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Progress and obstacles in vaccine development for the ehrlichioses

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Abstract

Ehrlichia are tick-borne obligately intracellular bacteria that cause significant diseases in veterinary natural hosts, including livestock and companion animals, and are now considered important zoonotic pathogens in humans. Vaccines are needed for these veterinary and zoonotic human pathogens, but many obstacles exist that have impeded their development. These obstacles include understanding genetic and antigenic variability, influence of the host on the pathogen phenotype and immunogenicity, identification of the ehrlichial antigens that stimulate protective immunity and those that elicit immunopathology, development of animal models that faithfully reflect the immune responses of the hosts and understanding molecular host–pathogen interactions involved in immune evasion or that may be blocked by the host immune response. We review the obstacles and progress in addressing barriers associated with vaccine development to protect livestock, companion animals and humans against these host defense-evasive and cell function-manipulative, vector-transmitted pathogens.

Keywords

antibody epitope; antigenic variation; *Ehrlichia*; immune evasion; immunopathology; immunoprotection; tick; vaccine; zoonosis

Members of the genus *Ehrlichia* are tick-borne obligately intracellular bacteria that cause persistent infection of natural animal hosts, but are also associated with emerging human zoonoses of public health importance. Heartwater, one of the most economically important ehrlichioses, is a devastating endemic disease of livestock in sub-Saharan regions of Africa and a few eastern Caribbean islands [1]. *Ehrlichia canis* is globally distributed in tropical areas of the world and was relatively unstudied until an epizootic outbreak occurred in American military working dogs in Vietnam, a disease now recognized as canine monocytic ehrlichiosis [2]. Recently, *Ehrlichia chaffeensis*, *Ehrlichia ewingii* and *E. canis* have emerged and cause life-threatening zoonoses in humans [3–5]. *E. chaffeensis* is the most frequent cause of severe

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and potentially fatal ehrlichiosis in humans [6], *E. ewingii* has been associated with disease primarily in immunocompromised individuals [4,7] and *E. canis* has most recently been identified as the etiologic agent of human infections reported in Venezuela [8,5].

Although *Ehrlichia* are responsible for serious diseases of agricultural, veterinary and human importance, the only immunization strategy that is commercially available is one developed in 1945 for *Ehrlichia ruminantium*, which involves an infection and treatment strategy using cryopreserved blood from infected sheep [9]. However, this procedure has numerous biological and practical shortcomings including incomplete cross-protection and lethal infection if treatment is administered late [10]. Experimental vaccine development efforts have been focused on *E. ruminantium* because of the economic importance of the disease, the inability to improve production by introduction of highly susceptible exotic breeds, limitations on livestock export and the vast numbers of animals (150 million) that are potentially at risk [11]. Vaccines for *E. canis* are needed, but research funding available for this veterinary pathogen has been scarce. The emergence of *E. chaffeensis* as a human pathogen of public health importance has sparked new research and substantial advances in vaccine development for the ehrlichioses and our knowledge of host–pathogen interactions.

Agents, vectors & hosts

Ehrlichia are maintained in zoonotic cycles involving a variety of vertebrate animal hosts and are transmitted by tick vectors. Most ehrlichiae exhibit tropism for hematopoietic cells, including monocytes and granulocytes, but one species (*E. ruminantium*) primarily targets endothelial cells.

Ehrlichia chaffeensis is the etiologic agent of human monocytotropic ehrlichiosis (HME) [12], the most severe of the human ehrlichioses. In humans, many (40–60%) *E. chaffeensis* infections require hospitalization [13,14], and the case fatality rate is 3% owing to the difficulty in making an accurate diagnosis and delays in treatment. White-tailed deer are the primary reservoir for *E. chaffeensis*, but dogs and coyotes may also be significant natural reservoirs [15]. *E. chaffeensis* is transmitted primarily by the lone star tick, *Amblyomma americanum* [15,16]. The emergence of *E. chaffeensis* is attributed to demographic and ecologic factors, including increased human contact with natural foci, immunocompromised and aging human populations, increases in vector and mammalian host populations and availability of improved diagnostic methods and mandated reporting [15].

Ehrlichiosis *ewingii* caused by *E. ewingii* is the most recently described emerging human ehrlichiosis [4]. This agent also infects canines causing two distinct clinical syndromes, anemia and polyarthritis [17]. *E. ewingii* exhibits host cell tropism for granulocytes (neutrophils) and is also transmitted by the lone star tick, *A. americanum* [18]. Deer and dogs are the main reservoirs for *E. ewingii*, and many cases of human disease are associated with canine companions [4]. *E. ewingii* appears to be an opportunistic pathogen that has emerged in immunocompromised patients [7,4].

Ehrlichia ruminantium is strictly a veterinary pathogen that causes severe acute infection known as heartwater in domestic ruminants in Africa, which can result in high mortality (50–90%) [19]. *E. ruminantium* is responsible for major production losses to the African livestock industry, and is also considered to pose a potential danger to the southeastern USA where competent native tick vectors are present [20–22] and the climate is appropriate for introduction of exotic tick species capable of transmitting the disease [23–25]. In Africa, *E. ruminantium* is transmitted by *Amblyomma variegatum* and *Amblyomma hebraeum*, but other *Amblyomma* spp. ticks, including two North American species, *Amblyomma maculatum* and *Amblyomma cajennense*, appear to have similar transmission efficiency [20]. Notably, a closely related

organism (by *gltA* and *map1* sequence analysis) has recently been discovered in the USA in a naturally infected goat and in ticks collected from ten states [26,27].

Ehrlichia canis is the type strain for the genus *Ehrlichia* and is the primary etiologic agent of canine monocytic ehrlichiosis, a serious and sometimes fatal, globally distributed hemorrhagic disease of dogs [28]. *E. canis* is transmitted by the brown dog tick, *Rhipicephalus sanguineus* [29], and infects monocytes/macrophages. The clinical manifestations of acute human infection with *E. canis* are similar to those observed with *E. chaffeensis* [8,5].

Vaccine interest & feasibility

Commercial interests in developing and marketing vaccines for canine ehrlichiosis and heartwater exists, and interest in a vaccine for HME has the potential to develop if the actual incidence (greater than Lyme disease) and lethality were more widely recognized and more prominently elucidated. Development of vaccines depends on our understanding of pathogenesis, antigenic composition, protective and pathologic immune responses, and identification of components of the organism that stimulate protective immunity and molecular host-pathogen interactions that are subject to immune disruption. *Ehrlichia* have evolved mechanisms that allow them to persistently infect mammalian hosts by subverting the innate and adaptive immune responses. Thus, the organism is maintained in nature through subclinical infections of vertebrate hosts (carriers) as well as ticks for long periods of time, avoiding immune clearance [30]. However, effective immune responses leading to the elimination of organisms without treatment have been documented in *E. canis*-infected dogs [30,31], and infection and treatment strategies in Africa for *E. ruminantium* have been used for decades that provide protection against challenge [32]. Furthermore, various experimental *E. ruminantium* vaccines (inactivated, live-attenuated and recombinant) have demonstrated varying levels of protection against homologous challenge, although less protection has been achieved against natural field challenge [10]. Cell-mediated immune responses and IFN- γ production correlate with protection against *E. ruminantium* and other *Ehrlichia* spp. [33,34]. Although, a conclusive role for antibodies in protection against *E. ruminantium* has not been established [35,36], they appear to play an important role in immunity to *E. chaffeensis* and other *Ehrlichia* spp. [37-39]. Thus, vaccines that stimulate humoral and cell-mediated immune responses, prevent disease or minimize clinical signs, shorten duration of illness and/or prevent progression to a chronic infection appear to be feasible.

Genetic & antigenic heterogeneity

A major obstacle to vaccine development for *E. ruminantium* has been defining the relevant genetic and antigenic heterogeneity among strains circulating in Africa. Until recent genome sequences were obtained from three different strains, *E. ruminantium* was considered to be relatively homogenous [40,41]. It is evident that *E. ruminantium* exhibits substantial genetic heterogeneity, and this may have contributed to the inconsistent efficacy of various vaccines for heartwater [42-44]. In fact, eight different 16S rRNA (*rrs*) genotypes are well documented, and there are probably many more [11,45]. Genome sequences have revealed genomic modifications, such as high synonymous substitution rates, truncated genes and plasticity, due to active expansion and contraction of the genome through addition and deletion of tandem repeats (TRs) [41]. Some co-circulating *E. ruminantium* strains appear to be in genomic stasis, while others are in a divergent state defined by active recombination [46]. Multilocus sequence typing has revealed that eight strains in one small African locality had different phylogenies for each gene target examined, providing an example of the heterogeneity that occurs in the natural population [46]. Similar findings for the *MAP* genes suggest a high level of diversity that reveals divergent evolution and no correlation among *MAP* genotypes, geographic distribution and timing of strain introduction [44,47,48]. The inconsistency of any single strain

to protect against experimental needle challenge is at least partially associated with the antigenic differences in the challenge strain.

Genetic diversity among strains of *E. chaffeensis* has been noted in the major outer membrane protein (OMP) genes and other major immunoreactive proteins that are considered as priority vaccine candidates [49,50]; however, *rrs* gene sequences are identical among all US isolates. Variations in the number of repeats present in TR protein (*TRP*) genes such as *TRP47*, *TRP120* and *TRP32* (VLPT) have also been demonstrated in different *E. chaffeensis* isolates [50-53]. Although variations in TR number are well defined, complete differences in the TR sequences have only been identified in the *TRP47* gene of *E. chaffeensis* strains [50]. Furthermore, diversity in the number of TRs does not correlate with geographic distribution. The OMP-1 family of major outer membrane genes has been examined, and three genotypes have been identified based on diversity of *OMP-1* gene sequences, associated promoter regions and locus organization [49,53]. The molecular variation of the OMP-1-classified genetic groups appears to be due to mutations related to selective pressure and not recombination [54]. Although *E. chaffeensis* does exhibit some genetic diversity, it appears to be much more conserved than *E. ruminantium*, and the diversity does not appear to be associated with recombination, but rather selective pressure that results in point mutations. Therefore, the limited diversity of *E. chaffeensis* strains suggests that, although antigenic variation is a factor, vaccines could be developed that represent the three main genotypes.

Interestingly, *E. canis* strains in the Americas appear to be highly conserved [55], suggesting that genetic and antigenic heterogeneity is not a major consideration, at least for North American continent-specific vaccine development. The 16S rRNA sequences of *E. canis* are 99.9–100% identical, and analysis of major immunoreactive protein genes from North and South American strains demonstrates a high level of conservation [50,56,57]. However, a recent study has examined major immunoreactive protein genes from *E. canis* strains distributed on three continents and demonstrated for the first time that an Israeli strain of *E. canis* has significant genetic and antigenic differences within the antibody epitope-containing region of the *TRP36* gene compared with American *E. canis* [55]. These changes resulted in alteration of the antibody epitope such that it did not react with anti-TRP36 antibodies from heterologous strains. Other differences, such as fewer TRs in the Israeli TRP140 and amino acid substitutions in the ankyrin repeat protein, Ank200, were also noted [55]. One major immunoreactive protein gene (*TRP19*) is highly conserved in all *E. canis* strains examined to date [55,58], and thus could be a component of a vaccine with broad efficacy. The fact that *E. canis* primarily infects canines and is transmitted by only a single tick species vector may explain the lack of diversity observed in this organism. However, the diversity of *TRP36* appears to be related to immune pressure [55]. The considerable conservation among North American *E. canis* strains substantially increases the opportunity for successful development of a broadly effective vaccine for canine ehrlichiosis.

Economical & relevant animal models

Vaccine development requires an understanding of host–pathogen interactions involved in pathology, immunopathology and protective immune mechanisms in order to block critical host–pathogen interactions that favor the pathogen, avoid immune-mediated mechanisms and effectively limit bacterial growth. *Ehrlichia* spp. of human and veterinary importance naturally infect ruminants and other large animal hosts. Such animals are more costly to buy and maintain and thus dramatically increase the expense of vaccine development. Furthermore, they are less well characterized immunologically than the usual mouse models. Identification of protective antigens and immune mechanisms requires appropriate murine models that are well characterized and economical to use and mimic natural disease and immunity.

There are important animal model barriers that have restricted *Ehrlichia* research and continue to be impediments to understanding infection and immunity. The ability of *Ehrlichia* spp. to infect laboratory mice is variable. In the case of *E. ruminantium*, the organism readily infects many inbred strains of mice, and the murine model has been used to study vaccine candidates and determine vaccine efficacy [59,60]. However, different outcomes in protection observed between murine models and the natural hosts have been reported and indicate that these models may have limitations for assessing *E. ruminantium* vaccine efficacy [61,62]. *E. chaffeensis* only causes transient infections in immunocompetent mice [63]. Hence, murine models using closely related surrogate agents (e.g., *E. muris* and *Ixodes ovatus Ehrlichia*) that naturally infect mice have been effectively used for determining the immune mechanisms relevant to *E. chaffeensis*. These have provided important insight in understanding the immune mechanisms, and immunopathologic and protective immune responses associated with the human disease [64-68]. Although much can be learned using the available murine models, utilization of surrogate pathogens and inconsistent comparative immunity suggests that a substantial barrier exists related to small animal models and vaccine development.

Influence of the host on *Ehrlichia* phenotype & immunogenicity

The ability of *Ehrlichia* vaccines to provide protection against experimental needle challenge with ehrlichiae propagated in mammalian cells, but the apparent lack of consistent protection against field challenge by naturally infected ticks has suggested that natural antigenic diversity in addition to other vector-associated factors, including the *Ehrlichia* phenotype in the invertebrate host, have contributed to disappointing results of experimental vaccines under natural field conditions [69]. A significant gap in our understanding exists regarding the influence of the host environment (invertebrate/vertebrate) on the molecular phenotype of *Ehrlichia* and how this relates to transmission, immunogenicity and ultimately vaccine composition. The influence of the host environment on pathogen phenotype is becoming more appreciated, as shown in recent studies that have identified some differences in gene and protein expression of major OMPs of *E. chaffeensis* and *E. canis* in mammalian and tick-derived cell lines [70,71], *E. canis* in dogs, ticks and cell culture [72], and host cell-specific protein expression of *E. ruminantium in vitro* [73,74] and in ticks [75]. These studies have focused on the major OMP family and suggest that these proteins are differentially regulated in invertebrate and vertebrate hosts. Other data suggesting that *Ehrlichia* phenotypes are different and result in differential clearance by the host is provided by studies that have reported delayed clearance of *E. chaffeensis* that was tick cell (ISE6)-derived compared with macrophage (DH82)-derived organisms [76]. There are differences in antibody reactivity with tick- and macrophage-derived proteins, and in total antibody responses and subclasses [76]. The functional significance of this differential expression is not understood. Although these studies provide the first insight into differential gene expression by *Ehrlichia* in different hosts, comprehensive genome-wide studies are needed to fully understand the differences in gene expression in various hosts. Genome-wide *E. chaffeensis* expression has been investigated in mammalian cells, and several vaccine candidate genes were highly expressed in mammalian cells, including secreted effector proteins TRP47 and TRP32 [77]. Indeed, *TRP47* was the most highly expressed gene in mammalian cells. Future studies that compare genome-wide expression profiles will be invaluable for fully understanding pathogen genes important for survival in the invertebrate and mammalian hosts, transmission, protective immunity and vaccine development.

Virulence factors & variations in infectivity & pathogenicity

Variations in infectivity and pathogenicity of *Ehrlichia* spp. are well described, and understanding the molecular and phenotypic differences that result in such variation can provide needed insight into microbial factors that may contribute to effective vaccines. The

best-characterized examples of infectivity and pathogenicity variations are described among *E. ruminantium* strains in various hosts (e.g., cattle, sheep/goats and mice) [45,78], in which wide variations in pathogenicity are reported. Most recently, nonpathogenic *E. ruminantium* variants were detected in goats from a heartwater- and *Amblyomma*-free area, but the variants had genetic markers similar to virulent strains [78]. Similarly, an *Ehrlichia* spp. most closely related to *E. ruminantium* has been detected in white-tailed deer and ticks in the USA, but has not been associated with disease in domestic livestock [27,26]. Moreover, differences in the intrinsic pathogenicity of *E. chaffeensis* have recently been reported among three isolates in immunodeficient mice [79,80]. The differences in pathogenicity of *E. chaffeensis* isolates (Wakulla, Liberty and Arkansas) representing the three divergent genetic groups were associated with three polymorphic chromosomal regions, with variations concentrated in genes that encode OMPs (OMP-1), ankyrin proteins, *hemE* and a small subset of hypothetical protein genes [79]. In severely immunodeficient mice, the Liberty strain did not cause liver pathology, but caused more severe clinical signs and higher bacterial loads in spleen and liver than the Arkansas strain, which caused more severe histopathologic lesions [79]. However, the lack of a functional adaptive immune response in these mice suggests that these differences may be related to ehrlichial susceptibility to innate immunity or innate immune-mediated pathology. Furthermore, although differences in pathology were observed among the three *E. chaffeensis* strains in mice, increased severity of disease in humans has not been associated with differences in genotype [51,81]. Nevertheless, associating differences in virulence with specific genetic differences is an important step in the development of molecularly engineered attenuated vaccines and is also critical for the identification of vaccine candidates.

Immunoprotective proteins

Successful trials with live and attenuated vaccines indicate that vaccine-induced immunity is feasible and encourages the pursuit of recombinant vaccines for *Ehrlichia* spp. containing defined immunoprotective proteins. Some ehrlichial proteins targeted by the host immune response are well defined, such as major OMPs (MAP1, MAP2 and OMP-1), and others have only recently been molecularly defined. Newly identified targets of the host immune response have been characterized in *E. chaffeensis* and *E. canis*, and most of these proteins contain TRs or ankyrin repeats [58,82-85]. Moreover, many of these proteins are secreted effector proteins that have major species-specific antibody epitopes [58,83,85-88]. However, there is relatively little information regarding the protective efficacy of specific immunoreactive proteins, and not all of these proteins have been molecularly characterized.

One of the most extensively studied vaccine candidates is a family of major OMPs (MAP, OMP, P28 and P30) ranging from 16 (*E. ruminantium*) to 25 (*E. canis*) members that are present in all species. A small group of major immunoreactive proteins of *E. chaffeensis* and *E. canis* has been identified on the basis of immunoblot reactivity, but the protective efficacy and functions of these proteins remain largely unknown. Major immunoreactive *E. chaffeensis* proteins are 200, 120, 88, 55, 47, 40, 28 and 23 kDa [89,90]; *E. canis* 200, 140, 95, 75, 47, 36, 28 and 19 kDa [91]; and *E. ruminantium* 160, 85, 58, 46, 40, 32 and 21 kDa [92]. *E. chaffeensis* immunoreactive proteins (Ank200, TRP120, TRP47, TRP32 [VLPT], OMP-1 family [22 genes] and MAP2) have been molecularly characterized as well as the corresponding orthologs in *E. canis* (Ank200, TRP140, TRP36, TRP19 [VLPT], OMP-1 family [25 genes] and MAP2, respectively). Fewer of these orthologs have been molecularly identified and characterized in *E. ruminantium* (MAP1 family [16 genes], MAP2 and mucin-like protein [clone hw26; TRP36/47 ortholog]) [92-94], but additional immunoreactive proteins have been identified in *E. ruminantium* that have not been described in *E. chaffeensis* or *E. canis* [59]. Immunoreactivity of the *E. chaffeensis* and *E. canis* MAP2 is primarily dependent on a major conformational epitope that does not react by western immunoblot [95,96], while *E. ruminantium* MAP2 appears to have a linear B-cell epitope [92].

Although the identification of immunoprotective proteins is limited, two immunoprotective proteins have been identified in *E. ruminantium*, including the major OMPs (MAP1 and MAP2), which elicit strong T-cell and antibody responses in cattle infected with *E. ruminantium* and are associated with protection [59,94,97-99]. Furthermore, protective antibody epitopes have been mapped to the MAP1 ortholog (OMP-19) in *E. chaffeensis* [89, 100,101], and OMP-19 protects immunocompetent mice against fatal infection with the surrogate highly pathogenic *Ixodes ovatis Ehrlichia* (IOE) [102]. However, this protein exhibits substantial diversity (see genetic diversity section) in *E. ruminantium* and in *E. chaffeensis*. Other less-studied proteins appear to provide some protection against challenge. In mice, protection is stimulated by a mixture of proteins including GroEL, MAP2 and the TRP36/47 ortholog (Erum1110) and others [59]. Some protection has also been demonstrated with GroEL and GroES individually [60], and with a family of ABC transporter proteins [103]. However, numerous candidates that induce T-cell proliferation are not protective against challenge. Additionally, some low-molecular-weight proteins (<15–18 kDa) of *E. ruminantium* that have remained molecularly uncharacterized stimulate T-cell proliferation and IFN- γ production [104].

Complete molecular characterization of proteins that elicit cellular and antibody responses has been a major challenge to address for vaccine development. Many newly identified immunoreactive proteins of *E. chaffeensis* and *E. canis* remain to be tested for protective efficacy. Furthermore, little information regarding the immune response to the orthologs of these proteins in *E. ruminantium* is available. Nevertheless, recent advances in the molecular characterization of major immunoreactive proteins of *Ehrlichia* spp. are an important advance towards molecularly defining immunoprotective components, and understanding the antigenic diversity that exists in the vaccine candidates.

Protective & pathologic immune mechanisms

Critical to vaccine development is understanding the adaptive immune mechanisms involved in immunity, identification of important immunoprotective proteins and developing immunization strategies that maximize the induction of protective immune responses and immunologic memory, and avoid an undesired immune response. Such information has been delayed by the lack of small animal models and resistance of inbred mice to infection with *Ehrlichia* of human and veterinary importance [63]. However, it is becoming evident that both humoral and cellular immunity play a role in host defense against ehrlichiae. Recently, the role of specific immune system elements has been investigated with inbred mice deficient in various immune system components, including MHC class II, Toll-like receptors, Fc γ RI and B cells, and in mice with severe combined immunodeficiency disease (SCID). Mechanisms involved in immunity to *E. ruminantium* have been investigated in natural hosts and in immunocompetent mice [34-36,105-110]. All of the available models utilizing natural infections, established murine models and newly developed murine models have provided insight into protective immune responses necessary for immunity to *Ehrlichia*.

Numerous studies with multiple *Ehrlichia* spp. indicate that IFN- γ is an essential mediator of protection [111-115]. Moreover, CD4⁺ and CD8⁺ T cells both contribute to IFN- γ production [33,67,110,116]. Notably, similar conclusions regarding the importance of MHC class I, CD4⁺ and CD8⁺ T cells, and the synergistic roles of IFN- γ and TNF- α have been reported in mice infected with *E. muris* [39]. An important role for CD4⁺ T cells in immunity to *E. ruminantium* and IOE has been suggested [34,33,105]. Similarly, mice lacking functional MHC class II genes are unable to clear *E. chaffeensis* infection, suggesting that CD4⁺ T cells are essential for ehrlichial clearance [117]. The intradermal environment (natural route of inoculation) appears to promote the induction of protective type 1 responses characterized by increased CD4⁺ and CD8⁺ T cells, and IFN- γ -producing CD4⁺ T cells [118]. This study

suggests that intradermal inoculation may enhance the efficacy of vaccines by promoting effective immune responses.

Antibody-mediated immunity appears to play a significant role in successful clearance of *E. chaffeensis* infection. Infection of SCID mice (B- and T-cell deficient) with *E. chaffeensis* results in an overwhelming infection [37,119,120]. Furthermore, mice lacking B cells or Fc γ RI are unable to resolve an ordinarily sublethal infection by IOE, and passive transfer of antibodies in these mice results in significant reduction of bacterial load [38]. Similarly, passive transfer of anti-*E. muris* antibodies, but not Fab fragments, also protect mice against lethal infection [39]. The specific anti-ehrlichial antibody-mediated mechanism is not fully understood, but appears to involve binding of antibody to the Fc receptor [38,121], and subsequent generation of a proinflammatory cytokine response [121] and oxidative defenses [38]. Conversely, the role of antibodies in immunity to *E. ruminantium* is less clear. Some studies suggest that antibodies are irrelevant [36,122], while others demonstrated the ability of antibodies to neutralize *E. ruminantium in vitro* [35]. Nevertheless, there are overwhelming data indicating that antibodies are important for protective immunity against *Ehrlichia*.

The relatively low bacterial burden in the blood and tissues in nonimmunocompromised patients with HME suggests that the pathogenesis of ehrlichiosis may involve immunopathologic responses that are manifest as a toxic shock-like syndrome [3,123]. The first murine model of fatal human ehrlichiosis has been instrumental in understanding the mechanisms behind the toxic shock-like syndrome of severe and fatal ehrlichiosis [65]. This model has recently been used to investigate the immunopathologic mechanisms involved in the development of severe monocytotropic ehrlichiosis. Mice inoculated with IOE develop histopathologic lesions resembling those observed in HME patients, and a similar disease course is observed in the IOE murine model. Lethal infections with IOE are accompanied by extremely high levels of serum TNF- α , a high frequency of TNF- α -producing CD8⁺ splenic T cells, decreased *Ehrlichia*-specific CD4⁺ T-lymphocyte proliferation, low IL-12 levels in the spleen, and a 40-fold decrease in the number of ehrlichial antigen-specific IFN- γ -producing CD4⁺ Th1 cells [67,124]. Furthermore, mice lacking TNF receptors I/II are more resistant to IOE-induced liver injury (an apparent effect of reduced immunopathology), but exhibit higher bacterial burdens (indicating reduced protective immunity) [68]. Others have also demonstrated immunopathologic responses linked to CD8 T cells [125]. Interestingly, fatal memory responses against homologous but not heterologous challenge are associated with decreased bacterial burden, enhanced inflammatory response in the liver, decreased T-cell responses and defective maintenance of IFN- γ -producing T cells [126]. CD1D-restricted natural killer T cells appear to be instrumental in the induction of immunopathologic responses [127]. Thus, the specter of adverse effects of the immune response is a potential obstacle to be avoided in ehrlichial vaccine development. The mechanisms that result in such immunopathologic responses following infection and challenge must be understood in order to identify protective vaccines that do not elicit undesired responses.

Molecular host–pathogen interactions, persistence & immune evasion

The obligately intracellular life of *Ehrlichia* spp. involves complex molecular interactions between the pathogen and host in order to ensure ehrlichial survival in the host cell and avoid innate and adaptive immune mechanisms [128]. Understanding these molecular host–pathogen interactions involved in immune evasion is necessary for rational development of effective vaccines and therapeutics. Yet there has been a lack of knowledge with regard to specific molecular host–pathogen interactions involved. Most *Ehrlichia* spp. exhibit tropism for phagocytes, and have evolved sophisticated molecular mechanisms to reprogram host cell defenses. Many host cell processes have been identified that are manipulated by *Ehrlichia*, including gene transcription, apoptosis, superoxide generation, lysosomal fusion, cell cycle

regulation and IFN- γ responsiveness [129-133]. However, the information that has been elusive is the pathogen effector proteins involved in reprogramming these host cell processes and the host cell targets.

The search for potential effector proteins has been facilitated by the recent completion of genome sequences of several *Ehrlichia* spp. [134-136]. *Ehrlichia* have small genomes, but several interesting genomic features associated with host-pathogen interactions have been identified. These include small families of genes that contain tandem and/or ankyrin repeats, a multigene family that encodes OMPs and a group of poly (G-C) tract (short sequence repeat) containing genes [135]. Furthermore, ehrlichial effector protein delivery mechanisms have been identified including components of the type 4 secretion system and a type 1 secretion system [134]. Three two-component systems that allow bacteria to sense signals and respond to changes in their environment through activation and repression of gene expression have been identified [134].

Many of the proteins associated with host-pathogen interactions are strongly recognized by the host immune response, as determined in studies with *E. canis* (and *E. chaffeensis*) including TRP19 (TRP32), TRP36 (TRP47), TRP140 (TRP120) and Ank200, suggesting that they may be immunoprotective. These proteins have been molecularly characterized and are high-priority targets for vaccine development, but information on their role in pathobiology has only recently emerged. Furthermore, information with respect to these protein orthologs and the immune response to *E. ruminantium* or their potential as immunoprotective vaccine candidates has lagged behind the major OMPs, MAP1 and MAP2.

A significant theme that underlies *Ehrlichia* survival and persistence is its ability to modulate host gene transcription. *E. chaffeensis* significantly alters the transcriptional levels of approximately 5% of the host genes within 24 h of infection [129]. Evading the innate immune response appears to be partly related to the lack of ehrlichial pathogen-associated molecular patterns (lipopolysaccharide and peptidoglycan) [137], but also by direct suppression of cytokines involved in innate and adaptive immune responses [129]. How the organism directs the cellular reprogramming strategy has remained an enigma. However, progress has recently been reported in studies describing novel molecular host-pathogen interactions with *E. chaffeensis* TRP47 involving host proteins involved in cell signaling, transcriptional regulatory mechanisms, vesicle trafficking and apoptosis [130]. Moreover, Ank200 is translocated to the nucleus where it binds *Alu* elements located in the promoter region of many genes associated with ehrlichial pathobiology including the IFN- γ signaling pathway (Jak/Stat) [138]. In addition, TRP120 has been associated with attachment and/or entry of *E. chaffeensis* [139]. However, the functions of some TRP and Ank proteins are only beginning to emerge, while the function of many others is still unknown. Studies to determine the immunoprotective ability of these virulence-associated proteins are needed to confirm that these host immune response-targeted effectors are protective.

Expert commentary

Ehrlichioses are significant veterinary diseases that cause rapid death in production livestock, hemorrhagic disease in companion animals and emerging life-threatening human zoonoses. In humans, ehrlichiosis is difficult to diagnose and can be fatal if not accurately diagnosed clinically and treated promptly and appropriately. Heartwater is widespread on the continent of Africa, and current methods of control are ineffective and/or expensive. Furthermore, there is only one effective antibiotic family, and development of antibiotic resistance could render treatment ineffective in the future. Vaccines are the most cost-effective means of preventing the ehrlichioses. Although there are still significant barriers to overcome, substantial scientific progress towards an effective vaccine has been made in the last 20 years with respect to

understanding the role of antigenic variation, developing relevant animal models, defining immunoprotective and immunopathologic mechanisms, identifying immunostimulating components of the organism and elucidating the molecular basis of infection.

Five-year view

The prospects for effective vaccines have been greatly enhanced as genome sequences have become available, molecular characterization of major immunoreactive proteins has been accomplished and animal models have been developed. In the next 5 years, new technologies, such as next-generation sequencing, will provide researchers with the capability to rapidly and fully explore ehrlichial heterogeneity in the field and examine pathogen gene expression in order to define the dynamics of pathogen phenotype in invertebrate and vertebrate hosts. Ultimately, new vaccine candidates will likely be identified through this exploration. New insights into immunoprotective and immunopathologic mechanisms and molecular pathogen–host interactions have provided encouraging signs of progress that have addressed key gaps in our knowledge that are required to make effective vaccines. It is likely that effective first-generation multisubunit vaccines containing molecularly characterized proteins will be developed and experimentally validated.

Key issues

- Vaccination is the most cost-effective long-term means of controlling the ehrlichioses, and economic factors are driving substantial commercial interest in developing vaccines for heartwater and canine ehrlichiosis. Protective immune responses have been demonstrated in response to infection and recombinant vaccines, indicating that developing a consistently effective vaccine is feasible.
- *Ehrlichia ruminantium* exhibits high genetic and antigenic diversity in contrast to *Ehrlichia canis* and *Ehrlichia chaffeensis*, which are more conserved. Defining antigenic heterogeneity and the mechanisms involved is important for developing effective vaccines for heartwater.
- Low-cost and immunologically well-characterized murine models have recently been developed that are defining protective and pathologic immune mechanisms and will be beneficial for validating the efficacy of vaccine candidates. Avoiding enhanced immunopathologic mechanisms must be considered in developing ehrlichial vaccines.
- Ehrlichiae exhibit different phenotypes in mammalian and arthropod host environments. Defining pathogen phenotype and mechanisms involved in differential protein expression in these hosts will enhance the prospects for vaccines.
- Many of the major immunoreactive proteins of *Ehrlichia* have recently been molecularly characterized and contain tandem or ankyrin repeats with major continuous species-specific antibody epitopes. At least two of these proteins (TRP47 and Ank200) are involved in complex molecular host interactions that may contribute to immune evasion mechanisms. In addition, these pathogen–host interactions could be blocked by the host immune response. Future investigations will determine their molecular roles and immunoprotective capability.

Acknowledgments

This manuscript summarizes the information provided by many investigators covering decades of scientific inquiry. Although all relevant scientific discoveries were not cited in this article, due to the limited space available, we dedicate

this article to all the investigators who have made important contributions towards the development of vaccines for the ehrlichioses.

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