INTRACELLULAR PATHOGENS II

Rickettsiales
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Cover image: Confocal fluorescent microscopic image of GFPuv-expressing *A. phagocytophilum* infecting a rhesus cell (RF/6A) expressing DsRed2, a red fluorescent protein. Courtesy of Ulrike Munderloh (see chapter 14).

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Just over a century ago, Sir Arnold Theiler reported what came to be known as the first rickettsial pathogen, *Anaplasma marginale*. With this report in 1910, Theiler ushered in a dramatic era of discoveries of rickettsiae as pathogens with a series of independent investigations by scientists who became recognized as giants in the field: Cowdry, Nicolle, and Ricketts, among others. Their investigations of the fundamental epidemiology, immunology, and pathogenesis of rickettsial infections have stood both the test of time and the subsequent reanalysis of their work using tools unimaginable in their era. In the process, their original monographs have become classics in the study of infectious diseases.

Following this initial burst of creative investigation in rickettsiology during roughly the first 3 decades of the 20th century, there have been quantum leaps in knowledge. Some have been spurred by the development of powerful new tools, such as the electron microscope in the 1950s, which confirmed that rickettsiae were indeed bacteria. Other gains in knowledge, more tragically, have come from armed conflicts, which led to the discovery of multiple previously unknown pathogens and understanding of the role that host susceptibility plays in disease epidemiology and severity.

The development of molecular biology as both a field and a corresponding set of tools ushered in a new era of quantum gains in rickettsiology. There has been a virtual explosion of genome sequences, from the first sequencing of *Rickettsia prowazekii* in 2005, through 2012, when not only have species in all six genera of the order Rickettsiales been sequenced, but multiple strains with unique phenotypes have now been sequenced for several key pathogens. This knowledge has transformed the field: the discovery of extrachromosomal elements among the Rickettsiaceae and the use of lateral gene transfer to acquire genetic material is just one example of major shifts in the paradigm of rickettsial biology. Importantly, rickettsial genome data are publicly and freely available, thus allowing scientists worldwide, especially those in low- and
middle-income countries that suffer a disproportionate burden of rickettsial disease, to access sequence data and progress in investigation.

The sudden richness of the genome data and the ability of scientists worldwide to identify rickettsiae molecularly also bring challenges to the field. While the seminal taxonomic reorganization of *Anaplasma*, *Ehrlichia*, and *Neorickettsia* in 2001 brought sense to the family *Anaplasmataceae* and promoted comparative investigations that addressed major knowledge gaps, the criteria required for species definition are increasingly problematic, especially for the family *Rickettsiaceae*, challenging the classical descriptions of spotted fever group versus typhus group rickettsioses. Similarly, the widespread availability and ease of molecular identification can lead to the misplaced assumption that a molecular “tag” is sufficient to infer epidemiologic and pathogenic behavior identical to that of a type strain or species. Indeed, recent work on several rickettsial pathogens has highlighted strain differences in phenotype—hence the continuing need for isolation and phenotypic characterization of a diversity of strains.

The ability to now ask heretofore unanswerable questions regarding rickettsial epidemiology, immunity, and pathogenesis has been accompanied by an influx of new investigators into the field of rickettsiology. It is these individuals, as much as any molecular tool, who have given rise to the next quantum leap in the field that is the subject of the current volume. These young scientists have brought new perspectives, from both within and outside the field, to the study of rickettsiae. To the greatest degree possible, we have selected among these young investigators, now firmly established as independent scientists, to author the chapters in this volume. Their achievements are recent, their viewpoints fresh, and their writing unencumbered by prior authorship of similar chapters. Consequently, this book, *Intracellular Pathogens II: Rickettsiales*, is not an updating of any prior edition but rather a new text that links the quantum increase in our knowledge of rickettsiae over the past decade with the future perspectives of the cohort of scientists who will lead the field forward in the decades to come. Equally we wish to acknowledge the superb contributions of the many superb rickettsiologists, junior and senior, who have not authored a chapter in the current text—we have endeavored to present your achievements faithfully and appreciate your notable contributions.

The authors have been asked to focus on the most exciting advances in their respective topic areas rather than attempt to be encyclopedic. Thus, individual chapters often center on only a few of the rickettsial species, based on where the leading edge is being pursued. As a result, however, five of the six genera in the order *Rickettsiales* are covered extensively in multiple chapters. The exception is *Wolbachia*—this is by no means to diminish the importance of these remarkable bacteria, but rather in acknowledgment that their biology has been discussed in several recent texts, and to allow us to focus on those rickettsiae that cause disease in mammalian hosts.

One of the giants of the past 40 years in rickettsiology, Herb Winkler, cleverly paraphrased Jacques Monod by noting that “what is true for *E. coli* may well be true for elephants—but not for rickettsia.” Indeed, what is now happening is that rickettsiology is making novel contributions to microbiol-
ogy and to biology in general by expanding our understanding of the roles of convergent and divergent evolution in the remarkable diversity of life. With respect for both Monod and Winkler: rickettsiae, \textit{E. coli}, and elephants all teach the same lesson of adaptation to their unique niches on the planet.

\textbf{GUY HUGHES PALMER}

\textbf{ABDU AZAD}
INTRODUCTION
The order Rickettsiales includes a diverse group of bacteria with an obligatory intracellular existence and a mandatory transmission cycle that includes arthropods as hosts, reservoirs, and vectors (Dumler and Walker, 2005a). While the entire range of bacteria and their underlying genetic diversity have not been fully characterized, considerable insight into their ability to cause disease has been gleaned from advanced studies of genomes of an increasing number of Rickettsia, Orientia, Anaplasma, Ehrlichia, and Neorickettsia species (Georgiades et al., 2011; Gillespie et al., 2008). There is substantial controversy over the capacity of species in the Rickettsiales to cause disease, and examples of both human and veterinary pathogens exist for the vast majority of the species described in all of the genera in both the families Rickettsiaceae and Anaplasmataceae. In fact, a number of species are also recognized to be pathogenic for their arthropod hosts or to alter these hosts in ways that promote survival of the bacterium (Saridaki and Bourtzis, 2010). Yet species not associated with disease in any vertebrate or invertebrate system exist, and evidence of infection in humans and animals without accompanying evidence for clinical disease, even with established pathogenic rickettsiae, is clear (Bakken et al., 1998; Marshall et al., 2003). The extent to which disease is caused; the penetrance of the diseased phenotype for each species, strain, or subspecies; and mechanisms that govern the degree of pathogenicity are areas of vigorous investigation.

Despite the extensive recent study regarding disease in humans infected by members of the family Anaplasmataceae, infections in animals are the precedent and basis for all of the work. The vast majority of clinical, ecological, and epidemiological information described for humans is likely applicable to animals. Moreover, animals sustain infections and variations on disease not yet described in humans (Rikihisa, 2006). This is likely in part due to the greater likelihood of an animal encountering an infected tick, and perhaps because of tick-pathogen-animal coevolution. One hypothesis also maintains that coevolution contributes to the reduction in apparent acute virulence and promotion of sustained persistent bacteremia in animals that develop minimal clinical signs and disease. This hypothesis in part stems from the observation that transovarial transmission...
in ticks is very inefficient (Long et al., 2003). This would imply greater fitness for those Anaplasmataceae that maintain high blood microbe loads, longer intervals of patent infection, and reduced disease costs to the infected mammalian host (Dumler, 2001). A complete discussion of disease processes that occur in animals is beyond the scope of the current chapter, so comments are largely confined to information relevant to human infection and disease, except where animal models inform human disease as well.

Thus, the purpose of this chapter is to review and discuss (i) the human disease manifestations for major pathogenic groups, with some attention to detail on how these differ between genera and species within a single genus; (ii) the evolution of treatment for rickettsial infections; (iii) the existing challenges for treatment and management in human infections; and (iv) corroborated or suspected treatment failures or persistent clinical complaints.

CLASSIFICATION AND PATHOGENS

The order Rickettsiales is divided into two families, Rickettsiaceae and Anaplasmataceae (Dumler and Walker, 2005a). Pathogenic species are recognized in both. In general, pathogenic members of the Rickettsiaceae family, including spotted fever and typhus groups of Rickettsia (SFGR and TGR, respectively) and Orientia tsutsugamushi, target infection predominantly in endothelial cells in mammals, including humans. In contrast, pathogens in Anaplasmataceae have a wider variety of in vivo targets, ranging from hematopoietic cells including professional phagocytes like monocytes, macrophages (Ehrlichia canis, Ehrlichia chaffeensis, Ehrlichia muris, Ehrlichia ruminantium, Anaplasma bovis, Neo rickettsia helminthoea, Neo rickettsia se mtus, Neo rickettsia ristia i), and neutrophils (Anaplasma phago cytphilum, Ehrlichia ewingii); to platelets (Anaplasma platys) or erythrocytes (Anaplasma marginale, Anaplasma centrale); to endothelial cells (E. ruminantium) and even intestinal epithelial cells (N. ristia i) (Dumler and Walker, 2005a).

Rickettsiaceae

There are only two recognized genera in the family Rickettsiaceae: Rickettsia and Orientia. The genus Rickettsia has classically been divided into the SFGR and TGR based on serological reactions, for which the basis is expression of surface antigens encoded by the outer membrane protein A (ompA) and B (ompB) genes, the former possessed uniquely by the SFGR species (Dumler and Walker, 2005a). However, recent characterization and full genome sequences illustrate genetic resolution that allows the SFGR to be divided into up to four clades (Gillespie et al., 2008). This division provides for both SFGR and TGR clades, but also one clade that is sometimes called “ancestral, nonvirulent,” including R. bellii and R. canadensis, and another that appears to be “transitional” between SFGR and TGR and includes R. akari and R. felis. From a phylogenetic standpoint, such genetic methodology defines evolutionary relationships that may or may not have any bearing on pathogenicity or the ability to cause mammalian infection and disease.

To address this issue and the difficulties in investigating and classifying rickettsiae that may not yet be cultivated, one proposal examines the sequences of five genes present in SFGR species as a surrogate for the genetic relatedness established by complete genome sequencing (Fournier et al., 2003a). By coupling phenotypic data like serological responses and clinical features, this approach has been used to define a large number of new species (Fournier and Raoult, 2009). The controversy with this approach stems from the fact that very little is known about the molecular basis for rickettsial virulence and how this relates to pathogenicity in the genus Rickettsia (Fournier et al., 2009; Walker and Ismail, 2008). Moreover, this approach is necessarily confounded by phenotypic data that are in part host- and not pathogen-related. The former approach has resulted in an expansion of the list of accepted and candidate SFGR species, accurately reflecting some genetic diversity that may or may not relate to
disease--causing ability. In fact, genetic diversity among SFGR, based on sequence analysis of \textit{ompA}, is similar to that of the major 56-kDa surface protein gene of \textit{O. tsutsugamushi}, the cause of scrub typhus, yet only a single species is proposed and recognized for that microbe (Fournier et al., 2008). One result has been an increased level of confusion regarding rickettsial diseases, as busy practitioners attempt to understand the changing bacterial nomenclature and disease names (Walker et al., 2008). The resolution to this controversy will be assisted by studies that employ genomic data and modern molecular methods to delineate the contributions of candidate virulence genes in \textit{Rickettsia} species and isolates.

In contrast to the marked expansion of SFGR species, the TGR group is represented by only two species: \textit{R. prowazekii}—the cause of louse-borne or epidemic typhus; and \textit{R. typhi}—the cause of murine, flea-borne, or endemic typhus. Both are well-recognized pathogens of humans, and in general have a high degree of genetic homogeneity (Walker and Yu, 2005). The genetic bases for virulence and pathogenicity are not well understood.

\textit{O. tsutsugamushi}, the cause of scrub typhus, is transmitted by larval trombiculid mites and is sufficiently unique at the genetic level that it occupies a distinct clade and genus (Georgiades et al., 2011). While considerable genetic diversity exists, it is still considered a single species with at least several characteristic strains (Fournier et al., 2008). As for other rickettsiae, little is known regarding the molecular bases for virulence and pathogenicity.

\section*{Anaplasmataceae}

There are currently five genera classified within the \textit{Anaplasmataceae} family, including \textit{Ehrlichia}, \textit{Anaplasma}, “\textit{Candidatus Neoehrlichia},” \textit{Neorickettsia}, and \textit{Wolbachia} (not including \textit{Aegyptianella}, which is likely within the genus \textit{Anaplasma}) (Dumler and Walker, 2005a; Kawahara et al., 2004). Members of this family were first recognized because of veterinary disease, but human disease is now well recognized for \textit{Ehrlichia}, \textit{Anaplasma}, and \textit{Neorickettsia} (Dumler et al., 2007b; Newton et al., 2009); a few cases of infection with “\textit{Candidatus Neoehrlichia}” are also published (Fehr et al., 2010; Pekova et al., 2011; von Loewenich et al., 2010; Welinder-Olsson et al., 2010), while \textit{Wolbachia} may be a significant cofactor for promoting inflammatory disease in humans with some forms of filariasis (Shakya et al., 2008; Turner et al., 2009). For the purposes of this chapter, discussion is limited predominantly to the former two genera.

Complete genome sequences of \textit{Ehrlichia} and \textit{Anaplasma} species demonstrate their close relationships and genomic synteny (Dunning Hotopp et al., 2006). In contrast, as anticipated from sequence analysis of individual genes, \textit{Neorickettsia} species are more divergent (Dunning Hotopp et al., 2006). The genus “\textit{Candidatus Neoehrlichia}” is now validly published, but lack of a genome sequence confounds appropriate evaluation of its taxonomic position (Kawahara et al., 2004). The current organization of these genera and their species was based on comparisons of partial sequences of \textit{rrs} (16S rRNA genes) and the \textit{groESL} heat shock operons (Dumler et al., 2001). All members described in the genera \textit{Ehrlichia}, \textit{Anaplasma}, “\textit{Candidatus Neoehrlichia},” and \textit{Neorickettsia} are capable of causing disease in humans, animals, or both. Significant pathogens of humans include \textit{E. chaffeensis}, \textit{E. ewingii}, and \textit{A. phagocytophilum}, all of which also cause veterinary disease (Little, 2010; Thomas et al., 2009). Recent investigations implicate an \textit{E. muris}-like bacterium as a human pathogen (Pritt et al., 2011), and individual reports suggest human pathogenicity for \textit{Anaplasma bovis} (J. S. Chae, personal communication) and \textit{E. ruminantium} or a similar organism as well (Reeves et al., 2008). While \textit{E. canis} can cause disease in humans, it is best known as a pathogen of dogs, as is \textit{E. ewingii} (Liddell et al., 2003; Little, 2010; Perez et al., 2006). Except for \textit{A. phagocytophilum} and possibly \textit{A. bovis}, all other \textit{Anaplasma} species, including the erythrovircytic \textit{A. marginale} and \textit{A. centrale}, as well as \textit{A. ovis} and \textit{A. platys}, have been recognized as
veterinary pathogens only (de la Fuente et al., 2006; Kocan et al., 2010). The precise taxonomic position of the avian/amphibian/reptile anaplasmas currently listed in the Aegyptianella genus (e.g., A. pullorum) is still not entirely settled, but molecular evidence suggests a close relationship to the genus Anaplasma (Rikihisa, 2006). Human infection has not been reported. All members of the Ehrlichia and Anaplasma genera are transmitted by ticks, usually acarid ticks. The “Candidatus Neoehrlichia” genus forms a clade intermediate between Anaplasma and Ehrlichia (Kawahara et al., 2004). The first example of DNA from this group was obtained from Dutch Ixodes ricinus ticks (Schotti vari- ant) and among I. ricinus ticks in northeastern Italy (“Ehrlichia walkeri”) (Brouqui et al., 2003; Dumler et al., 2001), but it was first isolated from Rattus norvegicus and Ixodes ovatus ticks in Japan (Naitou et al., 2006). A second species, “Candidatus Neoehrlichia lotoris” (Yabsley et al., 2008), was identified in raccoons in the United States.

The genus Neorickettsia is more distantly related to Ehrlichia and Anaplasma (Dumler et al., 2001). N. helminthoea was the first species described, and disease is apparently limited to dogs and bears that consume fish infested by infected trematodes, the major vector for this genus (Pretzman et al., 1995). N. sennetsu, previously classified as Rickettsia sennetsu, then Ehrlichia sennetsu, is the first and so far only human pathogen in this genus (Newton et al., 2009; Tachibana, 1986). Although only rarely diagnosed, it also is likely to be vectored by consumption of fluke-infested fish (Tachibana, 1986). The major pathogen in this genus, N. risticii, is very closely related to N. sennetsu, but has never been reported as a cause of human disease (Rikihisa, 2006). The complex life cycle of N. risticii serves as a model for understanding transmission in this genus, where infected trematode cercariae are vectors for fish, possibly other aquatic animals like waterfowl, and insects and their larvae that breed in water containing infected cercariae (Pusterla et al., 2003). Adult insects, such as mayflies, can then carry the infectious agents to terrestrial environments, where they are inadvertently consumed by animals and establish infection in intestinal epithelial cells before disseminating in phagocytes like macrophages.

**RICKETTSSIOSES**

**Pathogenesis of Vasculotropic Rickettsial Infections**

The underlying theme for disease manifestations in the Rickettsiaceae is systemic infection of endothelial cells, leading to increased vascular permeability. Although the presence of the bacterium and its microbe-associated molecular pattern structures likely trigger fever, the predominant clinical manifestation, increased vascular permeability, is likely to be the major pathogenic component, especially for those with complicated or severe disease or in those who die (Walker and Ismail, 2008). Where examinations of human or animal tissues have been conducted, both Rickettsia and Orientia target endothelial cells in a variety of tissues, after dissemination from the dermal inoculum via tick, mite, flea, or louse bites (Moron et al., 2001; Walker and Ismail, 2008). It is clear that these vasculotropic rickettsiae initially interact with other cells at the site of inoculation, since most inoculation sites become inflamed and large numbers of defense cells, including macrophages, dendritic cells, lymphocytes, and neutrophils, are recruited in response to both pathogen- and arthropod-associated products. It is likely that the earliest interactions with rickettsiae occur with resident dendritic cells and macrophages that both initiate early immune responses but perhaps are also the vehicles by which the bacteria disseminate, first to local draining lymph nodes and then to blood and all tissues and organs (Fang et al., 2007; Murphy et al., 1978). The ability of rickettsiae to disseminate, infect endothelial cells, and induce localized microvascular alterations competes with the waxing immune responses that generally lead to infection control.

Although vasculotropic rickettsiae can kill endothelial cells, whether this is the main determinant of increased vascular permeability or
whether induced changes in cytoskeletal structure are major contributors is not well investigated (Walker and Ismail, 2008; Woods and Olano, 2008). In vitro investigations suggest that the combined effects of early rickettsial infection with increasing nitric oxide production and a proinflammatory cytokine milieu all contribute to the increased microvascular permeability (Walker et al., 1997; Woods et al., 2005). These observations are consistent with the concept that infection and host response are in part the cause of early vascular compromise in vasculotropic rickettsial infections. In time, rickettsiae alone have the capacity to kill endothelial cells, but the majority of in vivo lesions examined in humans and animal models have a significant degree of inflammation and, infrequently, thrombosis (Walker et al., 2003). Infection in animal models devoid of the capacity to induce protection, e.g., major histocompatibility complex class I knockout mice, illustrates the beneficial role of the immune and inflammatory response, although contributions to early pathogenesis seem likely (Walker et al., 2001).

Dissemination to and infection of endothelium in diverse anatomical compartments often explains many significant complications, severe disease, and death (Walker et al., 2003). The disseminated rash is the result of rickettsial infection of dermal capillaries and venules, initially resulting in vascular dilatation (macular rash), followed by transudation of edema fluid and exudation of inflammatory cells to create an elevated erythematous (maculopapular) rash (Dumler and Walker, 2005b). Not all infections advance to this state before control by increasing immunity or antimicrobial treatment. However, in some cases, particularly with the severest forms of rickettsial infections like Rocky Mountain spotted fever (RMSF), Mediterranean spotted fever (MSF), louse-borne typhus, murine typhus, and scrub typhus, vascular permeability owing to cytoskeletal retraction and loss of intercellular junctions is replaced by frank endothelial cell injury or death and extravasation of vascular contents, including erythrocytes, into dermal tissues to create a nonblanching lesion called a petechia. Petechiae are considered a hallmark of many rickettsial infections and generally indicate an advanced stage of vascular injury or vasculitis, as illustrated in histopathologic studies of these diseases (Kao et al., 1997). The range of histopathologies characterized by examination of skin biopsies from patients with documented vasculotropic rickettsial infections extends from leukocytoclastic vasculitis with significant degrees of associated necrosis and formation of nonocclusive fibrin-platelet thrombi, to lymphocytic capillaritis and venulitis, to simple vascular dilatation with peri-vascular inflammatory cell infiltrates that represents the earliest change. Epithelial cells are not infected in vivo, but are affected by the vascular compromise. Basal vacuolization and apoptotic keratinocytes are observed in skin biopsies of RMSF patients, likely a response to local inflammatory mediators and/or ischemic changes with altered local microvascular circulation.

These vascular changes also underlie the systemic manifestations of severe vasculotropic rickettsial diseases. Major complications include gastrointestinal symptoms and signs, such as abdominal pain, nausea, vomiting, and diarrhea, likely owing to similar vascular changes that are documented in autopsy studies. Acute respiratory distress syndrome and noncardiogenic pulmonary edema are life-threatening events with these infections, and occur as a result of infection of the massive microvascular network of the lung (Walker, 2007). As vascular permeability increases, loss of intravascular fluids into pulmonary alveoli leads to significant respiratory compromise that requires very careful hemodynamic management to avoid iatrogenic complications. Similarly, increasing vascular permeability with infection of the meninges or cerebral capillaries can lead to degrees of cerebral edema that could cause brain-stem herniation and death. Even in the absence of generalized cerebral edema, the compromise in vascular integrity and moderate levels of ischemia lead to ataxia, seizures, coma, and auditory deficits acutely (Sexton and Kirkland,