Immune control of *Babesia bovis* infection

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Abstract

*Babesia bovis* causes an acute and often fatal infection in adult cattle, which if resolved, leads to a state of persistent infection in otherwise clinically healthy cattle. Persistently infected cattle are generally resistant to reinfection with related parasite strains, and this resistance in the face of infection is termed concomitant immunity. Young animals are generally more resistant than adults to *B. bovis* infection, which is dependent on the spleen. Despite the discovery of *B. bovis* over a century ago, there are still no safe and effective vaccines that protect cattle against this most virulent of babesial pathogens. Immunodominant antigens identified by serological reactivity and dominant T-cell antigens have failed to protect cattle against challenge. This review describes the innate and acquired immune mechanisms that define resistance in young calves and correlate with the development of concomitant immunity in older cattle following recovery from clinical disease. The first sections will discuss the innate immune responses by peripheral blood- and spleen-derived macrophages in cattle induced by *B. bovis* merozoites and their products that limit parasite replication, and comparison of natural killer cell responses in the spleens of young (resistant) and adult (susceptible) cattle. Later sections will describe a proteomic approach to discover novel antigens, especially those recognized by immune CD4+ T lymphocytes. Because immunodominant antigens have failed to stimulate protective immunity, identification of subdominant antigens may prove to be important for effective vaccines. Identification of CD4+ T-cell immunogenic proteins and their epitopes, together with the MHC class II restricting elements, now makes possible the development of MHC class II tetramers and application of this technology to both quantify antigen-specific lymphocytes during infection and discover novel antigenic epitopes. Finally, with the imminent completion of the *B. bovis* genome-sequencing project, strategies using combined genomic and proteomic approaches to identify novel vaccine candidates will be reviewed. The availability of an annotated *B. bovis* genome will, for the first time, enable identification of non-immunodominant proteins that may stimulate protective immunity.

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1. Introduction

*Babesia* are unique among the apicomplexan parasites because the majority of the species invade and replicate exclusively within erythrocytes, whereas related parasites in the genera *Plasmodium* and *Theileria* additionally infect nucleated cells. Babesiosis occurs primarily in tropical and semitropical areas, where the ixodid tick vectors, predominantly *Boophilus* spp., are found. All babesial parasites cause anemia, but *Babesia bovis* causes the most virulent disease resulting from sequestration of infected erythrocytes to microcapillary endothelia of vital organs and a hypotensive shock syndrome. *B. bovis* infection has a rapid onset, and primary infections are often fatal in susceptible cattle, typified by cerebral, renal, and pulmonary dysfunction or failure (Wright and Goodger, 1988; Wright et al., 1988; Clark and Jacobsen, 1998; Schetters and Eling, 1999). The severe pathogenesis is thought to be partially immune mediated, and overproduction of soluble mediators including IFN-γ, TNF-α, and nitric oxide (NO) that are associated with protective immunity against many intracellular pathogens has been implicated (Wright et al., 1988; Clark and Jacobsen, 1998). In support of this, Hemmer et al. (2000) demonstrated that TNF-α played a significant role in the pathogenesis of virulent WA-1 *Babesia* infection of TNF-α receptor deficient mice.

Cattle that do survive initial infection with *B. bovis*, either naturally or following chemotherapy, remain persistently infected and resistant to clinical disease. This immunity to clinical disease in the face of persistent infection is termed concomitant immunity. Because *Babesia* parasites only infect erythrocytes, the adaptive immune response to subsequent infection and protection against clinical disease is dependent on presentation of parasite antigens by antigen presenting cells to CD4+ T lymphocytes (reviewed in Brown and Palmer, 1999; Homer et al., 2000; Brown, 2001). Control of infection is likely mediated by destruction of infected erythrocytes by activated splenic macrophages (Brown and Palmer, 1999; Brown, 2001) and by neutralizing antibodies directed against extracellular merozoites and the infected erythrocyte surface variable erythrocyte surface antigens, defined as VESA1 (Allred et al., 1994, 2000). Both of these immune mechanisms depend on CD4+ T cells. The importance of the spleen in controlling infection has been demonstrated, as splenectomy results in recrudescence of parasitemia and clinical disease in persistently infected cattle (Wright and Goodger, 1988).

Interestingly, young calves are relatively resistant to developing the severe form of disease typically observed in susceptible adults upon initial infection with *B. bovis* (Trueman and Blight, 1978; Goff et al., 2001). This age-related resistance is not solely due to the protective effects of maternal antibody, as the duration of resistance exceeds that of passively transferred antibody and calves born in disease-free regions that were experimentally infected with the parasite were also resistant (Goff et al., 2001). Understanding the mechanisms of resistance in young animals to acute *B. bovis* infection (innate immunity) and of controlling parasitemia to persistent levels in adult cattle that survive infection (adaptive immunity), is critically important for devising strategies to induce a protective immune response by vaccination. This review will describe the innate and acquired immune mechanisms that define resistance in young calves and correlate with the development of concomitant immunity in older cattle immune to challenge. We describe the role of CD4+ T cells in macrophage-mediated inhibition of *B. bovis* replication and the immune response in the spleen of calves that are resistant to infection with the virulent Texas T2-Bo isolate of *B. bovis*. We then review strategies used to identify novel *B. bovis* protein antigens and the limitations of these strategies, which can now hopefully be overcome following the completion of the *B. bovis* sequencing project. A combined genomic and proteomic approach to identify novel vaccine antigens will enable the identification of subdominant antigens; those antigens against which little or no immune response is made during infection. Subdominant antigens may prove to be more effective as vaccine candidates than immunodominant antigens. Logically, for a parasite to survive, it would not be beneficial for it to express immunodominant antigens that would be targeted by a protective immune response, an idea that was put forth by Byron Waksman in the 1970s and referred to as the “Waksman Postulate” (Sher, 1988). A combined genomic and proteomic approach to antigen discovery, coupled with the ability to enumerate and track CD4+ T lymphocytes in vivo
following immunization by use of major histocompatibility class (MHC) II tetramers, will facilitate a better understanding of the reasons behind vaccine success or failure.

2. Cellular effector mechanisms of parasite clearance

The mechanisms of immunity to babesial parasites are hypothesized to require both innate and adaptive responses that include both CD4+ T cells and neutralizing antibody. Several reviews have described our model of protective immune mechanisms for B. bovis (Goff et al., 1998; Brown and Palmer, 1999; Brown, 2001). In this model, resolution of an acute infection in immunologically naïve animals infected with virulent B. bovis parasites depends on a sufficiently strong innate immune response that leads to activation of macrophages via IFN-γ and parasite derived products, and results in killing of the organisms by phagocytosis and production of toxic macrophage metabolites, including NO. In persistently infected animals that have controlled parasitemia, or in successfully immunized animals, antigen-specific CD4+ T cells are central to the adaptive immune response through production of IFN-γ. In addition to activating macrophages for efficient organism clearance, IFN-γ enhances production of the neutralizing IgG2 antibody (Estes and Brown, 2002), which when mixed with IgG1, was shown to passively protect cattle against homologous strain challenge (Mahoney, 1986). Thus, resolution of acute B. bovis infection in naïve animals is largely achieved by activation of the innate immune response in the spleen, whereas protection against clinical disease in persistently infected cattle (concomitant immunity) or vaccinated cattle relies upon rapid activation of memory and effector CD4+ T cells that secrete IFN-γ and provide help for the production of protective antibodies.

Studies performed in mice with the human and mouse babesial parasites B. microti and the WA-1 Babesia support this model. Briefly, studies with the virulent WA-1 babesial parasite using SCID mice and gene knockout mice on a genetic background of relatively resistant mouse strains, have shown that recovery from primary infection is independent of CD4+ T cells and antibody, but rather is dependent on macrophages and NK cells (Aguilar-Delfín et al., 2001, 2003). This innate response required production of type 1 cytokines IL-12 and IFN-γ. This importance of innate immunity in control of the virulent WA-1 parasite is similar to what we propose for virulent B. bovis infections of cattle. Experiments with the self-limiting B. microti in mice demonstrated the importance of CD4+ T cells in protective immunity. Partial protection against challenge was achieved by adoptive transfer of CD4+ T cells to immunodeficient mice (Hanafusa et al., 1998). Furthermore, studies using mice depleted of T-cell subsets or IFN-γ demonstrated that CD4+ T cells and IFN-γ, but not CD8+ T cells, were essential for resistance to challenge (Igarashi et al., 1999). A temporal study of cytokine responses during infection showed that recovery from infection correlated with early production of IFN-γ and IL-2, followed later by production of IgG that was associated with an IL-4 and IL-10 response (Chen et al., 2000).

2.1. Role of macrophage-derived NO in control of B. bovis infection

Soluble products from macrophages cultured in vitro inhibited B. bovis (Montealegre et al., 1985), and TNF-α was itself not directly babesiacidal (Tambrallo et al., 1992). The importance of NO in parasite growth inhibition was demonstrated in two separate studies where chemical donors of NO were shown to inhibit B. bovis replication in a dose-dependent manner, and parasites were observed to undergo degeneration and display “crisis forms” (Johnson et al., 1996; Shoda et al., 2000). To determine if the NO mediated killing had any relevance to innate immunity during infection, we examined whether B. bovis-infected erythrocytes, purified merozoites, or components of the parasite could elicit NO from bovine monocytes or monocyte-derived macrophages, and whether the levels of NO induced were sufficient to inhibit parasite growth. In the presence of IFN-γ, B. bovis merozoites or infected erythrocytes elicited NO production in the range of 10–40 μM (Shoda et al., 2000; Goff et al., 2002a). Furthermore, a merozoite membrane and organelle-enriched fraction (CM) was more effective than soluble parasite antigen (HSS) in inducing NO production and the CM fraction...
stimulated the production of NO in a dose-dependent manner (Stich et al., 1998). The NO response required co-stimulation with IFN-γ, but only small, biologically relevant (1 unit/ml) amounts were needed (Stich et al., 1998). Additional studies showed that a crude parasite lipid extract and purified B. bovis DNA, that contains unmethylated CpG motifs, also stimulated production of NO in the presence of IFN-γ (Shoda et al., 2000, 2001).

2.2. Production of inflammatory cytokines in response to B. bovis

In addition to NO, other products secreted by activated macrophages are important for activation of the innate and acquired immune response. These include IL-12, TNF-α, and IL-18. IL-12 activates natural killer (NK) cells to produce enhanced levels of IFN-γ and activated effector function, and additionally primes Th1 cells that produce IFN-γ upon antigenic stimulation and stimulates IFN-γ production by differentiated Th1 cells and γδ T cells (Brown et al., 1996a). TNF-α, in concert with IFN-γ, activates NO production by macrophages (Adler et al., 1994; Goff et al., 1998). IL-18 also acts synergistically with IL-12 to promote IFN-γ production (Shoda et al., 1999). TNF-α transcript levels and biologically active TNF-α were significantly elevated in macrophages stimulated with B. bovis-infected erythrocytes (Shoda et al., 2000). Similarly, IL-1β transcripts and IL-12 p35 and p40 transcripts and IL-12 biological activity were upregulated in macrophages exposed to B. bovis-infected erythrocytes (Shoda et al., 2000). We were unable to measure an effect on IL-18, which is post-translationally modified.

2.3. Induction of B. bovis growth-inhibitory NO by B. bovis-infected erythrocytes

The next question we asked was whether culturing macrophages in vitro with B. bovis-infected erythrocytes could inhibit parasite replication under conditions where parasites and macrophages were either in contact, permitting phagocytosis, or not in contact. Using contact conditions, replication of B. bovis measured by tritiated hypoxanthine incorporation was inhibited by 57–89%, and this inhibition was only partially dependent on NO and independent of exogenous IFN-γ (Shoda et al., 2000). Similarly exposed parasites were also visibly seen to undergo death (crisis forms) within erythrocytes (Goff et al., 1998). When macrophages were separated from infected erythrocytes by a membrane, parasite replication was inhibited by 16–35% in two experiments, and the effect was again partially, but significantly blocked by inhibitors of NO. Thus, these studies suggest that B. bovis does stimulate the production of NO that is partially responsible for its own growth inhibition in vitro. However, additional macrophage products that have not been identified must also contribute to the babesioidal effect. Years ago, Levy et al. (1982) showed that blood from young animals contained a soluble factor that caused inhibition of parasite growth in vitro. This factor cannot be NO, which has a very short half-life.

2.4. Role of antigen-specific CD4+ T cells in macrophage activation

To investigate the role of antigen-specific CD4+ T cells in activation of macrophages to kill B. bovis, in vitro studies were performed, as complete depletion of specific T cells or cytokines in vivo has proven problematic (Valdez et al., 2001). CD4+ T-cell lines were established from cattle immune to B. bovis following infection and treatment (Brown et al., 1993). These cell lines secreted IFN-γ and TNF-α in response to B. bovis CM and HSS antigens (Brown et al., 1993; Stich et al., 1998). Supernatants from these cell lines that contained significant levels of IFN-γ and TNF-α stimulated macrophages to produce NO, and the NO response correlated with cytokine levels in the supernatants (Stich et al., 1998). However, the NO response to T-cell culture supernatants was significantly greater than the response to seven-fold higher levels of IFN-γ alone, suggesting that additional cytokines or parasite products from CM or HSS used to stimulate the T cells contributed to macrophage activation.

3. Age-related resistance to infection with B. bovis

Cattle less than 6 months of age are resistant to clinical disease following exposure to B. bovis
(Truman and Blight, 1978; Levy et al., 1982). This age-related resistance is somewhat counter-intuitive, as in general the innate immune system of infants is less developed than that of adults (Johnston, 1998; Petty and Hunt, 1998; Reen, 1998; Suen et al., 1998). Possible explanations for the increased resistance of young animals is the abundance of γδ T cells, which in ruminants can comprise over 70% of circulating T lymphocytes (Hein and Mackay, 1991), or a decreased pro-inflammatory response that has been shown in other hemoparasite infections, such as malaria, to contribute to the disease pathogenesis (Clark and Jacobsen, 1998). Furthermore, the spleen is particularly important in controlling infection as splenomegaly occurs during acute babesiosis, and splenectomized adult and young animals experience much higher levels of parasitemia (Wright and Goodger, 1988; Goff et al., 2001).

3.2. Identification of splenic cells involved in the innate immune response to B. bovis

It was demonstrated earlier that splenic monocytes stimulated ex vivo with B. bovis plus IFN-γ expressed NO (Goff et al., 2002a). However, it was also of interest to determine which cells produce IFN-γ in the spleen, as αβ and γδ T lymphocytes, as well as NK cells and some types of dendritic cells, can all produce this cytokine. In the first set of experiments, spleen cells from calves taken pre-infection or at the peak of infection with B. bovis were gated for activated cells stained with monoclonal antibodies specific for CD2, CD3, CD8, and the γδ TCR (Goff et al., 2003). The predominant population of cells that were activated following infection was CD2+ CD8+ cells, which is the phenotype of bovine NK cells (Storset et al., 2004). In contrast, the percentage of activated CD3+ αβ and γδ T cells did not appreciably change following infection. Although the NK-like cells were not stained for intracellular IFN-γ in this experiment, the activation of NK-like cells in the spleen was coincident with peak plasma levels of IFN-γ (Goff et al., 2003). We have recently confirmed that splenic CD2+ CD8+ cells express the NK cell marker NK-p46 (Goff et al., 2006), that splenic NK cells produce IFN-γ in the presence of IL-12 and IL-18 and in the presence of B. bovis-exposed mononuclear phagocytes (Goff et al., 2006), and in vivo studies are in progress to show that these cells are the source of IFN-γ during B. bovis infection of calves.
immune response to *B. bovis* infection, a rational strategy to identify and select candidate proteins for use in vaccination was to select those that stimulated a strong CD4+ IFN-γ secreting effector/memory T-cell response. Additional attributes of vaccine antigens are the ability to stimulate an anamnestic response upon exposure to the parasite, the presence of epitopes conserved among different parasite strains, the presence of surface-exposed epitopes that could be targeted by neutralizing antibody, and the ability of the antigen to be recognized broadly in the population of outbred cattle (Brown and Rice-Ficht, 1994; Brown and Palmer, 1999). Because serologically immuno-dominant antigens were not shown to be protective in vaccine trials, whereas weakly reactive antigens of low abundance were reported to be protective (Wright et al., 1992; Hines et al., 1995), we elected to select *B. bovis* antigens that elicited recall CD4+ T-cell proliferative and IFN-γ responses, rather than those that were serologically dominant. Furthermore, we chose those antigens that elicited recall T-cell responses from multiple animals that expressed different genetic backgrounds and MHC class II haplotypes (Brown et al., 1995; Stich et al., 1999; Brown and Palmer, 1999). Briefly, cattle immune to *B. bovis* (Mexico or Texas strains) following infection and treatment with Berenil were used as a source of CD4+ T lymphocytes. Short-term CD4+ T-cell lines and treatment with Berenil were used as a source of bovis (Mexico or Texas strains) following infection haplotypes (Brown et al., 1995; Stich et al., 1999; different genetic backgrounds and MHC class II responses from multiple animals that expressed those antigens that elicited recall T-cell that were serologically dominant. Furthermore, we chose those antigens that elicited recall T-cell responses from multiple animals that expressed different genetic backgrounds and MHC class II haplotypes (Brown et al., 1995; Stich et al., 1999; Brown and Palmer, 1999). Briefly, cattle immune to *B. bovis* (Mexico or Texas strains) following infection and treatment with Berenil were used as a source of CD4+ T lymphocytes. Short-term CD4+ T-cell lines were established from these animals by stimulation with merozoite extracts, and tested for proliferation to soluble parasite antigens subjected to size based fractionation by continuous flow electrophoresis (Brown et al., 1995; Stich et al., 1999). Immunostimulatory fractions were shown to contain known immunogenic proteins, such as rhoptry associated protein-1 (RAP-1), by immunoblotting with specific monoclonal antibodies, providing proof of concept (Stich et al., 1999). Fractions with peak stimulatory activity that were recognized by the majority of immune animals were selected and used to immunize rabbits for production of monospecific immune sera. The sera were then used to screen a *B. bovis* expression library, and 14 clones were expressed and tested for T-lymphocyte recognition. This approach, which was very labor-intensive, identified several novel immunostimulatory antigens including a 20-kDa member of the small heat shock protein (hsp) family (Hsp20; Brown et al., 2001b), a ribosomal phosphoprotein P0, and a long-chain fatty acyl coA synthetase (ACS) (Norimine et al., 2006). These three proteins were interesting because homologous proteins in other protozoa are considered vaccine or chemotherapeutic targets. A related *Toxoplasma gondii* small hsp (Hsp30/bag1) was reported to induce protection in a mouse immunization model (Mun et al., 1999; Mohamed et al., 2003) and antibodies raised against the ribosomal phosphoprotein P0 of *Plasmodium yoelii* conferred passive protection against parasite challenge in mice (Chatterjee et al., 2000). Because ACS enzymes are essential for parasite survival, and differ in structure from the mammalian enzymes, these may be useful chemotherapeutic targets (Matesanz et al., 1999).

The well-characterized immunostimulatory rhoptry protein RAP-1, and the novel Hsp20 were tested as vaccine antigens using RIBI adjuvant combined with IL-12. IL-12 was shown to promote strong type 1 IFN-γ biased responses in cattle (Tuo et al., 2000; Zhang et al., 2003). These antigens were selected because RAP-1 was reportedly surface expressed, and monoclonal antibodies specific for RAP-1 prevented binding of merozoites and sporozoites to erythrocytes (Yokoyama et al., 2002; Mosqueda et al., 2002), and the related *T. gondii* hsp30 was protective (Mun et al., 1999; Mohamed et al., 2003). Furthermore, both antigens were shown to be rich in CD4+ T-cell epitopes and elicited strong proliferative and IFN-γ responses in *B. bovis*-immune cattle (Brown et al., 1996b, 2001b; Norimine et al., 2002). All vaccinated animals developed high titers of *B. bovis*-specific IgG1 and IgG2 and strong, CD4+ T-lymphocyte recall responses to the respective immunogen and to *B. bovis* merozoites, including proliferation and IFN-γ secretion (Norimine et al., 2003, 2004, and unpublished observations). However, there was no protection against challenge 5 months later with the virulent Texas T2Bo strain in either group of vaccinates. At the peak of infection, all cattle required chemotherapy. The reasons for this vaccine failure are not known, but this total lack of protection in spite of strong cellular and humor immunity illustrates the complexity of developing vaccines for hemoproteozoa. One possibility is that challenge stimulated a population of T-regulatory cells that suppressed the pre-existing immune response to *B. bovis*. T-regulatory cells have been identified that downregulate CD4+ T-cell
responses in other protozoal parasite infections (Belkaid et al., 2002; McGuirk and Mills, 2002). However, T lymphocytes obtained from RAP-1 vaccinates after the resolution of disease responded normally to RAP-1, and recognized the majority of epitopes seen prior to infection, indicating that the response was intact (Norimine et al., 2003). Another possibility is that even though we were easily able to elicit strong proliferative and IFN-γ responses from short-term T-cell lines expanded for 2 weeks in vitro from PBMC obtained post-vaccination (Norimine et al., 2003, 2004), the number of antigen-specific T cells may have been insufficient to stimulate rapid anamnestic responses needed for protection. Use of assays that enumerate antigen-specific T cells in PBMC such as the IFN-γ ELISPOT (Abbott et al., 2004) or MHC class II tetramer staining (Klenerman et al., 2002; Mallone and Nepom, 2004) will facilitate accurate counting of antigen-specific T cells. It is also quite possible that a single antigen is insufficient to stimulate protection, or that these antigens were not appropriate ones to use. Although our selection strategy avoided the use of serologically immunodominant antigens, RAP-1 and Hsp20 may in fact be relatively immunodominant for CD4+ T cells, as these proteins were frequently stimulatory for T cells from B. bovis-immune cattle (Brown et al., 1996b, 2001b; Norimine et al., 2002). Thus, alternative approaches are needed that can more accurately measure the cellular immune response in vivo following immunization and challenge, and can identify subdominant protein antigens, which in other systems, have been shown to be superior to immunodominant proteins for invoking protective immunity (Oukka et al., 1996; van der Most et al., 1996; Gallimore et al., 1998a,b).

5. Enumeration of antigen- or epitope-specific T cells in vivo

T lymphocytes specific for a given protein antigen or epitope can be enumerated using intracellular cytokine staining and flow cytometry, ELISPOT assays, or by staining with MHC tetramers. Such approaches are very valuable when measuring antigen-specific T-cell responses directly from the animal, often without the need for in vitro expansion of the cells of interest. Using an IFN-γ ELISPOT assay, peptide epitopes of the major surface protein 2 (MSP2) of the rickettsial pathogen, Anaplasma marginale, were defined using either freshly harvested or cryopreserved PBMC (Abbott et al., 2004, 2005). The identity of the cells secreting IFN-γ as CD4+ T cells was determined by depleting specific cell subsets with monoclonal antibody-coated magnetic beads. In our experience, the assay could detect a minimum of 50 peptide-specific IFN-γ secreting cells per million lymphocytes, which is dependent on the variation among replicate cultures and the background levels of IFN-γ secreting cells (Abbott et al., 2004). This assay can be adapted to measure any secreted T-cell product, if appropriate pairs of high-affinity monoclonal antibodies are available. However, when the number of responding cells is limited, or the cells do not secrete the cytokine of interest, other approaches are required.

5.1. MHC class I and class II tetramers

The recent development of MHC tetramers to enumerate and track T cells during infection or autoimmune disease, or following immunization against tumors or infectious agents has greatly advanced our understanding of T-cell function following immunization or during disease (Klenerman et al., 2002; Mallone and Nepom, 2004). During infection, not only is the magnitude of the T-cell response important, but also the rapidity of the response can be critical, especially when dealing with virulent pathogens such as B. bovis that can cause acute disease by 9 days post-challenge. MHC class II tetramers can be used to track the development of antigen-specific T cells in vivo in immunized animals before and following challenge, and thus should be invaluable for understanding the reasons for vaccine success or failure. MHC tetramers consist of either soluble MHC class I and β-2 microglobulin or MHC class II DR or DQ α and β chains, linked by leucine zippers and exogenously loaded with a specific antigenic peptide epitope of interest. The dimers are biotinylated so that coupling these to strepavidin forms the tetrameric complex. The complex is labeled with a fluorochrome, such as phycoerythrin, so that the tetramers can be used in flow cytometry to stain T cells whose receptors specifically bind the peptide antigen of interest. MHC class I tetramers have been used to track the CD8+
T-cell response of humans and mice following viral infection or immunization (Klenerman et al., 2002). MHC class II tetramers have been used more recently in humans to enumerate antigen-specific CD4+ T-cell responses following infection with a variety of pathogens, such as Epstein-Barr virus (EBV) and Borrelia burgdorferi (Amyes et al., 2003; Meyer et al., 2000). Whereas the frequency of viral epitope-specific CD8+ cytotoxic T lymphocytes is relatively high, that of MHC class II restricted CD4+ T cells is relatively low, and was reported to be in the range of <0.005 to 0.1% of circulating PBMC in the Lyme disease study (Meyer et al., 2000), and 0.04–5.2% of CD4+ T cells in the EBV study (Amyes et al., 2003). Importantly, the use of MHC tetramers has facilitated a comparison of epitope-specific T cells with functional T cells that secrete cytokines, determined by intracellular cytokine staining or ELISPOT assay (Klenerman et al., 2002; Mallone and Nepom, 2004). For construction of effective tetramers, high avidity and immunodominant T-cell epitopes must be used to obtain efficient T-cell binding.

To develop the technology for constructing MHC class II tetramers for use in enumerating and tracking bovine CD4+ T-cell responses following B. bovis infection or immunization and challenge, we have made use of several immunodominant T-cell epitopes identified in the conserved regions of B. bovis RAP-1 and Hsp20 proteins. Several epitopes under investigation are recognized by CD4+ T cells from B. bovis-immune cattle that express common MHC class II haplotypes (Norimine et al., 2002, 2003, 2004), so that in future experiments the haplotype of the animals could be selected with logical prediction of a response to a given epitope. Furthermore, the MHC restriction elements must be determined for each epitope of interest, as both DR and DQ molecules present epitopes to bovine CD4+ T cells (Glass et al., 2000; Brown et al., 2001a, 2002, 2003; Norimine et al., 2002, 2004) and duplication in DQ alleles with documented inter-haplotype pairing of DQA and DQB chains suggests a potentially large number of DQA and DQB pairs can present an epitope (Glass et al., 2000; Brown et al., 2002; Norimine and Brown, 2005). The precise pairs of DRA–DRB3 or DQA–DQB chains that present a known immunodominant epitope have been determined for several B. bovis and A. marginale proteins using human embryonic kidney cells transfected with cDNAs encoding these proteins to present peptide antigen to specific CD4+ T cells (Norimine and Brown, 2005). We are in the process of developing tetramers using these well-defined class II molecules and the peptide epitopes that they present. Hopefully, these technologies will allow us to better understand the dynamics of the effector and memory CD4+ T-cell response to B. bovis following immunization with specific antigen or experimental infection.

MHC class II tetramers have also been used to map T-cell epitopes on large, complex immunogenic proteins (Novak et al., 2001; Reijonen and Kwok, 2003). This technology has been designated tetramer-guided epitope mapping, and requires knowledge of an immunogenic protein and the MHC haplotype of the individual. The advantage of this approach over conventional epitope mapping strategies is that the assay does not rely upon function (i.e. proliferation or IFN-γ secretion) to identify the epitope of interest, but rather relies on epitope–T-cell receptor interaction. This approach can identify subdominant as well as immunodominant epitopes. Tetramers are constructed with pools of 5–10 overlapping peptides that span a given protein and that are exogenously loaded onto soluble MHC tetramers. These pooled peptide tetramers are then used to stain T cells from an individual using short-term (2 weeks) T-cell lines generated against the whole protein of interest. When a pool tests positive, individual tetramers are then tested by cell staining to identify the immunostimulatory peptide epitope. Additionally, individual T cells can be cloned, expanded, and tested for specificity to the epitope used to isolate the clone.

6. Combined genomic and proteomic approach to identify novel and subdominant B. bovis antigens

Matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry is a powerful technique that can be used to identify membrane proteins or other proteins of a given organism (Molloy et al., 2000; Montigiani et al., 2002; Piubelli et al., 2002). Antigens from the pathogen are subjected to some form of fractionation and functional analysis, such as continuous flow electrophoresis and testing with T cells, or two-dimensional gel electrophoresis.
and screening with immune sera. Selected stimulatory fractions or excised protein spots are then subjected to trypsin digestion, MALDI-TOF mass spectrometry, and in silico analysis of tryptic peptides to identify the protein, if the genome sequence is known. Additionally, tandem mass spectrometry can be used to analyze a tryptic peptide of interest to obtain more specific peptide sequence information, which increases the specificity of correctly identifying the antigen of interest (Figey et al., 2001).

In conjunction with the Institute for Genome Research (TIGR), the USDA-ARS Animal Disease Research Unit in Pullman, WA, and the Washington State University Department of Veterinary Microbiology and Pathology have nearly completed sequencing and annotation of the genome of the highly virulent T2Bo Texas isolate of *B. bovis*. Completion of this genome project will greatly facilitate identification of novel antigens, including those that are immunologically subdominant, and which may be useful vaccine candidates according to the Waksman Postulate (Sher, 1988). The approach that will be used is to combine our previous proteomic approach of fractionating merozoite antigen and testing immunostimulatory fractions (Brown et al., 1995; Stich et al., 1999) with mass spectrometric analysis of stimulatory protein fractions to identify the proteins present in the fraction based on the genome sequence. We can additionally identify proteins by two-dimensional gel electrophoresis and screening with immune bovine sera or purified IgG2 (Barigye et al., 2004) to identify type 1 T-cell-dependent proteins, and subject individual spots to liquid chromatography fractionation and tandem mass spectrometry (Tang et al., 2005). This latter approach was feasible because sequencing and annotation of the genome of *A. marginale* was recently completed (Brayton et al., 2005). We successfully identified over 60 antigenic membrane or membrane-associated proteins of this bovine pathogen (Lopez et al., 2005). Furthermore, a genome search for genes that encode proteins with protective function in other protozoa or for specific proteins, such as merozoite outer membrane proteins, using algorithm prediction programs, can be used to identify other potentially interesting vaccine antigens. The strength of this approach is that it combines functional assays (T-cell recognition or antibody-binding) and identification of the functional antigens using the genome sequence information with genome analysis alone to identify additional putative vaccine antigens. These proteins can be systematically expressed and tested for T-cell immunogenicity or antibody reactivity. Thus, the ability to detect functional, subdominant antigens will be increased by making use of genomic information for *B. bovis*.

7. Concluding remarks: immune response versus pathogenesis

Developing protective non-living vaccines against complex protozoan pathogens is extremely difficult and complicated by the heterogeneity of the MHC in those non-rodent human and veterinary species for which vaccines are desperately needed. Although logic dictates that stimulation of the predicted protective immune response (type 1 cytokine and IgG2) using immunodominant vaccine antigens that contain conserved T-cell epitopes should prove somewhat efficacious against challenge, our experience with such vaccines for *B. bovis* has proven otherwise (Norimine et al., 2003). The availability of the genome sequence will undoubtedly increase the number of antigens that can be tested for generation of long-lived memory T-cell responses that may contribute to protective immunity against this very virulent babesial parasite. Furthermore, developing improved techniques for quantifying antigen-specific T cells in vivo following immunization or infection will also provide informative knowledge of the cellular events that occur in the host leading to protection or susceptibility to infection. However, it may be that with a pathogen as virulent as *B. bovis*, strategies other than generating conventional protein antigen-based vaccines will be needed to prevent the severe pathology associated with primary infection of naïve and susceptible animals from killing the host before the requisite memory T cell and antibody responses are expanded to sufficient levels to retard parasite replication and disease. One strategy would be to target the antigenically variant erythrocyte surface VESA1 antigens responsible for adhesion to the microcapillary endothelia (Allred et al., 1994, 2000; O’Connor and Allred, 2000; Allred and Al-Khedery, 2004). Conserved T-cell epitopes on these proteins could be used, possibly with a cocktail of known variable epitopes involved in adhesion. This
approach could, at least, accelerate development of antibody responses against novel, variant B-cell epitopes by priming T helper cells against conserved regions of the protein. At best, this approach could outsmart antigenic variation by the pathogen by priming T- and B-cell responses against possible VESA1 variants as well. Identity of the sequences of all of the ves genes and pseudogenes in B. bovis genome would facilitate such an approach, unless the proposed mechanism of antigenic variation by segmental gene conversion is correct (Allred and Al-Khedery, 2004). In this case, it may be impossible to predict the sequence composition of all potential variant epitopes generated by segmental gene conversion of a large number of pseudogenes and short gene segments into a single expression site.

A second strategy would be to vaccinate against parasite products that are not necessarily protein in nature, but that cause pathology by presumably activating macrophages and other leukocytes to secrete high levels of inflammatory mediators that promote parasite-infected erythrocyte cytoadherence and other toxic sequellae (Clark and Jacobsen, 1998; Scragg et al., 1999). Such “anti-disease” vaccines have proven efficacious against mouse malaria (Taverne et al., 1990) and B. canis infection in dogs (Schetters, 2005). It is likely that a protective vaccine against virulent B. bovis infection will need to target multiple antigens, including those related to virulence.

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