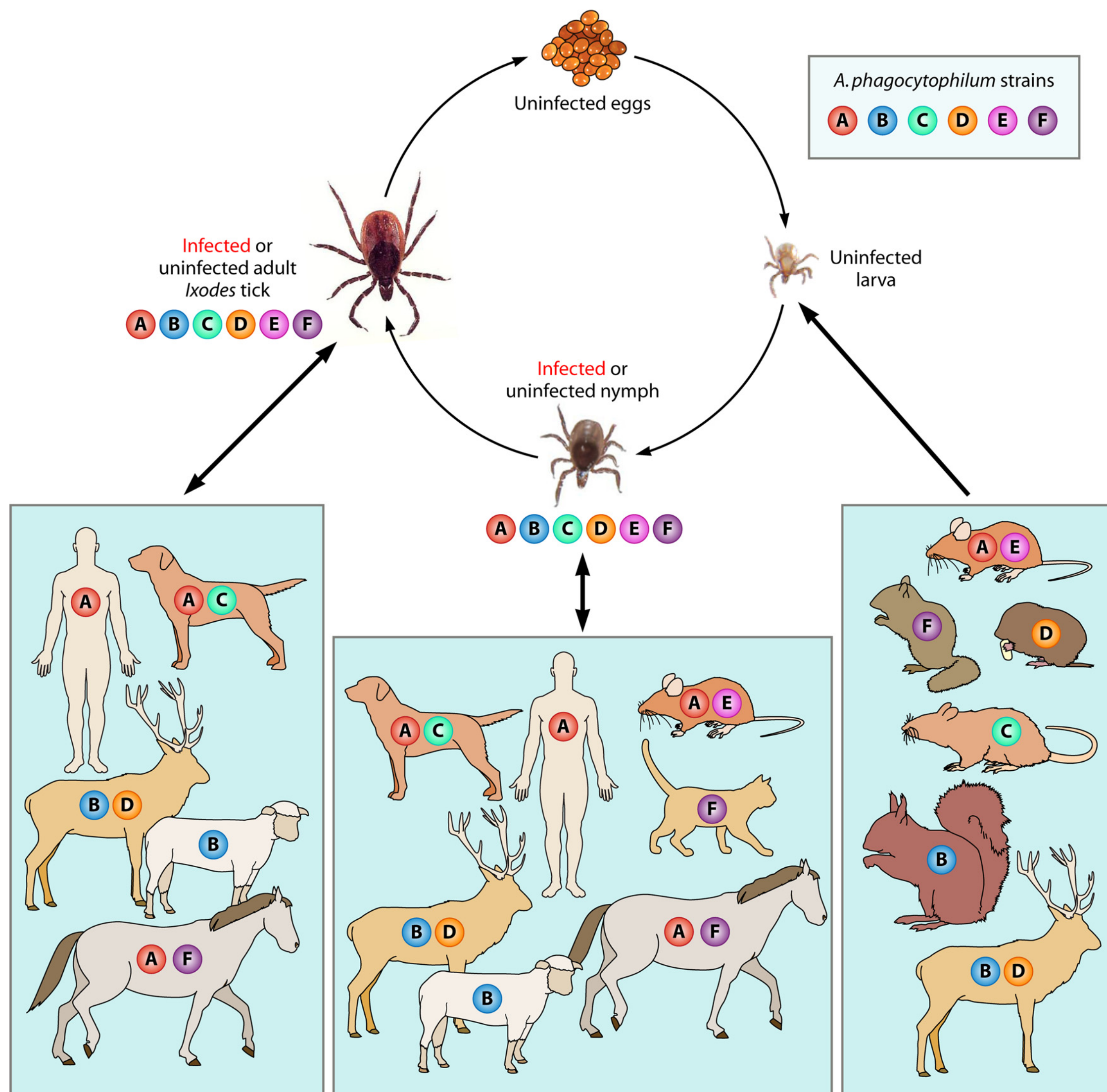

FIG. 2. Proposed life cycle of *A. phagocytophilum*. There are diverse strains of *A. phagocytophilum* in nature, and the susceptibilities of mammalian species to *A. phagocytophilum* strains vary. *A. phagocytophilum* cannot be passed effectively from infected adult *Ixodes* sp. ticks to eggs. Thus, larvae are not infected. Ticks at the larval, nymphal, or adult stage acquire *A. phagocytophilum* strains through blood feeding on infected animals. Once infected at the larval or nymphal stage, *A. phagocytophilum* is maintained in ticks through metamorphosis and molting to the next life stage and transmitted to the animals via blood feeding when the animal host is susceptible to the particular strain. Humans are susceptible to only limited strains, are the dead-end host of *A. phagocytophilum*, and are not a normal part of the life cycle of *A. phagocytophilum* or ticks. The animal species susceptibility to putative *Anaplasma* strains shown is a proposal, most of which has not been proven experimentally.

in broad distributions across the United States, Canada, South America, and Europe. Feline granulocytic anaplasmosis is less common but has been increasingly reported in Europe and the United States (8, 28, 31, 122, 202).

The present review primarily describes our current understanding of the molecular mechanisms of intracellular infection by *A. phagocytophilum* isolates from HGA patients in the United States, since *A. phagocytophilum* from other sources has not been sufficiently studied in this aspect. For clinical and laboratory diagnosis, treatment, epidemiology, and the prevention of HGA, please refer to recent reviews and guidelines (15, 59, 218, 226, 236).

GENOMIC FEATURES AND GENE REGULATION OF *A. PHAGOCYTOPHILUM*

Genomic Features

The genome size (1.47 Mb) of *A. phagocytophilum* strain HZ, isolated from a patient in the state of New York (199), is approximately one-quarter of the size of the *Escherichia coli* genome, the model prokaryote. The number of open reading frames (ORFs) (1,369) is also about one-quarter of that found in *E. coli* (61). The G-C content of the DNA in *A. phagocytophilum* strain HZ is 41.6 mol%, which is lower than the *E. coli*

TABLE 2. Selected characteristics different between the *Anaplasmataceae* and the *Rickettsiaceae*

Characteristic	*Anaplasmataceae*	*Rickettsiaceae*
Replication site	Membrane-lined vacuole	Cytosol
Cofactor synthesis	Yes (less in *Wolbachia*)	Limited
Purine, pyrimidine	Yes	No
NAD synthesis	Yes (except *Wolbachia*)	No
ATP/ADP translocase	No	Yes
LPS	No	Yes (no for *Orientia*)
Peptidoglycan	Minimum	Yes
Glycolysis pathway	Partial	No
Two morphotypes, DC and RC	Yes	No

average G-C content (~50 mol%) but higher than the ~29.1% of *Rickettsia prowazekii* (10). No plasmids, intact prophages, or transposable elements are present. The genome of *A. phagocytophilum* has numerous repeats (12.7% of the genome), as seen in over 100 *p44* (*msp2*) genes, type IV secretion (T4S) genes, and genes containing tandem repeats (61, 207, 248). Among genes that are conspicuously absent from *A. phagocytophilum* are those genes required for the biosynthesis of lipopolysaccharide and peptidoglycan (61, 130). Approximately 747 ORFs (55%) have an assigned function, encoding mostly housekeeping genes found in other bacteria (61), and 82 ORFs encode conserved hypothetical proteins (meaning that they are found in other bacteria as well but do not have an assigned function). Approximately 458 ORFs encode unique hypothetical proteins that are not found in other organisms. A recent global proteomic analysis revealed a total of 1,212 *A. phagocytophilum* proteins in infected human leukocytes, representing 89.3% of the predicted bacterial proteomes. Nearly all bacterial proteins (≥99%) with known functions are expressed, whereas only approximately 80% of "hypothetical" proteins were detected in infected human cells (132).

Several hypothetical proteins in *A. phagocytophilum* were found to be surface-exposed proteins, inclusion membrane proteins, and T4S substrates that confer unique phenotypes to this bacterium (74, 99, 100, 166, 195, 210). *A. phagocytophilum* and *Rickettsia prowazekii* share 469 genes (61), and both bacteria cannot use glucose as a carbon or energy source. However, unlike *Rickettsia*, *A. phagocytophilum* has a partial glycolysis pathway starting with fructose 1,6-biphosphate. Like *R. prowazekii*, *A. phagocytophilum* has pathways for aerobic respiration, including the metabolism of pyruvate, the tricarboxylic acid cycle, and the electron transport chain. Unlike *R. prowazekii*, however, *A. phagocytophilum* does not encode a cytochrome *d*-type oxidase (*cydAB* [cytochrome *d* ubiquinol oxidase]), which has a high affinity for oxygen that is useful for microaerophilic respiration; *A. phagocytophilum* does not have ATP/ADP translocase, which assists in moving ADP out of the bacterial inner membrane while assisting ATP to enter the inner bacterial membrane to supply the bacteria with host-synthesized ATP; and *A. phagocytophilum* contains genes for the biosynthesis of all of the necessary nucleotides and most vitamins and cofactors, including biotin, folate, flavin adenine dinucleotide (FAD), NAD, coenzyme A, thiamine, and protoheme, which might benefit the tick vector. Selected characteristics that are different between the *Anaplasmataceae* and the *Rickettsiaceae* are summarized in Table 2. *A. phagocytophilum* has a minimal coding capacity for central intermediary metabolism, and it has the ability to make only four amino acids, glycine, glutamine, glutamate, and aspartate (61); therefore, *A. phagocytophilum* must acquire the remaining amino acids and other compounds from the host. *A. phagocytophilum* has 41 genes that belong to the category of transport and binding proteins, which is lower than the number in free-living bacteria but is critical for the survival of *A. phagocytophilum*.

Major Surface Antigen P44

The P44/Msp2 proteins are major surface antigens, and therefore, they are useful for serodiagnosis (56, 105, 214, 249, 251). They are transmembrane β-barrel proteins that exhibit porin activity to allow the passive diffusion of hydrophilic small-molecule nutrients across the outer membrane (101). The *A. phagocytophilum* genome contains 113 *p44* (*msp2*)-paralogous genes encoding P44/Msp2 proteins (61). The *p44* (*msp2*) genes are comprised of a central hypervariable region that is flanked with 5′ and/or 3′ conserved sequences. In order to conserve the genomic space, the majority of the *p44* genes are shorter pseudogenes that are truncated partially at the 5′- and/or 3′-terminal sequences (248–250). A single full-length *p44* (*msp2*) locus is remarkable, as it is highly polymorphic and similar to the *A. marginale msp2* expression locus (22, 24, 136), and it is designated *p44ES* (*msp2ES*). It was recently shown that this locus is also conserved in *A. platys* (121). The remaining *p44* genes, including the *p44* pseudogenes in the chromosome, function as donor *p44* genes to recombine at *p44ES* to be expressed from this genomic locus as a full-length recombined protein. *p44ES* recombination occurs via a gene conversion mechanism, by which the DNA sequence information of the donor *p44* gene (which remains unchanged) is copied and transferred to *p44ES*, whose sequence is altered (24, 133, 136, 137). In fact, almost all *p44* mRNAs and P44 proteins have a base or amino acid sequence in the hypervariable region almost identical to the corresponding DNA sequence of the donor *p44* genes found somewhere else in the genome (74, 136, 138, 248, 249). *A. phagocytophilum* lacks the homologous RecBCD recombination pathway that repairs bacterial DNA brakes and uses the RecFOR pathway for *p44* gene conversion (24, 133, 136, 137). This mechanism allows a large repertoire of P44 antigens to be rapidly exchanged while transcriptional regulation from the same promoter is maintained. The conserved 5′ and 3′ ends of the *p44* genes are likely required for annealing with homologous sequences of *p44ES* for recombination, and the N- and C-terminal conserved regions form the β-barrel P44 protein structure to preserve porin function. Antigenic variation is found on the predicted central external loop (101). Indeed, a recent global proteomic analysis revealed that 110 P44 (Msp2) proteins are expressed in infected HL-60 cells and that 88 of them are encoded by the pseudogenes (132). The composition of the *A. phagocytophilum p44ES* allelic population at a given time and in a particular tissue environment is expected to be determined by immune pressure and bacterial physiological conditions. Consequently, the antigenic variation of P44 proteins contributes to the evasion of the

host immune system and the persistence of *A. phagocytophilum* infection.

Type IV Secretion System

In order to secrete proteins, enzymes, DNA, or toxins from the cytoplasm to the exterior of bacteria across the cytoplasmic membrane, bacteria use at least six distinct extracellular protein secretion systems, referred to as type I to type VI secretion systems (172). Homologs of type I secretion system (T1SS) and T4SS components were found in the *A. phagocytophilum* genome (61). The T4SS forms a transmembrane channel composed of a multiprotein complex, which delivers the effector protein and DNA, and a nucleoprotein complex in an ATP-dependent manner. Recently, T4SSs have been classified into four groups: groups F, P, I, and GI (107). *A. phagocytophilum* has P-T4SSs (previously known as type IVA, the prototype encoded by plasmid pTi of *Agrobacterium tumefaciens*). Interestingly, despite the genome size reduction, *A. phagocytophilum* has up to eight nonidentical copies of these genes. The genes are distributed into three major genomic islands: *sodB-virB3-virB4-virB6-1-virB6-2-virB6-3-virB6-4*, *virB8-1-virB9-1-virB10-virB11-virD4*, and *virB2-1-virB2-2-virB2-3-virB2-4-virB2-5-virB2-6-virB2-7-virB2-8-virB4-2*. Between these genomic islands are *virB8-2*, *virB9-2*, and a putative *virB7* (no ORF number assigned; positions 1033978 to 1034181). The significance of the duplicated *virB* genes is currently unknown, but distinct sets of *virB2* genes, which encode a surface-exposed pilus, are expressed in ticks and mammalian cell cultures (164). It is critical to identify substrates that are delivered by the T4SS to understand its role in bacterial infection. So far, two T4SS substrates, ankyrin-repeat-rich protein A (AnkA) and *Anaplasma* translocated substrate 1 (Ats-1) (128, 166), have been identified and are discussed below. Unlike several other bacteria, genes encoding these proteins are not closely associated with *virB* or *virD* genomic islands; therefore, a global genomic search is required to find remaining potential substrates, as was performed for Ats-1 (166).

Two-Component System and Transcriptional Regulation

Two-component systems (TCSs) serve as a basic stimulus-response-coupling mechanism to allow organisms to sense and respond to changes under many different environmental conditions (145). They typically consist of an inner membrane-bound histidine kinase that senses a specific environmental stimulus and a corresponding response regulator that mediates the cellular response, mostly through the differential expression of target genes. The *A. phagocytophilum* genome encodes three pairs of TCSs: PleC/PleD, CckA/CtrA, and NtrY/NtrX (49). PleC, CckA, and NtrY are sensor histidine kinases which are essential for infection (49). CtrA, NtrX, and PleD are response regulators. CtrA and NtrX have a C-terminal DNA-binding domain and are believed to function as transcription factors. PleD has a C-terminal GGDEF domain associated with a diguanylate cyclase that produces cyclic di-GMP (c-di-GMP) (120). The expression of PleC and PleD is upregulated during an exponential growth stage (120). A hydrophobic c-di-GMP analog, 2′-*O*-di(tert-butyldimethylsilyl)-c-di-GMP, can serve as a functional antagonist in well-defined c-di-GMP-regulated phenomena in *Salmonella enterica* serovar Typhimurium, including cellulose synthesis, clumping, and the upregulation of *csgD* and *adrA* mRNAs (119). The analog competitively inhibits c-di-GMP binding to recombinant PleD and to native proteins of *A. phagocytophilum* and inhibits the infection of HL-60 cells with *A. phagocytophilum* (120).

The differential expression of *A. phagocytophilum* genes in HL-60 cells and ISE6 tick cells (164, 228) and the temporal transcriptional and protein expression profiles during *A. phagocytophilum* infection in HL-60 cells and neutrophils (120, 128, 166, 167) imply that the host environment and bacterial concentration regulate the transcription of *A. phagocytophilum*. However, genes encoding the nutritional stress response protein RelA/SpoT and proteins required for the biosynthesis of a quorum-sensing pheromone have not been found in *A. phagocytophilum*. *A. phagocytophilum* encodes only two sigma factor homologs: a constitutive factor, σ^{70}, and a single alternative factor, σ^{32} (RpoH). The paucity of alternative sigma factors suggests that *A. phagocytophilum* relies on the regulation of constitutive σ^{70}-type promoters by transcription factors and posttranscriptional regulation.

Mass spectrometry analysis of the DNA affinity-purified *A. phagocytophilum* protein identified a 12.5-kDa hypothetical protein (GenBank accession no. YP_505110.1). We named this protein A. phagocytophilum expression regulator (ApxR). ApxR is an unique DNA-binding protein that can regulate the transcription of downstream genes in *A. phagocytophilum* (229). Interestingly, ApxR is upregulated in HL-60 cells, whereas a putative transcriptional regulator, Tr1, a protein of 21 kDa which is predicted to have a DNA-binding motif, is upregulated in ISE6 tick cell cultures, suggesting that these proteins might be involved in the expression of mammal- and tick-specific genes (164, 228). Both ApxR and Tr1 homologs are uniquely evolved in members of the family *Anaplasmataceae* but are not found in the family *Rickettsiaceae* or in other bacterial families. In infected ticks, other than the data available from transcript analyses of *p44*, *tr1*, *msp2*, and *apxR* (63, 134, 228, 250), little information is available on the expression of *A. phagocytophilum* genes.

REPLICATION OF *A. PHAGOCYTOPHILUM* IN EUKARYOTIC CELLS

Intracellular Developmental Cycle

Electron microscopy identified distinct morphotypes of *A. phagocytophilum* inside neutrophil inclusions from infected sheep (235). Two morphotypes of bacteria can also be observed in both mammalian and tick cell cultures, a larger reticulate (RC) form and a smaller dense-core (DC) form that contains condensed protoplasm (158, 185, 223), and are considered to be normal developmental stages similar to those of other intracellular bacteria, including *Chlamydia* species (157) and *Coxiella burnetii* (91). In addition, a variety of aberrant bacterial forms have been reported (158, 185, 223). The biological significance of these aberrant forms is unknown, but they may reflect an unfavorable or unbalanced physiological state of the bacteria. Electron microscopy indicated that only the DC form binds to HL-60 cells (223), but both the DC and RC forms bind to and enter ISE6 tick cells (158). Only the DC

form binds to Chinese hamster ovary cells that are transfected to express P-selectin glycoprotein ligand 1 (PSGL-1), and binding is inhibited by the preincubation of the cells with an anti-PSGL-1 antibody (223). Troese and Carlyon (223) reported that internalized DC bacteria transition to RC bacteria in HL-60 cells within 12 h and that the RC bacteria initiate replication. By 24 h, large numbers of RC bacteria can be observed within individual inclusions, and by 36 h, the reinfection of already infected cells occurs, with individual, vacuole-enclosed DC and RC bacteria within the same cell (223). Although the proposed developmental cycle of the DC and RC forms morphologically resembles that of *Chlamydia* species, genes encoding histone H1-like proteins involved in chromatin condensation in *Chlamydia* (88) have not been identified in *A. phagocytophilum*.

Immunofluorescence showed that only small ($<$1-μm) bacteria corresponding to the DC form bind to and enter human peripheral blood neutrophils (167). In HL-60 cells, however, either small or large ($>$1-μm) bacteria are taken up, and most large bacteria, but not most small bacteria, are rapidly delivered to lysosome-associated membrane protein 1 (LAMP1)-positive compartments (167), implying that different developmental stages of *A. phagocytophilum* have distinct surface or biological properties that allow them enter host cells through different receptors and internalization pathways. Except for a T4S apparatus protein, VirB9 (167), molecular markers that differentiate the DC form from the RC form have not been reported.

The ultrastructures of the DC and RC forms of *A. marginale* and *Ehrlichia muris* in tick tissues have been reported (117, 186). *A. phagocytophilum* has been detected in the salivary glands of experimentally infected ticks and in field-derived adult deer ticks as Feulgen-positive inclusions (216), but the bacterial ultrastructure in ticks remains to be studied.

Host Cell Receptor, Site of Entry, and Internalization Signal

A. phagocytophilum is maintained mostly in circulating mature neutrophils, rather than in peripheral tissues, including hematopoietic tissue (25, 94, 243), although *A. phagocytophilum* can infect bone marrow progenitors and endothelial cells (92, 116). While skin lesions have not been reported for HGA, during field transmission from ticks to lambs, *A. phagocytophilum* is found primarily in association with neutrophils and macrophages in skin lesions, suggesting the role of peripheral leukocytes in tick transmission (83).

The ability to attach to and enter susceptible host cells is essential for infection. Cell lines, rather than human neutrophils, have been used in most studies of the binding and internalization of *A. phagocytophilum*. The tetrasaccharide sialyl Lewis^x (sLe^x) on P-selectin glycoprotein ligand 1 (PSGL-1) is required for the binding and infection of HL-60 cells by *A. phagocytophilum* (81, 93, 190, 191, 201, 244). Similar to the binding of P-selectin, the binding of *A. phagocytophilum* to HL-60 cells activates the PSGL-1 signaling pathway, which results in the tyrosine phosphorylation of ROCK1, an effector kinase of the GTPase RhoA by spleen tyrosine kinase (Syk). PSGL-1-blocking antibodies and Syk small interfering RNA (siRNA) inhibit ROCK1 phosphorylation in *A. phagocytophilum*-infected HL-60 cells (220). The treatment of HL-60 cells with piceatannol, a Syk inhibitor, or the knockdown of either Syk or ROCK1 impairs infection by *A. phagocytophilum* (220). ROCK1 is a serine/threonine kinase and is a key regulator of actin organization, suggesting that bacterial binding to PSGL-1 activates actin cytoskeleton reorganization through ROCK1 activation to facilitate bacterial invasion.

A naturally occurring subpopulation of *A. phagocytophilum*, termed NCH-1A2, has been selected through cultivation in sLe^x-defective HL-60 cells (HL-60 A2 cells). NCH-1A2 binds to HL-60 cells in a sialic acid- and PSGL-1-independent manner (190, 201). In agreement with the PSGL-1 independence, Syk is also not essential for NCH-1A2 infection of HL-60 cells (191). Whether NCH-1A2 is a genetic or phenotypic variant and whether an alternative receptor exists are unknown. Nonetheless, these studies suggested that *A. phagocytophilum* can infect HL-60 cells through at least two independent receptors and signaling pathways.

PSGL-1 is not required for infection of mice because *A. phagocytophilum* binds effectively to PSGL-1$^{-/-}$ murine neutrophils, and it infects PSGL-1$^{-/-}$ mice. Fucosyl transferases are, however, required for infection of mice, because the binding of *A. phagocytophilum* to Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ neutrophils and the infection of Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ mice are significantly reduced (40). Tick colonization with *A. phagocytophilum* was recently shown to involve α1,3-fucosylation on N-glycan, as determined by using the *I. ricinus* tick embryonic cell line IRE/CTVM19 and siRNA in *I. scapularis* ticks (179). The binding and/or infection of *A. phagocytophilum* appears to involve the major surface proteins Msp2 (P44), Asp55, and Asp62, as shown by the results of neutralization studies (74, 173, 230). It is important to identify the bacterial ligands that interact with PSGL-1 and other potential receptors to elucidate the specific ligand-receptor interactions that mediate bacterial attachment and entry.

Entry and intracellular infection by *A. phagocytophilum* require lipid rafts, which are signaling platforms (131). Caveolin-1, which is concentrated in lipid microdomains (9), was colocalized with early inclusions of *A. phagocytophilum* in HL-60 cells (131). Whether caveolin-1 is required for infection by *A. phagocytophilum* is unknown. Glycosylphosphatidylinositol (GPI)-anchored proteins (GAPs) and flotillin 1, both of which are associated with lipid rafts, are required for binding and infection by *A. phagocytophilum* (131, 240). In contrast, clathrin, a protein involved in endocytosis, is not associated with the internalization of *A. phagocytophilum* (131, 154). *A. phagocytophilum* concentrates in lipid rafts, but the signaling cascades are still poorly understood. One signal shown to be rapidly induced upon *A. phagocytophilum* binding is the phosphorylation of protein tyrosine, which is required for internalization and infection with *A. phagocytophilum* (103, 128, 131, 220). Tyrosine-phosphorylated proteins and phospholipase C γ2 (PLC-γ2) accumulate in *A. phagocytophilum*-infected cells within 5 min of infection (131). ROCK1 is tyrosine phosphorylated after *A. phagocytophilum* infection, although it is not known how long after infection this occurs (220). In addition, the tyrosine phosphorylation of AnkA, a bacterial T4S effector protein, occurs 5 min after infection of HL-60 cells (103). Tyrosine-phosphorylated proteins recruit active signaling molecules to create multiprotein signaling networks (177). In order to understand how bacteria enter, it is necessary to eluci-

date the downstream signaling pathways that become active after tyrosine phosphorylation.

Anaplasma Inclusion Biogenesis

Contribution of host cells. *A. phagocytophilum* multiplies strictly within membrane-bound inclusions. As bacteria divide and proliferate, the inclusions expand to occupy most of the cytoplasm of infected cells. The characteristics of the inclusions, including the membrane and luminal components, are unique and largely undetermined, and they appear to evolve over time as the *A. phagocytophilum* infection cycle progresses. A recent global proteomic analysis revealed an upregulation of human proteins involved mostly in cytoskeleton components, vesicular trafficking, cell signaling, and energy metabolism in HL-60 cells infected with *A. phagocytophilum* compared with uninfected cells (132).

Neutrophils kill invading microorganisms by sequestering vital nutrients (e.g., iron) or by fusing the phagosomes that contain the invading microorganisms with granules that contain both antimicrobial peptides (e.g., defensins and lysozymes) and lysosomal hydrolytic enzymes (52). *A. phagocytophilum* interferes with vesicular trafficking to avoid lysosomes. The *A. phagocytophilum* inclusion compartment does not resemble an endosome because it lacks transferrin receptor (TfR), early endosomal antigen 1, Rab5, α-adaptin, clathrin heavy chain, and annexins I, II, IV, and VI (154). The inclusion compartment also is not acidic and lacks V-type H^+ ATPase, and it does not acquire the late endosomal/lysosomal markers, including myeloperoxidase, CD63, and LAMP1 (154, 231). The inclusion also avoids fusion with secretory vesicles and specific and tertiary granules harboring NADPH oxidase (39, 104, 154). Additionally, unlike *Chlamydia* species (89), the growth of *A. phagocytophilum* is not impaired by brefeldin A (BFA), the Golgi apparatus-destabilizing agent, and the *A. phagocytophilum* inclusions do not acquire the Golgi vesicular marker β-coat protein (β-COP) or C6NBD {6-[*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl) amino]}-sphigomyelin (154). Certain *A. phagocytophilum* inclusions contain major histocompatibility complex class I (MHCI) and MHCII, and these may be involved in bacterial antigen presentation (154). *A. phagocytophilum* inclusions were reported to colocalize with green fluorescent protein (GFP)-Rab4A, GFP-Rab10, GFP-Rab11A, GFP-Rab14, RFP-Rab22A, GFP-Rab35 (all of which regulate endocytic recycling), and GFP-Rab1 (98). Interestingly, the treatment of infected cells with tetracycline for 30 min results in the loss of these GFP-Rab proteins and the acquisition of GFP-Rab5, GFP-Rab7, and the lysosomal markers (78, 98), suggesting that the inhibition of the endosome-lysosome pathway requires new bacterial protein synthesis. Several indicators of early autophagosomes were detected in *A. phagocytophilum* replicative inclusions, including a double-lipid bilayer membrane and the colocalization of GFP-tagged LC3 and Beclin 1, the human homologs of the yeast *Saccharomyces cerevisiae* autophagy-related proteins Atg8 and Atg6, respectively (168). *A. phagocytophilum* autophagosomes do not mature into degradative autolysosomes. The inhibition of the autophagosomal pathway by 3-methyladenine (3-MA), an inhibitor of class III phosphatidylinositol 3-kinase (PI3KC3), inhibits *A. phagocytophilum* infection by preventing bacterial growth but not bacterial internalization (168).

A. phagocytophilum requires cholesterol for survival and infection, and unlike *Escherichia coli*, *A. phagocytophilum* is capable of incorporating a substantial amount of host cholesterol into its membrane (130). The expansion of the inclusion membrane during infection also requires a lipid source, including cholesterol. In fact, *A. phagocytophilum* infection is facilitated in mice with high blood cholesterol levels (243). Infection with *A. phagocytophilum* results in an upregulation of host cell cholesterol levels and transportation to the inclusion (241). This occurs by the upregulation of low-density lipoprotein (LDL) receptor and LDL vesicular transport, not by *de novo* biosynthesis in host cells, and it requires bacterial protein synthesis (241). Cholesterol is then incorporated into the anaplasma membrane. Importantly, the inhibition of the LDL cholesterol uptake pathway blocks bacterial infection (241).

Overall, these studies imply the contribution of at least three pathways of cytoplasmic vesicular transport in the biogenesis of *A. phagocytophilum* inclusions: recycling endosomes, autophagosomes, and the LDL uptake pathway. These pathways enable *A. phagocytophilum* to acquire host nutrients while avoiding lysosomes and NADPH oxidases. Figure 3 illustrates some features of *A. phagocytophilum* internalization and inclusion biogenesis.

Modification of the inclusion membrane by anaplasma proteins. Three *A. phagocytophilum* proteins associated with the inclusion membrane have been reported: APH_0233, APH_1387, and APH_0032. APH_0233 (also called *A. phagocytophilum* toxin A [AptA]) surrounds the *A. phagocytophilum* inclusion and interacts with vimentin, the cytoplasmic intermediate filament protein that surrounds the inclusion (210) (Fig. 3).

Two acidic proteins containing tandem repeats, APH_1387 (previously named P100 [207]) and APH_0032, localize on the surface of intravacuolar *A. phagocytophilum* and on the matrix side of the inclusion membrane (99, 100). Similar acidic tandem repeat proteins, TRP120 and TRP47, localize on the bacterial surface of *Ehrlichia chaffeensis* (75, 187). TRP120 is associated with the filamentous matrix of the inclusion (187), and TRP47 was proposed to interact with multiple eukaryotic cell signaling proteins (225). There is no evidence that any of these acidic tandem repeat proteins are secreted via a bacterial secretion system, including the T4S system. Tyrosine-phosphorylated AnkA, another tandem repeat protein and a T4S substrate, is also associated with the inclusion at certain stages of infection (Fig. 3) (103, 128). The membrane localization of these proteins suggests their role in inclusion membrane biogenesis, which needs to be determined.

SUBVERSION OF NEUTROPHIL ANTIMICROBIAL DEFENSES

A. phagocytophilum infects granulocytes by subverting their powerful innate antimicrobial defenses (194), which also makes infected humans and animals more susceptible to opportunistic infection (13, 72, 232). Neutrophils express pattern recognition surface receptors, such as transmembrane Toll-like receptors (TLRs) and a nucleotide-binding oligomerization

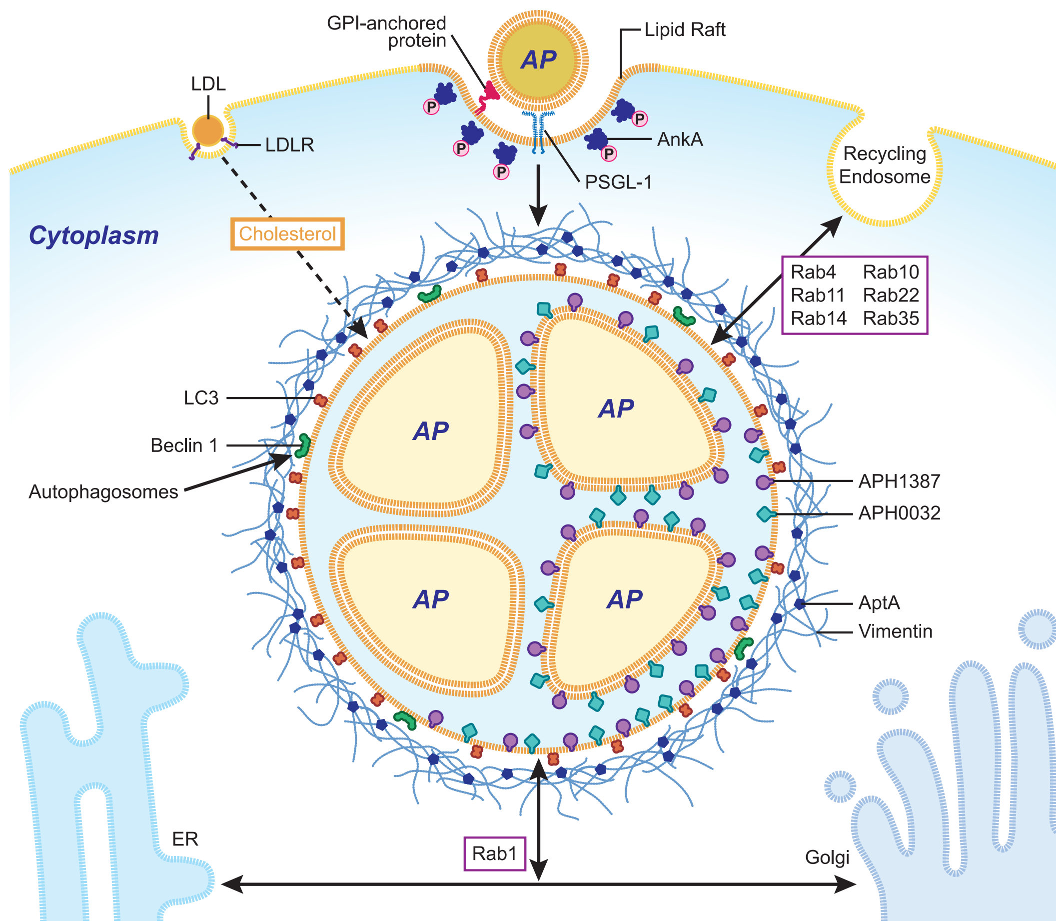

FIG. 3. *A. phagocytophilum* inclusion biogenesis. *A. phagocytophilum* binds to PSGL-1, a GPI-anchored protein, or an unidentified host cell receptor(s) that is localized in cholesterol-rich membrane microdomains (yellow). Three pathways of eukaryotic vesicular transport converge during *A. phagocytophilum* inclusion biogenesis: recycling endosomes (several Rab proteins), autophagosomes (LC3 and Beclin 1), and LDL uptake pathways (free cholesterol). This process creates a safe haven that allows *A. phagocytophilum* to acquire nutrients while remaining secluded from lysosomes and NADPH oxidases. Three *A. phagocytophilum* proteins are associated with the inclusion membrane: APH_1387, APH_0032, and APH_0233 (also called *A. phagocytophilum* toxin A or AptA). Vimentin is also remodeled to surround the inclusion membrane and interact with AptA. Tyrosine-phosphorylated AnkA is associated with bacterial inclusion at an early stage of infection. *AP*, *A. phagocytophilum*; LDL, low-density lipoprotein; LDLR, LDL receptor; ER, endoplasmic reticulum.

domain (NOD) that contains cytoplasmic protein receptors that can recognize and bind to conserved pathogen-associated molecular patterns (PAMPs). The binding of PAMPs elicits profound innate immune responses, including the generation of reactive oxygen species (ROS), the fusion of lysosomes, the activation of mitogen-activated protein kinases (MAPKs), autophagy, cellular apoptosis, and the induction of cytokines/chemokines. These responses generally result in the elimination of invading microorganisms at the site of infection by the neutrophils themselves or by neutrophil-recruited cells (62, 215). *A. phagocytophilum* lacks the major PAMPs LPS and peptidoglycan (130). A recent global proteomics study showed that *A. phagocytophilum* infection of HL-60 cells downregulates TLR1, which recognizes peptidoglycan and (triacyl) lipoproteins in concert with TLR2 (as a heterodimer), and macrophage mannose receptor 2, which binds to high-mannose structures on the surface of viruses, bacteria, and fungi so that they can be engulfed by the cell (132). Furthermore, *A. phagocytophilum* evolved “regulatory hijacking” as its mode of subversion of host cells by targeting pleiotropic host kinases, transcription factors, and histone-modifying enzymes (as illustrated in Fig. 4).

Downregulation of Reactive Oxygen Species Generation

Neutrophils and monocytes/macrophages are the primary mediators of an oxygen-dependent defense system that generates ROS (superoxide, hydrogen peroxide, and hydroxyl radicals) upon exposure to pathogens. In resting neutrophils, the components of NADPH oxidase are disassembled and dispersed among the membranes of secretory vesicles, specific granules, and cytosolic complexes (12). When neutrophils are activated, secretory vesicles and specific granules rapidly fuse with plasma or phagosomal membranes, and a complex of neutrophil cytosolic factor 1 (NCF1) (also known as $p47^{phox}$), NCF2 (also known as $p67^{phox}$), and, possibly, NCF4 (also known as $p40^{phox}$) translocates and associates with the cytochrome b_{245} light chain (CYBA [also known as $p22^{phox}$] and CYBB [also known as NADPH oxidase homolog 2 {NOX2} or

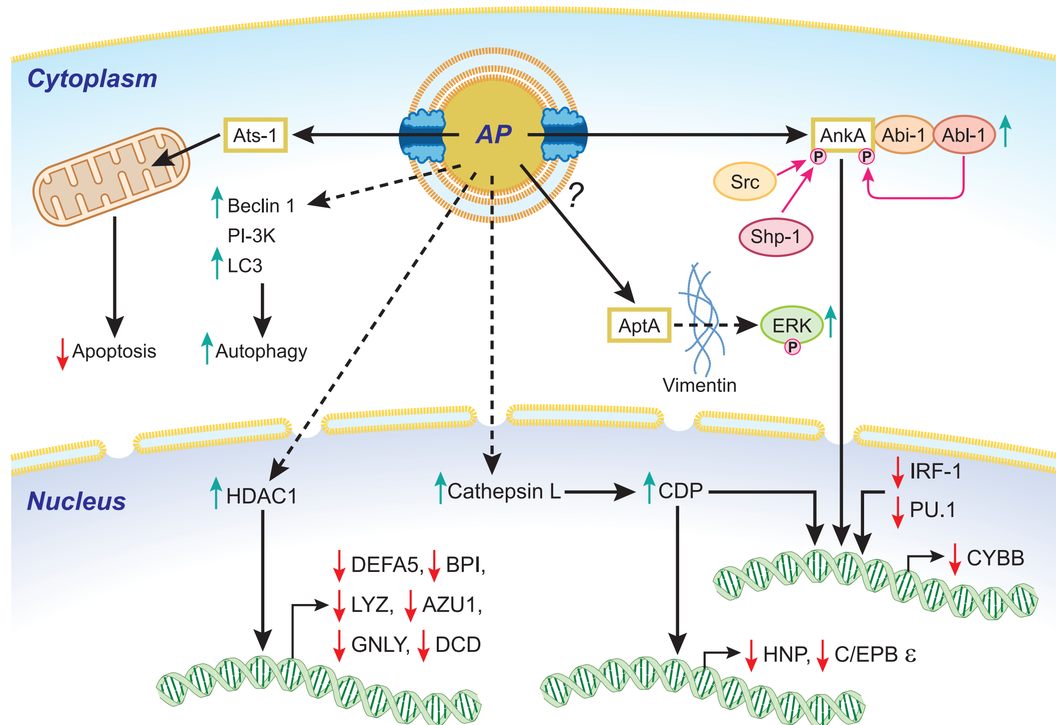

FIG. 4. *A. phagocytophilum* "regulatory hijacking." *A. phagocytophilum* dysregulates host cellular regulatory networks by targeting pleiotropic host kinases, transcription factors, and histone-modifying enzymes. In particular, *A. phagocytophilum* activates at least three distinct signaling pathways involving ERK1/2, Abl, and phosphoinositide 3-kinases (PI-3K). Some of the early phosphorylation substrates identified during *A. phagocytophilum* infection are the bacterial T4S substrate AnkA, which binds SHP-1 through its SH2 domain, and Abi-1, which activates Abl-1. *A. phagocytophilum* also upregulates nuclear cathepsin L, which cleaves CDP, and CDP binds to the promoter regions of several genes to downregulate gene transcription, including the genes for the transcription factors PU.1, c/EBPε, and IRF-1. The expression of HDAC1 is upregulated, and HDAC1 binds to the promoter region of target genes to suppress transcription. CDP, CCAAT displacement protein; c/EBPε, CCAAT enhancer binding protein epsilon; IRF-1: interferon regulatory factor 1; HDAC1, histone deacetylase 1; DEFAS, human α-defensin 5; BPI, bactericidal/permeability-increasing protein; LYZ, lysozyme; GNLY, granulysin; DCD, dermcidin; HNP, human neutrophil peptide 1. ↑, activated/upregulated; ↓, inhibited/downregulated.

gp91^{phox}]) (55). Unlike *E. coli*, *A. phagocytophilum* does not induce ROS upon interactions with human or murine neutrophils (21, 32, 104, 155, 227). *A. phagocytophilum* also does not induce the assembly of the NADPH oxidase subunits in the bacterial inclusion membranes in HL-60 cells (39, 104, 156). In agreement with these reports, the course of *A. phagocytophilum* infection in CYBB$^{-/-}$ mice does not differ from that in wild-type mice, indicating that NADPH oxidase does not help clear infection (20). This is not because *A. phagocytophilum* is resistant to ROS, since it is easily killed upon exposure to ROS (129). On the other hand, Carlyon et al. reported that neutrophil NADPH oxidase is activated when exposed to *A. phagocytophilum*, and this group proposed that one of the primary mechanisms by which *A. phagocytophilum* is protected from ROS is the scavenging of exogenous O_2^- (that cannot readily diffuse through the bacterial cytoplasmic membrane) (39).

Once *A. phagocytophilum* binds to human neutrophils, the neutrophils become refractory even to powerful exogenous stimuli, such as phorbol myristate acetate (PMA), LPS, *E. coli*, formyl-methionyl-leucyl-phenylalanine (FMLP), or Fc-Oxyburst immune complexes, indicating that this is active ROS inhibition by the bacteria (21, 155, 156, 227). Inhibition is specific to neutrophils, because human monocytes can respond to exogenous stimuli in the presence of *A. phagocytophilum* (155). *A. phagocytophilum* decreases protein levels of CYBA, but not other components of NADPH oxidase (CYBB, NCF1, NCF2, and NCF4), in human neutrophils within 1 h after exposure to the bacteria (156). Periodate oxidation and a heat-sensitive component, rather than *Anaplasma* protein synthesis or viability, are required for the inhibition of O_2^- generation in neutrophils (32, 155, 156). Cell contact with bacteria is presumably necessary, because the inhibitory factor does not diffuse through a 0.45-μm filter *in vitro* (155). Overall, these studies suggest that the destabilization of the complex of CYBB and CYBA (cytochrome b_{245}) is involved in the inhibitory effects of *A. phagocytophilum* on O_2^- generation by human neutrophils.

Not only preformed CYBA, as described above, but also the mRNA expression of Rac2 is downregulated in HL-60 cells and neutrophils after *A. phagocytophilum* infection (41). CYBB mRNA and surface protein levels are downregulated in infected HL-60 cells (21). CYBB downregulation seems to be a part of a generalized downregulation of host immune responses. In *A. phagocytophilum* strain Webster, a large proportion of AnkA, the bacterial T4S substrate, is contained within the nucleus and was proposed to induce CYBB downregulation (45, 71, 174). AnkA reportedly binds to a broad range of targets in the nucleus, including several nuclear proteins,

the internucleosomal region of chromosomes in HL-60 cells, ATC-rich sequences, and the transcriptional regulation regions of the CYBB locus (71, 174). Other researchers reported that the downregulation of the CYBB gene is associated with an increased binding of the repressive CCAAT displacement protein (CDP) to the promoter of the CYBB gene and with decreased levels of interferon regulatory factor 1 (IRF1) and the PU.1 protein in infected HL-60 cells (222). The relevance of these observations of HL-60 cells to human neutrophils is, however, questionable, since in human neutrophils during *A. phagocytophilum* infection, mRNA amounts of NADPH oxidase, including CYBB and Rac2, remain unchanged or are upregulated (32). Recently, *A. phagocytophilum* infection was shown to activate nuclear cathepsin L and trigger the cleavage of CDP, leading to increased DNA binding (221). *A. phagocytophilum* infection also enhances the binding of CDP to the promoters of the genes for human neutrophil peptide 1 and C/EBPε, two molecules that are important for neutrophil defense and maturation. Enhanced CDP activity, therefore, globally influences neutrophil function (221). The downregulation of the transcription factors IRF and PU.1 also globally influences neutrophil function. In addition, *A. phagocytophilum* infection of THP-1 cells, an acute monocytic leukemia cell line, increases the expression, activity, and binding of histone deacetylase 1 (HDAC1) (70). HDAC1 overexpression enhances infection, whereas the inhibition of HDAC1 by pharmacological agents or siRNA decreases the bacterial load (70). Since transcriptional patterns and regulations differ between immortal cell lines and neutrophil, it is important to test the involvement of cathepsin L, PU.1, IRF1, and HDAC1 in *A. phagocytophilum*-infected human neutrophils. Collectively, however, these data, as well as data from many microarray studies that are not elaborated in this review, suggest that *A. phagocytophilum* survives by modulating critical cell signaling mechanisms involved in phagocytic activation and the differentiation of infected neutrophils (Fig. 4).

Inhibition of Host Cell Apoptosis

Apoptosis, the process of programmed cell death, not only is important for removing damaged or no-longer-needed cells but also is an important innate cellular immune mechanism for killing intracellular pathogens. Particularly, peripheral blood neutrophils are already in the process of spontaneous apoptosis and readily accelerate the apoptosis in response to infection with microbes (54). *A. phagocytophilum* infection inhibits both spontaneous and induced apoptosis of isolated peripheral blood human neutrophils for up to 48 or 96 h, and this allows the bacteria sufficient time for replication, which takes places ~24 h after infection (76, 245). ROS limit the life span of neutrophils by activating death receptor signaling (204). In agreement with this, *A. phagocytophilum* itself does not induce NADPH oxidase activation in human and murine neutrophils (21, 32, 104, 155). *A. phagocytophilum* prevents human neutrophils from reducing mRNA for the antiapoptotic *bcl-2* family member *bfl-1* (*A1*), losing mitochondrial membrane potential and activating caspase-3 (73, 76). Several microarray studies showed that *A. phagocytophilum* infection upregulates the expression of the antiapoptotic *bcl-2* family members (32, 125, 180). *A. phagocytophilum* infection blocks the clustering of Fas on the cell surface during spontaneous neutrophil apoptosis (73). Furthermore, *A. phagocytophilum* blocks the anti-Fas-induced programmed cell death of human neutrophils (32, 73). *A. phagocytophilum* infection also inhibits the cleavage of procaspase-8, the activation of caspase-8, and the cleavage of Bid, a molecule that links the intrinsic and extrinsic pathways of apoptosis (73). Likewise, *A. phagocytophilum* infection inhibits the translocation of proapoptotic Bax to mitochondria, the activation of caspase-9, the activation of the initiator caspase in the intrinsic pathway, and the degradation of X-chromosome-linked inhibitor of apoptosis protein (XIAP), a potent caspase inhibitor (200a). In addition, the transcription of numerous genes related to apoptosis and differentiation is modulated in human neutrophils infected with *A. phagocytophilum* (32, 125). Choi et al. proposed that *A. phagocytophilum* inhibits human neutrophil apoptosis through the activation of p38 MAPK (50).

Anaplasma translocated factor 1 (Ats-1) was recently shown to be responsible in part for the antiapoptotic effects of *A. phagocytophilum* (166). Ats-1 is secreted by the T4SS and moves across two bacterial membranes and the inclusion membrane to the host cell cytoplasm, where it localizes in the mitochondrial matrix of infected human neutrophils, HL-60 cells, RF/6A monkey endothelial cells, HeLa cells, and even yeast cells. Ats-1 contains a cleavable N-terminal mitochondrion-targeting presequence, which directs the bacterial protein across two mitochondrial membranes by the mitochondrial protein transport system. This is an intrinsic property of Ats-1, because the *in vitro*-synthesized Ats-1 protein translocates into isolated mitochondria in a cell-free system (Fig. 4) (166). Mitochondrion-translocated Ats-1 inhibits etoposide-induced apoptosis in mammalian cells. Ats-1 also inhibits the docking of human Bax to mitochondria and the subsequent apoptosis of yeast, which lacks Bcl-2 members, after the induction of the human Bax protein (166).

Subversion of Autophagy

Autophagy is an essential eukaryotic cellular process to sequester and digest undesirable intracellular objects, including intracellular pathogens, to protect the whole organism and thus is considered one of the important innate immune response mechanisms (152). Several hallmarks of early autophagosomes are found in *A. phagocytophilum* replicative inclusions, including two lipid bilayers and colocalization with two critical autophagy proteins: LC3 (human homolog of yeast autophagy-related gene 8 [Atg8]) and Beclin 1 (human homolog of yeast Atg6) (168). The level of the membrane-associated form of LC3, LC3-II, is increased during *A. phagocytophilum* infection (Fig. 4). In agreement with the lack of lysosomal fusion found in previous studies (154, 231), *A. phagocytophilum* does not mature into autolysosomes. Importantly, instead of being killed, autophagosome formation favors *A. phagocytophilum* infection: the stimulation of autophagy by rapamycin enhances *A. phagocytophilum* infection (168). Furthermore, autophagy is required for *A. phagocytophilum* infection, because the inhibition of the autophagosomal pathway by 3-methyladenine (3-MA), an inhibitor of the class III phosphatidylinositol 3-kinase (PI3KC3), inhibits *A. phagocytophilum* infection (168). Interestingly, 3-MA does not inhibit the internalization of *A. phagocytophilum* but prevents its growth. This inhibition is reversible:

after the removal of 3-MA, bacteria resume replication (168). These data suggest that *A. phagocytophilum* subverts and actively induces cellular autophagy to remodel the host cell cytoplasm to make space available for their growth as well as to recycle host membranes and nutrients for bacterial growth. Taken together, *A. phagocytophilum* subverts two important innate immune mechanisms of neutrophils, apoptosis and autophagy, by inhibiting and inducing, respectively, to keep the host cell alive and create a safe haven.

Other Host Cell Signals Activated and Required for *A. phagocytophilum* Infection

Tyrosine kinase activation. *A. phagocytophilum* infection of human leukocytes requires protein tyrosine kinase activity, since bacterial infection is inhibited by the nonspecific protein tyrosine kinase inhibitor genistein and the host cytoplasmic delivery of antiphosphotyrosine antibody by the Chariot-mediated protein transfection system (128). The bacterial AnkA protein is the predominant phosphotyrosine protein in *A. phagocytophilum*-infected HL-60 cells and peripheral blood neutrophils (103, 128). The tyrosine phosphorylation of AnkA becomes evident as early as 5 min after *A. phagocytophilum* binds to host cells; bacterial internalization is not required for this early phosphorylation event, and AnkA is progressively phosphorylated throughout the intracellular growth of bacteria (103, 128). Host cytoplasmically delivered AnkA is required for *A. phagocytophilum* infection, as shown by Chariot-mediated anti-AnkA delivery (128). The N-terminal two-thirds of AnkA contains approximately 11 ankyrin repeats, and the C terminus of AnkA contains 6 to 7 tandem tyrosine phosphorylation sites (198). In *A. phagocytophilum*-infected HL-60 cells, the tyrosine phosphorylation of AnkA occurs after secretion into the host cytoplasm by two nonreceptor tyrosine kinases, Src and Abelson leukemia (Abl), which implies that these kinases are activated rapidly upon bacterial binding (Fig. 4) (103, 128). Following phosphorylation by Src, AnkA binds to the SH2 domains of the nonreceptor tyrosine phosphatase Src homology protein 1 (SHP-1) (103). SHP-1 generally interacts with and dephosphorylates a wide spectrum of phosphoproteins, and it primarily downregulates cellular activation (184). AnkA forms a complex with Abl-1 via Abl interactor 1 (Abi-1), activates Abl-1 kinase, and is phosphorylated by Abl-1 (128). Abl kinase activity is essential for *A. phagocytophilum* infection, as shown by a study using the Abl kinase-specific inhibitor STI571 (also known as imatinib mesylate [Gleevec]) or Abl-1 siRNA (128). In addition, as described above (see "Host cell receptor, site of entry, and internalization signal"), Syk is activated to tyrosine phosphorylate ROCK1 in a PSGL-1-dependent manner during *A. phagocytophilum* infection (220). Thus, multiple host tyrosine kinases are activated during *A. phagocytophilum* infection, and specific inhibitors of the tyrosine kinases, such as imatinib mesylate, currently used to treat patients with Bcr-Abl-positive chronic myelogenous leukemia, may be useful in reducing HGA clinical signs. To fully understand the roles of tyrosine kinase activation in *A. phagocytophilum* infection, further studies to determine these tyrosine kinase and phosphatase activation mechanisms and the downstream targets are needed.

ERK1/2 activation. Extracellular signal-regulated kinases (ERKs), also called mitogen-activated protein kinases, are widely expressed intracellular protein kinases that communicate a signal from a receptor on the surface of the cell to the DNA in the nucleus of the cell. The ERK/MAPK pathway, particularly ERK2, is activated in *A. phagocytophilum*-infected human neutrophils at 3 h after infection (126). ERK1/2 activation by *A. phagocytophilum* is evident in HL-60 cells at the mid-exponential growth stage, and activation is required for infection (241). The ectopic expression of the *A. phagocytophilum* AptA protein activates ERK1/2 in HL-60 cells (Fig. 4) (210). AptA interacts with the intermediate filament protein vimentin, which is essential for *A. phagocytophilum*-induced ERK1/2 activation and infection (210). Thus, AptA is another bacterial protein that activates the pleiotropic kinase pathway in the host. The molecular events linking cell surface receptors to the activation of ERKs are complex. The primary ERK activation pathway is the Ras GTP-binding protein activation of another protein kinase, Raf-1, which phosphorylates a "MAPK kinase," which then phosphorylates ERKs (Ras/Raf/ERK signaling cascade). It was found that the Ras prenylation inhibitor manumycin A effectively inhibits *A. phagocytophilum* infection (242). However, interestingly, manumycin A can directly inactivate the bacterium, resulting in reduced infection and ERK1/2 activation. Thus, the manumycin group of drugs may have a therapeutic potential for HGA (242).

PATHOGENESIS AND IMMUNE RESPONSES

Generation of Chemokines/Cytokines

Major clinical signs of HGA are fever, headache, myalgias, chills, and various combinations of leucopenia, anemia, and thrombocytopenia (3, 14). The pathogenesis of HGA is poorly understood; however, the small amounts of bacteria detected in the blood of patients and animals suggest that the clinical disease is mediated by proinflammatory cytokines. According to a single report available on cytokine expression in HGA patients, only gamma interferon (IFN-γ) and interleukin-10 (IL-10) protein concentrations are elevated in sera from patients with acute infection versus sera from convalescent patients or healthy subjects (60). Concentrations of tumor necrosis factor alpha (TNF-α), IL-1β, and IL-4 are not elevated (60). *A. phagocytophilum* is a natural equine pathogen known to cause equine ehrlichiosis (140, 142), and *A. phagocytophilum* strains isolated from HGA patients can infect and cause clinical disease in horses; the horse is therefore considered a valuable animal model of HGA (141). Four horses infected by intravenous inoculation with *A. phagocytophilum* or by the attachment of infected ticks showed an upregulation of mRNA expression levels of IL-1β and TNF-α in peripheral blood leukocytes (PBLs), and three of the four horses showed an upregulation of the mRNA expression level of IL-8. One horse showed weak mRNA expressions of IFN-γ, IL-10, and IL-12 p35. None of the horses had detectable mRNA expression levels of IL-2, IL-4, IL-6, or IL-12 p40 (112). *A. phagocytophilum* is also a natural pathogen of ruminants, and concentrations of TNF-α and nitrate in ovine sera are significantly increased in sheep infected with *A. phagocytophilum* (79). More studies are certainly needed to understand the roles of cytokines/chemokines in HGA pathogenesis *in vivo*.

In vitro, *A. phagocytophilum* and the recombinant P44 pro-

tein induce rapid and strong mRNA expressions of IL-1β, TNF-α, and IL-6 by human PBLs and monocytes within 2 h. The protein secretion of these proinflammatory cytokines occurs within 24 h of induction (113, 114). The addition of *A. phagocytophilum* to normal ovine PBLs enhances the *in vitro* production of TNF-α and nitric oxide (79). In contrast, only IL-1β is upregulated in human neutrophils, and none of these proinflammatory cytokines is upregulated in HL-60 cells (113). mRNAs for IL-8, IL-10, IFN-γ, IL-2, and transforming growth factor β (TGF-β) are not consistently upregulated in human PBLs after incubation with *A. phagocytophilum* for 2 h (113). These studies indicate that human monocytes, rather than human neutrophils, are responsible for proinflammatory cytokine production. Akkoyunlu et al. (6) reported that IL-8 is produced by retinoic acid-treated HL-60 cells after 24 h of culture and by *A. phagocytophilum*-infected human neutrophils after 7 h of culture, suggesting a relatively slow IL-8 induction. That same study did not detect IL-1α, IL-1β, or TNF-α in the culture supernatants of retinoic acid-treated or nontreated HL-60 cells 6 days after the addition of *A. phagocytophilum* to cultures. Klein et al. reported that the levels of IL-8 and other chemokines, but not IL-1, IL-6, or TNF-α, are significantly produced by infected, dimethyl sulfoxide-treated HL-60 cells or human bone marrow cells 24 h after infection (115). A microarray analysis showed that numerous cytokines, chemokines, and some of their receptors are induced in human neutrophils exposed to *A. phagocytophilum* for 6 to 9 h: TNF-α, IL-1β, IL-1ε, IL-6, CXCL1, CXCL2, CXCL3, CCL3, CCL4, CCL20, CD54, IL-1RN, IL-1R1, and orosomucoid (an acute-phase protein); however, IL-8 (CXCL8) is not strongly induced in human neutrophils. That same study showed that live bacteria induce a stronger response than heat-killed bacteria, suggesting a requirement for bacterial metabolism and growth (32). Another microarray study showed that several chemokines, cytokines, and their receptors are upregulated 1 h after infection in neutrophils and 2 h after infection in HL-60 cells (126). In contrast, a different microarray study did not detect an upregulation of cytokines/chemokines in HL-60 cells 3 days after infection with *A. phagocytophilum* (53) or in NB4 cells 4 h after infection (180). The secretion of IL-8 and CCL20 by neutrophils can be detected 4 and 7 h, respectively, after infection with *A. phagocytophilum* (6, 209). Although the level of IL-8 transcription is relatively low or undetectable in infected neutrophils (32, 209), IL-8 protein levels are significantly increased. It is important, therefore, to confirm results from mRNA analyses with data on protein levels and function. Overall, these studies indicate that *A. phagocytophilum* induces weak and/or delayed proinflammatory cytokine and chemokine responses by human neutrophils *in vitro*, which may be caused by the *A. phagocytophilum*-induced alteration of intracellular signaling and may aid bacterial infection.

The induction of proinflammatory cytokines by human monocytes in response to LPS involves the activation of MAPK and NF-κB (87). In agreement with the delayed and weak proinflammatory cytokine induction, human neutrophils do not show an activation of p38 MAPK or NF-κB within 1 h after infection with *A. phagocytophilum in vitro*; in contrast, human PBLs (buffy coat cells) and isolated human monocytes do show an activation of p38 MAPK and NF-κB within 1 h after incubation with *A. phagocytophilum* (114). This is also in agreement with strong IL-1β, TNF-α, and IL-6 responses by human PBLs and monocytes (114). Lee et al. showed that uninfected and *A. phagocytophilum*-infected human neutrophils produce little activation of p38 MAPK 1 h after infection and a subsequent downregulation of p38 MAPK 3 h after infection (126), corroborating data from a previous study (114). In contrast, according to data reported by Choi et al. (50), both uninfected human neutrophils and *A. phagocytophilum*-infected neutrophils 3 h after exposure show a profound activation of p38 MAPK, at a level similar to that observed after *E. coli* LPS stimulation. After 18 h of exposure, p38 MAPK is continuously upregulated in infected or LPS-stimulated neutrophils, but it is downregulated in uninfected neutrophils (50). The strong activation of p38 MAPK in uninfected neutrophils suggests a nonspecific, background stimulation of neutrophils, so these data should be interpreted cautiously. Nonetheless, the results indicate that *A. phagocytophilum* infection dose not activate p38 MAPK in human neutrophils compared to uninfected neutrophils at 3 h and between 3 and 18 h postexposure *in vitro*.

Since *A. phagocytophilum* lacks LPS and peptidoglycans, other PAMPs, such as lipoproteins, heat shock proteins, or unidentified molecules of *A. phagocytophilum*, may stimulate monocytes to induce proinflammatory cytokines. A global proteomic analysis demonstrated that the 15 predicted lipoproteins are expressed by *A. phagocytophilum* in HL-60 cells (132). The activation of NF-κB in monocytes by *A. phagocytophilum* infection was confirmed by using mouse macrophages *in vitro*, and TLR2, which can recognize bacterial lipoproteins, is involved in this effect (51). The lack of rapid activation in host neutrophils may be due to active suppression by *A. phagocytophilum*, since human neutrophils have functional TLR2. Proinflammatory cytokines generated by monocytes and perhaps other leukocytes, but not neutrophils, are expected to contribute to bacterial clearance, HGA pathogenesis, and disease manifestation. In fact, neutrophils do not seem to act as efficient killer cells during *A. phagocytophilum* infection, because mice deficient for antimicrobial effectors of neutrophils, such as myeloperoxidase, granulocyte elastase, and cathepsin G, were fully competent in pathogen elimination (29).

Roles of IFN-γ and IL-8

In immunocompetent mice (C3H/HeN, C3H/HeJ, C57BL/6, BALB/c, DBA/2, and CD1 strains), *A. phagocytophilum* causes a subclinical infection that peaks 4 to 7 days after infection and then spontaneously declines (5, 6, 34, 36, 94, 96, 143, 144, 213, 217). Despite the lack of clear clinical signs, infected mice do develop anemia, leucopenia, and thrombocytopenia, similar to HGA in humans. The mouse model of HGA is therefore useful to determine the suppression mechanisms of *A. phagocytophilum* proliferation and the causes of hematological abnormalities but is not useful for studying the pathogenesis of overt disease.

C3H mice that received an intraperitoneal inoculation of *A. phagocytophilum* showed a significant elevation in splenic IL-12 and IFN-γ mRNA levels and serum IFN-γ protein levels 2 days after infection (5). IFN-$\gamma^{-/-}$ mice showed a significant increase in the number of infected neutrophils 5 and 8 days after infection, but rickettsemia was resolved after 2 weeks, similar to what was observed for wild-type mice (5). Using several gene-deficient mouse strains, one study showed that IFN-γ

production by natural killer (NK) cells is important for initial defense, and subsequent bacterial clearance is strictly dependent on $CD4^+$ T cells, but perforin, Fas/FasL, and major Th1 cytokines, such as IL-12, IFN-γ, and monocyte chemoattractant protein 1 (MCP-1), do not appear to be important (29). In another study, IFN-γ secretion by an NKT cell was proposed to be important, as the administration of alpha-galactosylceramide, an NKT cell agonist, increased IFN-γ secretion and protected mice from *A. phagocytophilum* infection (178). c-Jun NH_2-terminal kinase 2 knockout ($JNK2^{-/-}$) mice produced increased levels of IFN-γ after *A. phagocytophilum* challenge and were more refractory to infection with *A. phagocytophilum* than wild-type mice (178). A recent study showed that an apoptotic speck-like protein with a caspase-activating recruiting domain (ASC/PYCARD) and caspase-1, but not the MyD88 or mitogen-activated kinase kinase 3 (MKK3) pathway, are critical for the IFN-γ-mediated control of *A. phagocytophilum* infection (181). The Nod-like receptor (NLR) family member IPAF/NLRC4, but not NALP3/NLRP3, is partially required for IFN-γ production in response to *A. phagocytophilum* infection. This is in stark contrast to infection with monocyte-tropic *Ehrlichia muris*, which causes chronic mouse infection, whereby $MyD88^{-/-}$ mice showed enhanced infection and reduced IFN-γ production in correlation with markedly lower levels of IL-12 production by dendritic cells (118).

CD11b/CD18 also appears to be important for early bacterial clearance and killing *in vivo* because the infection of CD11b/CD18 knockout mice results in an initial increase in the bacterial burden compared with that of wild-type mice (33). Similarly, *in vitro* culturing of neutrophils from infected CD11b/CD18 knockout mice resulted in a marked increase in bacterial proliferation compared with neutrophils from congenic controls (33).

Furthermore, impaired IFN-γ intracellular signaling was reported for HL-60 cells infected with *A. phagocytophilum*; unlike the impaired IFN-γ signaling observed after *E. chaffeensis* infection (124), the defect of IFN-γ signaling observed after *A. phagocytophilum* infection is not at the level of STAT1 phosphorylation but at the level of the binding of phosphorylated STAT1 to the promoter of the gene for interferon regulatory factor 1 (222). Infected neutrophils show a marked inhibition in the secretion of the IFN-γ-inducible chemokines IP-10/CXCL10 and MIG/CXCL9 (222). More recently, however, Bussmeyer et al. (38) reported that *A. phagocytophilum*-infected human neutrophils showed a decrease in the IFN-γ-induced phosphorylation of STAT1, a decrease in the level of expression of the IFN-γ receptor α-chain CD119, and an enhancement in the expression of suppressor of cytokine signaling 1 (SOCS1) and SOCS3. Overall, these studies suggest that IFN-γ production by NK and NKT cells is important for protection from *A. phagocytophilum* infection. However, *A. phagocytophilum* counteracts IFN-γ signaling in infected host cells.

IL-8 is a chemokine that induces neutrophil migration to sites of infection. *A. phagocytophilum* induces IL-8 production and upregulates the expression of its receptor CXCR2 (IL-8 receptor) in neutrophils and in a promyelocytic (HL-60) cell line that has been treated with retinoic acid to induce differentiation along the neutrophil lineage (6). The recombinant P44 protein also induces IL-8 secretion in neutrophils and in stimulated HL-60 cells (6). Human neutrophils migrate toward *A. phagocytophilum*-infected cells in a chemotaxis chamber assay, and this movement can be blocked with antibodies to IL-8 (6). Immunocompetent and severe combined immunodeficient mice that are administered CXCR2 antisera are resistant to *A. phagocytophilum* infection, as are $CXCR2^{-/-}$ mice (6). High blood cholesterol levels facilitate *A. phagocytophilum* infection and upregulate the expression of both macrophage inflammatory protein 2 (MIP-2) (the mouse IL-8 homolog) and CXCR2 in mice (243). Thus, IL-8 and CXCR2 appear to enhance *A. phagocytophilum* infection.

A. phagocytophilum infection in mice induces the delayed and weak expression of TNF-α and IL-6 and significant expression of MIP-2, KC (mouse IL-8 homologs), and JE (mouse MCP-1 homolog) in the bone marrow (106), similar to that observed for human bone marrow or HL-60 cells (115). As the exposure of bone marrow cells to chemokines (e.g., IL-8 and MIP-1) suppresses the proliferation of myeloid progenitor cells (35), alterations in chemokine levels in the bone marrow were proposed to be responsible in part for the hematological abnormality that accompanies *A. phagocytophilum* infection (106).

TICK FACTORS REQUIRED FOR TRANSMISSION

The tick is an essential biological vector in which *A. phagocytophilum* replicates and persists through molting between sequential mammalian transmissions (95, 110). The recent development of RNA interference (RNAi) techniques for ticks (189) led to several novel discoveries regarding the role of tick proteins in *A. phagocytophilum*. One of the salivary gland proteins from the *I. scapularis* tick, salp16, is induced during tick engorgement and is required for infection with *A. phagocytophilum* (211). This is because *A. phagocytophilum* migrates normally from *A. phagocytophilum*-infected mice to the gut of engorging salp16-deficient ticks, but up to 90% of the bacteria that enter the ticks are unable to successfully infect the salivary glands of *I. scapularis* ticks (211). As in mammalian hosts, *A. phagocytophilum* selectively modulates tick signaling by altering protein phosphorylation to aid *A. phagocytophilum* within ticks. Unlike in human cells, in tick cells *A. phagocytophilum* infection induces dramatic morphological changes by changing the G-actin/F-actin ratio, which is mediated by actin phosphorylation in a p21-activated kinase (IPAK1)-dependent manner (212). G-protein-coupled-receptor Gβγ subunits stimulate IPAK1 through the activation of phosphoinositide 3-kinase. Importantly, the silencing of *ipak1* using RNAi treatment reduces actin phosphorylation and *A. phagocytophilum* acquisition by ticks. Interestingly, phosphorylated actin translocates into tick cell nuclei, enhances the binding of the TATA box protein to RNA polymerase II (RNAPII), and selectively promotes the expression of salp16, a gene crucial for the survival of *A. phagocytophilum* (212). Those authors reported that actin phosphorylation was not induced in *A. phagocytophilum*-infected primary cultures of human neutrophils, suggesting that this is a specific signaling pathway activated in ticks by *A. phagocytophilum*. It is possible that salp16 may not be only tick gene activated by this pathway, and the activation of phosphoinositide 3-kinase may have pleiotropic effects on tick cells. It remains to be determined how *A. phagocytophilum* activates tick cell phosphoinositide 3-kinase.

A. phagocytophilum infection upregulates the expression of α1,3-fucosyltransferases in ticks, and gene silencing significantly reduces the colonization of ticks. The acquisition, but not the transmission, of *A. phagocytophilum* is inhibited when α1,3-fucosyltransferases are silenced during tick feeding (179). *A. phagocytophilum* therefore uses α1,3-fucose to colonize ticks, as in mammalian hosts.

Ticks need to endure desiccation, extreme environmental temperatures, and starvation, etc., throughout their life cycle. Interestingly, the presence of *A. phagocytophilum* in *I. scapularis* ticks increases their ability to survive in the cold by upregulating an *I. scapularis* antifreeze glycoprotein (IAFGP) as demonstrated via RNAi knockdown studies (163). IAFGP increases the viability of yeast cells in cold temperatures (163). The increased survival of infected ticks allows the bacteria to persist in the environment. Because ticks are not dependent on *A. phagocytophilum* for survival, but *A. phagocytophilum* infection appears to benefit ticks, these data suggest that *A. phagocytophilum* is considered "a facultative symbiont" for ticks. Overall, *A. phagocytophilum* modulates tick cells, but the infection does not seem to harm ticks, and especially the prolonged survival of infected ticks is advantageous for *A. phagocytophilum* to persist in nature and disseminate.

CONCLUSIONS

Through intensive research efforts on host-pathogen interactions, *A. phagocytophilum* emerged as an important model pathogen that can subvert the host innate immune response, particularly the antimicrobial mechanisms of neutrophils. Despite of the lack of effective genetic tools to analyze *A. phagocytophilum*, many fundamental questions concerning pathogen-host interactions could have been approached by using cell biological techniques, the targeted knockdown of genes in mammalian cell culture and in ticks, and knockout mouse strains. These studies would benefit from collaborative efforts of rickettsiologists and experts in the respective fields of eukaryotic cell biology and immunology. The Himar transposase system has been developed to randomly insert genes into the *A. phagocytophilum* genome (65), and studies using the cloned mutants are expected to generate a wealth of new information. Recently, the entire plasmid (4.5 kb) was inserted into the *Anaplasma marginale* chromosome by a single homologous crossover event not mediated by the transposase (64), suggesting the possibility of transforming *A. phagocytophilum* by using homologous recombination. The ongoing use of bacterial and tick genomics will no doubt continue to uncover novel virulence effectors of *A. phagocytophilum* and tick vector biology, which will deepen our understanding of the molecular pathogenesis of *A. phagocytophilum* infection.

ACKNOWLEDGMENTS

I acknowledge Tim Vojt for assistance in preparing the illustrations and Sam R. Telford III, Tufts Commings School of Veterinary Medicine, for helpful comments on tick transmission.

The work in my laboratory was supported by NIH grants R01AI030010, R01AI054476, and R01AI047885.

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