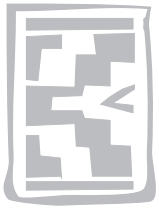

Persistence mechanisms in tick-borne diseases

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ABSTRACT

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The use of new, highly sensitive diagnostic methods has revealed persistent infections to be a common feature of different tick-borne diseases, such as babesiosis, anaplasmosis and heartwater. Antigenic variation can contribute to disease persistence through the continual elaboration of new surface structures, and we know in several instances how this is achieved. Known or suspected mechanisms of persistence in babesial parasites include cytoadhesion and rapid variation of the adhesive ligand in *Babesia bovis* and genetic diversity in several merozoite stage proteins of different *Babesia* spp. In *Anaplasma*, extensive variation in the pfam01617 gene family accompanies cycling of organism levels in chronic infection. One result from the pioneering research at Onderstepoort is the definition of a related polymorphic gene family that is likely involved in immunity against heartwater disease. We are beginning to understand the sizes of the antigenic repertoires and full definition is close, with the possibility of applying simultaneous high-throughput sequencing to the order of 1 000 small genomes. We also, for the first time, can consider modifying these genomes and looking at effects on persistence and virulence. However, important biological questions remain unanswered; for example, why we are seeing a new emerging *Anaplasma* infection of humans and is infection of endothelial cells by *Anaplasma* significant to persistence in vivo.

INTRODUCTION

Despite extensive investigation over the last 100 years, many vector-borne infectious diseases remain resistant to human solutions. Some of the most intransigent of these are infections that induce a 'carrier' state, where the animal remains infected, is still able to transmit organisms to the vector, but may show disease symptoms intermittently or not at all. Indeed, it may be difficult to detect the infection by classical means. Some examples are those infections caused by African trypanosomes, *Babesia* parasites, *Anaplasma* or *Borrelia* bacteria (Allred & Al-Khedery 2004; Horn 2004; Palmer, Futse *et al*. 2006; Bankhead & Chaconas 2007). In many cases, it is now evident through the use of sensitive molecular diagnostic methods that a typical infection course consists of numerous relapsing peaks of organisms separated by time periods when they are not detected (Burgdorfer & Mavros 1970; Kieser, Eriks *et al*. 1990; Calder, Reddy *et al*. 1996; Zheng, Sentsui *et al*. 1997; Granquist, Stuen *et al*. 2008). This has been known for many years with trypanosome infections but only more recently for some of the other infectious agents. Nevertheless, some molecular characteristics of these infections resemble those of trypanosomes, but with their own particular special features. I consider here our current understanding of some of these molecular characteristics.

DISCUSSION

Taking African trypanosomes as the original, most-studied paradigm, we know that the Variable Surface Glycoprotein (VSG) that covers the cytoplasmic

membrane is responsible for the ensuing battle with the host immune system and we have extensive information about its structure, variation and mechanisms of expression (Barry & McCulloch 2001; Vanhamme, Pays *et al*. 2001; Borst 2002; McCulloch 2004; Pays, Vanhamme *et al*. 2004). Despite data showing large differences in VSG structure as the trypanosome undergoes antigenic variation during infection, we know that VSG expression is not totally random and VSG repertoires are not entirely distinct in different places (Hutchinson, Picozzi *et al*. 2007). In fact, there are examples of shared variants between VSGs in East and West Africa and South America (Vervoort, Barbet *et al*. 1981; Magnus, Vervoort *et al*. 1982). Nevertheless, the repertoires are large (with > 1 500 genes in each trypanosome encoding VSGs) and largely divergent in different places and at different times (Barry, Crowe *et al*. 1983; Barry, Marcello *et al*. 2005; Berriman, Ghedin *et al*. 2005; Hutchinson, Picozzi *et al*. 2007). Moreover, the use of different segments of these >1500 genes to create novel trypanosome identities through recombination extends the repertoire size enormously (Roth, Bringaud *et al*. 1989; Kamper & Barbet 1992; Barbet & Kamper 1993; Marcello & Barry 2007) . This concept of antigenic repertoire size is useful in analyzing the epidemiology of many relapsing infectious diseases and the possibilities for control.

So, what do we know about tick-borne diseases important to animal production? *Babesia bovis* expresses a novel surface antigen, known as the Variant Erythrocyte Surface Antigen (VESA) 1, on the surface of infected erythrocytes (O'Connor, Lane *et al*. 1997). It is thought to mediate binding of parasitized erythrocytes to endothelial cells, thereby causing parasite sequestration in small capillaries (O'Connor, Long *et al*. 1999). This helps the parasite to avoid passage through the spleen and consequent removal. Sequestration in the microvasculature is an important feature of babesiosis and is often found in the dangerous cerebral form of the disease. VESA1 is strongly recognized during bovine infection, with variant-specific antibodies detected shortly after the respective parasitemic peaks containing the homologous organisms. In a recent study (Al-Khedery & Allred 2006), a genomic expression site for VESA1 was described which had acquired at least three different short-coding DNA segments from elsewhere in the genome, suggesting an analogous mechanism to amplify the VESA1 repertoire in *Babesia* parasites to that in trypanosomes. The VESA1 gene repertoire size in a single *Babesia* organism was estimated at > 350 (for one of the two VESA1 subunits) (Al-Khedery & Allred 2006), although genome sequence data put the repertoire size at closer to 150 copies (Brayton, Lau *et al*. 2007), without considering the effects of repertoire amplification by recombination. Therefore, in both trypanosomes and *Babesia,* which are protozoans with genome sizes of 35 Mb and 8.2 Mb, respectively, there are large, amplifiable repertoires of variant surface antigens.

One might expect the situation to be less complex in *Anaplasma* because of the smaller genome size of prokaryotes (1.2 Mb for *Anaplasma marginale*). In fact, this appears to be the case, with two variant outer membrane proteins (OMPs) identified, MSP2 and MSP3 (Barbet, Lundgren *et al*. 2000; Brayton, Meeus *et al*. 2003; Meeus, Brayton *et al*. 2003). Messenger RNA encoding these molecules is transcribed from single genomic expression sites to which approximately seven MSP2 pseudogenes and seven MSP3 pseudogenes contribute sequence by recombination (Brayton, Knowles *et al*. 2001). As with trypanosomes and *Babesia*, the variant repertoire is amplifiable in *A. marginale*, because of the use of different pseudogenes and pseudogene segments to create mosaic expression site sequences. Although this extends the repertoire of variant MSP2 and MSP3 enormously, the ultimate size is limited by the relatively small pool of donor pseudogenes. An important unknown question is how much this pool of donor sequences varies with time and location. We do know that many pseudogenes are shared by strains from diverse locations and that epidemic spread of the organism appears associated with the introduction of organisms carrying different MSP2 pseudogenes (Rodriguez, Palmer *et al*. 2005).

Anaplasma phagocytophilum is a related species *to A. marginale* which, although known for > 200 years as a pathogen of ruminants (cases in more than 300 000 Norwegian lambs annually) (Stuen 2007), is now causing severe infections in dogs and humans (Dumler, Choi *et al*. 2005; Lester, Breitschwerdt *et al*. 2005; Poitout, Shinozaki *et al*. 2005; Dumler, Barat *et al*. 2007; Jensen, Simon *et al*. 2007; Beall, Chandrashekar *et al*. 2008; Kohn, Galke *et al*. 2008). Interestingly, the *A. phagocytophilum* genome contains > 100 genes and pseudogenes homologous to MSP2 of *A. marginale* and likewise a single expression site to which they donate variant sequences (Barbet, Meeus *et al*. 2003; Wang, Rikihisa *et al*. 2004; Lin & Rikihisa 2005; Dunning Hotopp, Lin *et al*. 2006). As with *A. marginale*, the *A. phagocytophilum* ortholog (MSP2/P44) is immunoprotective against homologous variants (Palmer, Oberle *et al*.

1988; Wang, Kikuchi *et al*. 2006). Although the potential variant repertoire appears larger than *in A. marginale*, it is known that many similar variants are expressed in the multiple different animal species infected by *A. phagocytophilum* in the USA, possibly indicating a more recent introduction to the continent and less evolutionary diversification of the donor gene repertoire than in European strains (Barbet, Lundgren *et al*. 2006).

Of great significance to Africa is the tick-borne disease of heartwater and, here too, we now know much more of the potential variant OMP repertoire. This is, in large part, due to the pioneering research of the Onderstepoort Veterinary Institute which developed culture methods to grow *Ehrlichia* (formerly *Cowdria) ruminantium* (Bezuidenhout, Paterson *et al*. 1985) and then to obtain a complete genome sequence (Collins, Liebenberg *et al*. 2005). We are fortunate in this case to have more than one genome sequence, from both an African and a Caribbean strain (Frutos, Viari *et al*. 2006) of *E. ruminantium*. Although the ehrlichias express a related group of variable but immunoprotective (Nyika, Mahan *et al*. 1998; Nyika, Barbet *et al*. 2002; Yager, Bitsaktsis *et al*. 2005) OMPs to the MSP2s of the anaplasmas, they do not appear to have a similar system of antigenic variation that involves extensive recombination into a single genomic expression site. This is not to say that there are no antigenic differences in OMPs between strains and, in fact, breaks in protection against different strains have been a continual problem for development of vaccines against heartwater disease (Jongejan, Thielemans *et al*. 1991; Jongejan, Vogel *et al*. 1993; Mahan, Allsopp *et al*. 1999). The MSP2-related OMPs of *E. ruminantium* are encoded by a tandem group of 16 genes (Van Heerden, Collins *et al*. 2004) one of which appears to encode the major OMP expressed in mammals and a second the major OMP in ticks (Postigo, Taoufik *et al*. 2008). This leaves a question concerning the role of the remaining paralogs. In *Ehrlichia chaffeensis*, which has a similar genomic structure of MSP2-related paralogs to *E. ruminantium*, sensitive proteomic methods have detected expression of most paralogs despite the dominant expression of certain paralogs in ticks or mammals (Ge & Rikihisa 2007; Seo, Cheng *et al*. 2008). Analysis of RNA transcripts has suggested that the same may be true for *E. ruminantium* (Bekker, Postigo *et al*. 2005). It is also clear that both major and minor expressed paralogs are variant in different strains of *E. ruminantium* throughout Africa and the Caribbean (Reddy, Sulsona *et al*. 1996; Barbet, Byrom and Mahan, unpublished). Therefore, all paralogs could be involved in expression of the total antigenic identity of *E. ruminantium*, as they appear to be in *Anaplasma*.

In *A. marginale*, an interesting hypothesis has been proposed that the ability of strains to superinfect (and ultimately to cause epidemic spread and influence cross-protection by vaccines) depends on whether or not a superinfecting strain has a different donor pseudogene (for OMP expression) repertoire (Futse, Brayton *et al*. 2008). If it does, then the superinfecting strain can overcome pre-existing immunity developed over a long time period of infection in carrier animals. This is supported by data from the MSP2 pseudogene repertoire and, currently, the involvement of the other known variant OMP, MSP3, in this process is not clear. There are opportunities to test this theory in other systems. For example, despite the > 100 genes and pseudogenes potentially contributing to MSP2/P44 variants in *A. phagocytophilum*, there are two US strains, MRK and HZ, which differ in (presence or absence of) only two pseudogenes (Dunning Hotopp, Lin *et al*. 2006). One might predict heavy usage of these two pseudogenes in a superinfection of MRK-infected (long-term carrier) animals by HZ. If true for other tick-borne diseases, knowledge of the total repertoire of contributing variant genes to OMP diversity becomes of paramount importance to understand disease epidemiology and for vaccine development. Conceivably, this might be achieved for the smaller genome organisms using second and third generation genome-sequencing capable of obtaining, e.g. 1000 genome sequences of diverse *E. ruminantium* strains (Margulies, Egholm *et al*. 2005; Holt & Jones 2008).

Complementary to the new power of genome sequencing technology to evaluate variant repertoires in tick-borne diseases is the ability to introduce foreign DNA and investigate phenotypic effects. This has been achieved with *A. phagocytophilum* by transposon mutagenesis (Felsheim, Herron *et al*. 2006), but directed methods of gene knock-out in *A. marginale, Babesia* or *Ehrlichia* are still lacking, although standard in trypanosomes and other parasites and bacteria for many years. Potentially, these methods offer the ability to verify definitively the importance of OMP variation in persistent infections, as well as to use other approaches towards vaccines through deliberate attenuation. Despite the extensive work over the last 100 years, we are still discovering new and unforeseen aspects of these organisms, such as the ability to continuously culture *A. marginale*, thought to infect only erythrocytes, in endothelial cells (Munderloh, Lynch *et al*. 2004;

Wamsley & Barbet 2008). Therefore, although much is now known about these persistent tick-borne infections and there are good prospects for control through the application of new technology, we should recognize that there is much to be discovered about these fascinating organisms. The rigorous and critical application of the scientific method is as necessary now as it was in Theiler's day.

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