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The immunization-induced antibody response to the *Anaplasma marginale* major surface protein 2 and its association with protective immunity

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ABSTRACT

Many vector-borne pathogens evade clearance via rapid variation in their immunogenic surface expressed proteins. This is exemplified by *Anaplasma marginale*, a tick-borne bacterial pathogen that generates major surface protein 2 (Msp2) variants to provide for immune escape and allow long-term pathogen persistence. In contrast to persistence following infection, immunization with a surface protein complex, which includes Msp2, induces a response that prevents infection upon challenge. We hypothesized that the immune response induced by immunization altered the anti-Msp2 antibody repertoire as compared to that induced during infection, shifting the immune response toward conserved and thus broadly protective epitopes. The antibody response to the conserved (CR) and hypervariable (HVR) regions encoded by the full set of *msp2* variant alleles was determined for immunized animals prior to challenge and non-immunized, infected animals. While both groups of animals had a similar antibody repertoire in terms of breath and magnitude, the titers to the Msp2 CR were strongly correlated ($p < 0.005$) with control of bacteremia only in the infected animals. Among the immunized animals, there was no correlation between the breadth or magnitude of the anti-Msp2 antibody response and either complete protection from infection or control of bacteremia. This is consistent with separate immunologic mechanisms being responsible for control of bacteremia in infected animals as compared to immunized animals and suggests that conserved outer membrane proteins other than Msp2 are responsible for the complete clearance observed following challenge of vaccinees.

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1. Introduction

Infection with many vector-borne pathogens including *Theileria* spp., *Anaplasma* spp., *Babesia* spp., *Borrelia* spp., and *Plasmodium* spp. results in long-term persistent infection due to the pathogen's ability to evade the host immune response. This ability is in large part due to generation of outer membrane protein antigenic variants. For example, infection with *Anaplasma marginale*, a bacterial pathogen of cattle, generally results in life-long persistence in the mammalian host. Persistence is attributed primarily to rapid shifts in the surface coat structure and specifically variation in the highly immunogenic major surface protein 2 (Msp2). The expressed copy of Msp2 is composed of a central hypervariable region that is flanked by highly conserved regions (Fig. 1a and b). The variation is generated by gene conversion in which one of multiple *msp2* donor alleles is recombined into a single, operon-linked expression site [1–3]. The donor alleles have 5′ and 3′ regions which are identical to the expression site copy and flank a unique allele-specific hypervariable domain [1,4]. These donor alleles are termed functional pseudogenes as their 5′ and 3′ regions are truncated, they lack the function elements for *in situ* transcription, and are only expressed following recombination into the single expression site [1,4].

During infection, Msp2 represents dominant antigens recognized by sera from cattle infected with *A. marginale*. The anti-Msp2 specific antibody response is predominantly directed toward the hypervariable region rather than the flanking conserved regions [5,6]. However, the hypervariable region of newly emergent variants is not recognized by existing antibody [7,8]. Thus, generation of Msp2 variants allows for immune escape and long-term pathogen persistence [8,9]. In contrast to infection, where clearance does not occur, immunization with either purified *A. marginale* outer membranes or cross-linked outer membrane protein complexes induces complete protection against infection in 40–70% of vaccinees, and

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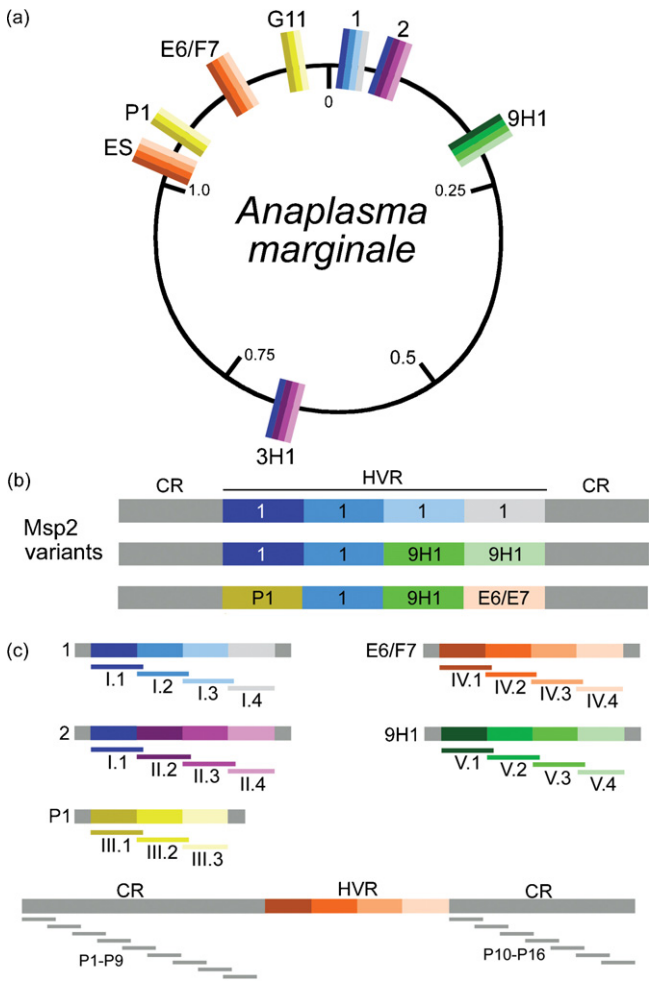

Fig. 1. The circular genome of the St. Maries strain of *A. marginale* (a) has one Msp2 expression site (ES), and five unique Msp2 pseudogenes which serve as donors for antigenic variation. Two of the Msp2 pseudogenes are duplicated (P1 and G11, 2 and 3H1) as indicated by matching colors. A linear depiction of the Msp2 expression site illustrates the source of variability within the Msp2 expression site (b). 30-mer peptides representing the CR and all possible pseudogene segments (c) were constructed and used to map the anti-Msp2 antibody response in this study.

protection against anemia and high-level bacteremia in nearly all animals [7,10,11]. Protection correlates with high IgG antibody titers against surface-exposed polypeptides, including Msp2 [7]. While protection associates with the IgG response to outer membrane proteins, the specific epitope targets and characteristics of this protective immune response remain unknown. In the experiments reported here we investigated the breadth and magnitude of the anti-Msp2 antibody response associated with the control of bacteremia in infection, and in the prevention of infection and control of bacteremia in immunized animals.

The first goal of these experiments was to determine if immunization altered the magnitude or epitope specificity of the anti-Msp2 responses as compared to infection; specifically whether immunization as compared to infection shifted the antibody response, in terms of the breadth or magnitude, toward the conserved regions of Msp2. This immunity against conserved region epitopes could prevent immune escape of new variants and result in the clearance observed following challenge of immunized animals but not during natural or experimental infection. The second goal of these experiments was to determine if the breadth or magnitude of the anti-Msp2 antibody response correlated with control of bacteremia in infected animals or prevention or control of bacteremia in immunized animals. To address these questions, animals were immunized with purified outer membranes or cross-linked surface proteins from the St. Maries strain of *A. marginale*, and the resulting specific antibody responses to the hypervariable (HVR) and conserved (CR) regions of Msp2 were mapped and titered. Vaccinees were then challenged with the homologous strain of *A. marginale*. Importantly, the St. Maries strain, for which the complete genome sequence is available, was used in these experiments, thus allowing mapping of the Msp2 expressed variants to their original donor pseudogene alleles, analysis of all possible combinations of the HVR, and comprehensive testing of the epitope specificity induced by immunization versus infection.

Table 1
Amino acid sequence of the peptides representing the conserved expression site domains of Msp2.

Locus	Peptides	Amino acid sequence
Expression site N-terminal domain	P1	MSAVSNRKLPLGGVLMALVAAVAPIHSLLA
	P2	AVAPIHSLLAAPAAGAGAGGEGLFSGAGAG
	P3	EGLFSGAGAGSFYIGLDYSPAFGSIKDFKV
	P4	AFGSIKDFKVQEAGGTTRGVFPYKRDAAGR
	P5	FPYKRDAAGRVDFKVHNFDWSAPEPKISFK
	P6	SAPEPKISFKDSMLTALEGSIGYSIGGARV
	P7	IGYSIGGARVEVEVGYERFVIKGGKKSNED
	P8	IKGGKKSNEDTASVFLLGKELAYDTARGQV
	P9	LAYDTARGQVDRLAAALGKMTKGEAKRWG
Expression site C-terminal domain	P10	VAGAFARAVEGAEVIEVRAIGSTSVMLNAC
	P11	GSTSVMLNACYDLLTDGIGVVPYACAGIGG
	P12	VPYACAGIGGNFVSVVDGHINPKFAYRVKA
	P13	NPKFAYRVKAGLSYALTPEISAFAGAFYHK
	P14	SAFAGAFYHKVLGDGDYDELPLSPISDYTG
	P15	PLSPISDYTGTAGKNKDTGIASFNFAYFGG
	P16	TAGKNKDTGIASFNFAYFGGELGVRFAF

2. Methods

2.1. Immunization and challenge

The immunization and challenge have been previously reported in detail [11]. Briefly, two groups of five calves each were immunized 5 times at 3-week intervals with approximately 35 μg of either *A. marginale* outer membranes or protein complexes suspended in 1 mg of saponin in a total volume of 1 ml administered subcutaneously. The third group of five calves was similarly immunized on the same schedule using only adjuvant. Four months after the last immunization, all calves were challenged intravenously with approximately 1×10^4 *A. marginale* (St. Maries strain) in 1 ml Hank's balanced salt solution. Starting 10 days post-challenge, the packed cell volume and bacteremia, as defined by the percent of infected erythrocytes, were determined daily for all the animals.

2.2. PCR to confirm infection status

PCR was used to confirm the lack of infection in the four challenged vaccinees that did not develop microscopically detectable bacteremia based on daily blood smear examination. DNA was isolated from whole blood using a Puregene DNA isolation kit (Qiagen, Valencia CA). Primers that specifically amplify *msp5*, a single copy gene, were used to detect *A. marginale*, as previously described in detail [12,13]. Amplification was performed in 50 μl volume with 35 cycles of melting at 94 °C for 15 s, annealing at 65 °C for 58 s, and extension for 71 s at 72 °C.

2.3. Measurement of segment specific antibodies

All conserved (Table 1) and hypervariable (Table 2) regions of Msp2 in the St. Maries strain were represented by the design and