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Babesia bovis-specific CD4+ T cell clones from immune cattle express either the Th0 or Th1 profile of cytokines

BROWN (W.C.), ZHAO (S.), WOODS (V.M.), DOBBELAERE (D.A.E.), RICE-FICHT (A.C.). Des clones de cellules T CD4+ spécifiques pour *Babesia bovis*, de bovins immunisés, expriment le profil de cytokines des cellules Th0 ou des Th1. *Revue Elev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 65-69

Le rôle central des cellules T dans la réponse immunitaire contre les hémoprotozooaires, aussi bien comme cellules "helper" pour la production d'anticorps sous dépendance de cellules T que comme cellules effectrices agissant directement ou indirectement sur les parasites intracellulaires par l'élaboration de cytokines, a conduit les auteurs à examiner la réponse immunitaire cellulaire chez les bovins aux antigènes de *Babesia bovis*. Des clones de cellules T produits à partir de quatre bovins immunisés contre *Babesia bovis* par stimulation *in vitro* par des antigènes solubles ou associés à la membrane des mérozoïtes, ont été caractérisés en ce qui concerne leur réactivité contre divers antigènes et des isolats de *B. bovis* et de *B. bigemina* d'origine géographique différente. Les clones ont été catégorisés dans sept groupes différents basés sur les divers types de réactivité. Ce tableau de clones de cellules T, ainsi que des clones additionnels spécifiques pour la protéine de 77 kDa associée au complexe apical des mérozoïtes (Bb-1) ou la protéine majeure de 42 kDa (MSA-1), ont été analysés pour des cytokines. Des tests biologiques pour mesurer IL-2/IL-4, IFN-γ et TNF-α/TNF-ß, et l'analyse par northern blot pour la détection d'ARN messager codant pour IL-2, IL-4, IFN-γ, TNF-ß and TNF-α bovins, ont montré la production différentielle de cytokines par des clones ayant des spécificités antigéniques différentes. Deux clones de cellules T spécifiques pour Bb-1 ont produit le profil de cytokines de Th1: IL-2, IFN-γ, TNF-ß et TNF-α, mais non IL-4. Des clones spécifiques pour la protéine 42 kDa produisaient des taux indétectables de tous les cytokines, mais ont exprimé un profil non restreint ou Th0 de cytokine ARN messager pour cytokines : IL-2, IL-4, IFN-γ, and TNF-α. Finalement, la majorité des clones Th réagissant avec des antigènes de mérozoïtes non définis exprimaient le profil Th0 de cytokines, et plusieurs clones avaient le phénotype Th1, tandis qu'aucun des clones n'a exprimé un profil de cytokines de Th2. Etant donné que dans d'autres infections à protozoaires, les cellules Th1, l'IFN-γ, le TNF-α, et le TNF-ß, mais non pas l'IL-4, sont associés au développement d'une immunité protectrice, le Bb-1 est un candidat logique pour un vaccin contre *B. bovis*.

Mots clés : Babesia bovis - Cellule T - Clone - Antigène - Immunité - Réponse immunitaire.

Babesia bovis causes a virulent form of babesiosis, characterized by fever, anaemia, anorexia, cachexia, low parasitemia and a generalized circulatory disturbance, often resulting in high mortality rates among non-immune cattle. Characteristic of this disease is the sequestration of parasitized erythrocytes in the capillary beds of the brain and lung, leading to cerebral babesiosis and respiratory distress syndrome (13). Similarity in the immunopathology caused by this and related malarial parasites has led to the hypothesis that the diseases caused by these hemoparasites share common mechanisms. Cytokines, including gamma interferon (IFN-γ) and tumor necrosis factor (TNF) released by parasite antigen-activated T cells and macrophages are implicated in anaemia, cytoadherence of parasitized erythocytes to the brain microcapillary endothelia and accumulation of infected erythrocytes and neutrophils in the pulmonary vasculature.

Protective immunity in experimental murine malaria and babesial infections is mediated by T cells and macrophages, and the same cytokines involved in immunopathology play a role in immunity. Although the T helper cell (Th) subsets that are involved in protective immunity to the intraerythrocytic stages of these parasites have not been conclusively identified, it has been suggested that both subsets of Th1 cells appear to be involved in protective immunity against this stage of malarial parasites, with Th1 cells appearing early and Th2 cells appearing late in the infection. IFN-γ and lymphotoxin, or TNF-ß, produced by both CD4+ T helper 1 (Th1) and CD8+ T cells are important for the resolution of hemoparasite infections. These cytokines inhibit parasites by direct toxic effects on the intraerythrocytic parasite, by inhibition of the intrahepatocytic development of exoerythrocytic stages, and through the activation of macrophages and neutrophils, resulting in enhanced phagocytosis of parasitized erythrocytes and the production of TNFα, which itself has antiparasitic properties *in vivo*. Th2 cells, through the elaboration of B cell growth and differentiation factors IL-4 and IL-5, probably function in maintaining an anti-parasitic antibody titer once the initial infection has been controlled. In other protozoal infections, the role of Th2 cells is not benign. In experimental leishmania infection for example, Th1 cells producing IFN-γ conferred protection, whereas Th2 cells, producing IL-4, exacerbated disease (10).

Identification of parasite antigens which evoke immunity and/or immunopathology during the course of hemopara-

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sitic infection is essential for vaccine development. Optimally, a vaccine would include proteins with both T and B cell epitopes that induce anamnestic cellular and humoral immunity upon natural exposure to the parasite. However, very little is known about the nature of either protective babesial antigens or the immune responses in cattle that they evoke. In studies with *B. bovis*, attempts to characterize protective antigens have historically relied upon the use of antibodies to identify immunodominant proteins. Although antibody may play a role in merozoite neutralization (8), it has become increasingly evident that serologically immunodominant antigens are not always protective against a challenge *B. bovis* infection; in some studies protective immunity to *B. bovis* was inversely related to antibody titer (13). These observations, together with the documented relevance of T cells and macrophages in both immunity and immunopathology associated with infection by related hemoparasites, have prompted a detailed investigation of cell-mediated immune responses in *B. bovis*-infected cattle. Because *B. bovis* has no known exoerythrocytic stage which could serve as a target for MHC-restricted cytotoxic T cells, this research has focused on the identification of babesial merozoite antigens which induce Th cell responses in immune cattle, and characterization of the cytokines produced by *Babesia*-specific Th cells. In addition, the authors were interested to determine if discreet subsets of Th cells, identified by distinct cytokine profiles, are present in ruminants as has been described for mouse and man.

Initial studies performed with cattle rendered immune to *B. bovis* either by application of *B. bovis* (Mexico)-infected ticks and treatment with Berenil, or by intravenous administration of avirulent, cultured *B. bovis* (Mexico) merozoites, revealed the preferential stimulation of $CD4^+$ T cells by unfractionated merozoite antigen (1, 2). Parasite-specific T cell lines were established from these cattle and additional cattle infected with a virulent blood stabilate prepared from the Texas isolate of *B. bovis*, and subsequently treated with Berenil. All animals were protected upon challenge infection with virulent organisms. The differential pattern of response by T cell lines derived from the immune cattle suggested the presence of multiple immunodominant epitopes (1). Several T cell lines were cloned by limiting dilution to obtain a panel of T cell clones with differing antigenic specificities (5). All of the clones were $CD4^+$ and MHC class-II restricted. To summarize these findings to date, Th clones stimulated with either soluble or membrane enriched fractions of *B. bovis* merozoites can be categorized into seven different groups (table I).

[An original description of five groups (5) has been revised as new Th clones have been characterized.] These distinctions are based on the differential patterns of proliferative responses to :

- different parasite isolates, including the Mexico, Texas and Australian isolates of *B. bovis* and a Mexico isolate of *B. bigemina* ;

- different forms of unfractionated antigen, including crude membrane (CM), soluble cytosolic (HSS), culture supernatant exoantigen (EXO) ;

- soluble cytosolic antigen fractionated by either anion exchange with a Mono Q column or gel filtration with a Superose-12 column by use of FPLC (Pharmacia).

The majority of Th clones obtained from animal C97 reacted with the membrane antigen only, and recognized all isolates of *B. bovis* tested, but not *B. bigemina* (group I). Some clones reactive with only CM did not recognise the Australian isolate (group II), and the remaining Th clones proliferated in response to soluble HSS antigen (groups III-VII). Clones in group VII, unlike the others, responded

TABLE I ***Helper T cell clones define seven antigenic epitopes in*** **Babesia bovis** ***merozoites.***

Group	Response of clones in each group to the following antigens								
	Unfractionated			Fractionated		Parasite isolate			
	CM	EXO	HSS	Mono Q (M NaCl)	Superose-12 (Size in kDa)	Tex	*B. bovis* Mex	Aust	*B. bigemina* Mex
I	+	—	—	NT[a]	NT	+	+	+	—
II	+	NT	—	NT	NT	+	+	—	—
III	+	—	+	Wash, 0.2-0.25	30-70	+	+	+	+
IV	+	—	+	0.35-0.45	25	+	+	+	—
V	+	—	+	NT	Vo	+	+	+	—
VI	+	—	+	0.35-0.45	50/NT	+	+	—	—
VII	+	+	+	0.35-0.45	NT	+	+	—	—

a. NT refers to not tested.

strongly to the soluble exoantigen. Other clones were grouped according to the soluble antigen fractionation pattern and response to Australian parasites and *B. bigemina.*

We have begun to characterize the antigens present in the fractionated soluble HSS analyzed by SDS-PAGE and silver staining (5). Three Th clones reactive with all babesial parasites tested (group III) recognized a common peak of activity in HSS fractionated by either gel filtration or anion exchange, suggesting the recognition of a common epitope. Upon gel filtration, activity eluted in a broad peak ranging from 30 to 70 kDa, and upon anion exchange, activity was present both in the unbound protein fractions and in fractions eluting at the beginning of the NaCl gradient, with 0.2-0.25 M NaCl. SDS-PAGE analysis of the fractions revealed a single band of approximately 43 kDa common to those fractions with antigenic activity (unpublished observations). However, additional experiments are needed to confirm the identity of the antigen that stimulates these T cell clones. In similar studies performed with *Theileria parva*, we found that antigenic activity in fractionated parasite extract was detected with T cell clones even when no protein bands could be visualized on silver-stained gels (3). Two additional Th clones reactive with all *B. bovis* isolates recognized distinct peaks of activity upon gel filtration (groups IV and V).

To determine whether different subsets of Th cells responsive to different *B. bovis* antigens were present, this panel of Th clones has been characterized for the expression of cell surface differentiation antigens indicative of a memory cell phenotype, and for the production of cytokines. Analysis of the cells using monoclonal antibodies (kindly supplied by C. HOWARD, Compton Laboratories, UK and N. MacHUGH, ILRAD, Nairobi, Kenya) and fluorocytometry revealed that all of the ten $CD4^+$ T cell clones examined expressed high levels of CD45RO, an isoform of the common leukocyte surface antigen, CD45, that is found on memory T cells. In contrast, expression of CD45R, an isoform of CD45 associated with naive T cells, was low. All clones also expressed high levels of L-selectin, a lymph node homing receptor. Biological assays for cytokines secreted into the supernatants of mitogen-activated T cell cultures revealed a differential production of T cell growth factor (IL-2/IL-4), IFN-γ and TNF-α/TNF-ß, suggesting functional differences among the Th clones. All clones in groups I-VI produced IFN- γ, and some clones in each group produced TNF and IL-2/IL-4 activities, whereas none of the clones in group VII produced any detectable cytokine (5, unpublished observations). Because bioassays that distinguish bovine IL-2 and IL-4 activities are currently not available, analysis of the expression by these cells of mRNA encoding IFN-γ, IL-2, IL-4, and TNFα was performed to determine whether these *B. bovis*-specific Th clones could be classified as Th0, Th1 or Th2 cells. Northern blotting revealed that the majority of Th clones in groups I-VI expressed an unrestricted, or Th0 profile of cytokine mRNA, whereas the three Th clones in group VII expressed a Th1-like profile of cytokine mRNA (unpublished observations).

Functional and phenotypic analysis of two sets of Th clones specific for two recombinant *B. bovis* proteins has similarly been performed. In the first study, the authors examined T cells from *B. bovis* immune cattle for responsiveness to the major merozoite surface antigen, MSA-1 (4). This antigen is a 42 kilodalton integral membrane glycoprotein previously shown to induce immunodominant antibody responses in cattle protectively immune to *B. bovis* and to induce neutralizing antibody (7, 8). Recent studies have also shown that MSA-1 B cell epitopes common to New World strains of *B. bovis* are not present in either Israel or Australia strains (8, 9). To understand the potential role of this protein in protective immunity, T helper cell responses specific for MSA-1 were characterized in *Babesia*-immune cattle. Peripheral blood mononuclear cells (PBMC) from immune cattle proliferated against affinity purified recombinant MSA-1 protein expressed in *E. coli.* MSA-1 preferentially stimulated the growth of $CD4^+$ T cells in cell lines cultured with antigen for 4 weeks. MSA-1-reactive cell lines responded to a membrane fraction of *B. bovis* merozoites, suggesting recognition of the native protein. However, the *B. bovis*-reactive T cell lines and Th clones established by stimulation with crude parasite membrane antigen described above failed to respond to recombinant MSA-1, indicating that this antigen is not immunodominant for T cells. The majority of MSA-1-specific Th clones reacted to unfractionated merozoite membrane antigen from New World *B. bovis* isolates, but none of the clones responded to Australia *B. bovis* or to a Mexico isolate of *B. bigemina*. Six clones tested did not secrete detectable levels of cytokines when stimulated with mitogen alone; however several Th clones produced low levels of cytokines when stimulated with mitogen and IL-2. Northern blot analysis revealed the expression of IL-2, IL-4, IFN-γ and TNF-α mRNA in mitogen-stimulated Th clones, showing that the clones examined expressed an unrestricted Th0 phenotype. These findings show that the MSA-1 protein, although serologically immunodominant and capable of inducing neutralizing antibodies as well as a T helper cell response, is not an immunodominant T cell antigen for the cattle used in this study. Furthermore, the parasite strain specificity of the Th clones supports previous findings of extensive polymorphism in the MSA-1 glycoprotein, and suggests that like B cell epitopes, T cell epitopes reside in a non conserved portion of the protein .

The authors also examined T cells from B. bovis-immune cattle for responsiveness to a recombinant form of the 77kDa merozoite protein, Bb-1 (6, 12), which was of interest for studies on T cell immunogenicity because :

- the Bb-1 gene is conserved among New World and Australian parasites ;

- the gene predominated during immunoselection with bovine sera obtained from cattle naturally infected with *B. bovis* ;

- affinity purified bovine antibody reacted with a 77 kDa merozoite protein on immunoblots, and with the apical end of the merozoites by immunofluorescence staining ;

- the Bb-1 protein is inducible by either nutritional or oxidative stress.

Together, these results suggested a functional importance for the Bb-1 protein and a logical target for immune intervention, and showed that the Bb-1 protein is immunogenic for naturally infected animals, where upon natural exposure it could presumably boost memory T cell and B cell responses in cattle vaccinated with this antigen. We first showed that recombinant Bb-1 induced proliferative responses of PBMC of two immune cattle. Antigenicity for PBMC resided in the N-terminal half of the protein. Both $CD4^+$ and $CD8^+$ Bb-1-specific T cell clones were obtained by cloning Bb-1-reactive cell lines. To map the T cell epitopes recognized by the Th clones, a nested set of truncated fusion proteins spanning the Bb-1 protein was prepared and tested for antigenicity in proliferation assays. Two Th clones recognized different T cell epitopes, which mapped to the N terminal half of the protein. Both clones expressed the Th1 profile of cytokines : IL-2, IFN-γ, TNF-β, and TNF-α, but not IL-4, suggesting the preferential induction of Th1 cells by the Bb-1 protein. The authors then characterized the antibody-reactive regions of the Bb-1 protein by Western blot analysis of the truncated recombinant fusion proteins using rabbit antiserum raised against intact Bb-1 protein. In contrast to the T cell epitopes, the antibody-reactive region mapped to the C-terminal half of Bb-1 which contains 28 tandem repeats of a tetrapeptide, PAEK or PAET.

In conclusion, the nature of the protective immune response against many hemoprotozoan parasites is not well understood, and *Babesia* is no exception. When T cells were selected in vitro with crude parasite extracts, the T cells that were subsequently cloned out of the population did not recognize the serologically immunodominant antigen, MSA-1. Furthermore, preliminary studies on fractionation of the soluble antigen of *Theileria* and *Babesia* parasites suggest that the immunodominant T cell antigens are not abundant. In agreement with these observations, WRIGHT and coworkers found that protective antigens of *B. bovis* were present in minute quanties in the organism, and did not stimulate strong antibody responses (13). These findings support the concept put forth by BYRON WAKSMAN, that the best choice of a vaccine antigen may be one which does not evoke a strong immune response during natural infection (11). In studies performed with *B. bovis*, the Th cell response generated against crude parasite extracts was comprised of Th0 and Th1 cells, but not Th2 cells. Interestingly, when a single antigen, Bb-1, was used to select T cells *in vitro*, Th1 cells were preferentially induced, providing a rationale for choosing Bb-1 as a potential vaccine antigen. The use of T cell clones as probes to identify parasite antigens and to characterize the immune response may facilitate the identification of protective parasite immunogens.

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BROWN (W.C.), ZHAO (S.), WOODS (V.M.), DOBBELAERE (D.A.E.), RICE-FICHT (A.C.). *Babesia bovis* -specific CD4+ T cell clones from immune cattle express either the Th0 or Th1 profile of cytokines. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 65-69

The central role of T cells in the immune response against hemoprotozoan parasites, both as helper cells for T-dependent antibody production, and as effector cells acting directly or indirectly on intracellular parasites through the elaboration of cytokines, has prompted us to investigate the bovine cellular immune response against *B. bovis* antigens. T cell clones generated from four *B. bovis*-immune cattle by *in vitro* stimulation with soluble or membrane associated merozoite antigen were characterized for reactivity against various forms of antigen and different geographical isolates of *B. bovis* and *B. bigemina*. The clones were categorized into seven different groups based on differential patterns of reactivity. This panel of T cell clones and additional clones specific for either the 77 kDa merozoite apical complex associated protein (Bb-1) or the 42 kDa major merozoite protein (MSA-1) were analyzed for cytokines. Biological assays to measure IL-2/IL-4, IFN-γ and TNF-α /TNF-β and Northern blot analysis to detect mRNA encoding bovine IL2, IL-4, IFN-γ, TNF-β and TNF-α revealed the differential production of cytokines by clones with different antigen specificities. Two Bb-1-specific T cell clones produced the Th1 pattern of cytokines : IL-2, IFN-γ ,TNF-β and TNF-α, but not IL-4. Clones specific for the 42 kDa protein produced undetectable levels of all cytokines, but expressed an unrestricted or Th0 pattern of cytokine mRNA : IL-2, IL-4, IFN-γ and TNF-α. Finally, the majority of Th clones reactive with undefined merozoite antigens expressed the Th0 pattern of cytokines, and several clones were of the Th1 phenotype, whereas none of the clones expressed a Th2 profile of cytokines. Because in other protozoal infections Th1 cells, IFN-γ, TNF-α, and TNF-β, but not IL-4 are associated with the development of a protective immunity, Bb-1 is a logical candidate for a *B. bovis* vaccine.

Key words : *Babesia bovis* - T cell - Clone - Antigen - Immunity - Immune response.

BROWN (W.C.), ZHAO (S.), WOODS (V.M.), DOBBELAERE (D.A.E.), RICE-FICHT (A.C.). Los clones de células T CD4+, de bovinos inmunes, específicos para *Babesia bovis*, se expresan de acuerdo a un perfil de citoquinas Th0 o de Th1. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 65-69

La razón que nos motivó a investigar la respuesta inmunológica celular en el bovino contra antígenos de *Babesia bovis*, es el papel central que juegan las células T en la respuesta inmunológica contra los hemoprotozoarios, tanto como células colaboradoras para la producción de anticuerpos T-dependientes, que como "células de efecto", actuando directa o indirectamente sobre los parásitos intracelulares, mediante la elaboración de citoquinas. Los clones de células T, provenientes de cuatro bovinos inmunizados contra *B. bovis* (por estimulación *in vitro*, con una asociación de un antigeno de merozoito soluble o de membrana), fueron caracterizados para la reactividad contra varias formas de antigeno y de aislamientos geográficos de *Babesia bovis* y *B. bigemina*. Los clones se clasificaron en siete grupos diferentes, basados en los patrones diferenciales de reactividad. El análisis de las citoquinas se hizo para esta muestra de clones de células T y otros clones específicos, ya sea para la proteína asociada al complejo 77 kDa del merozoito apical (Bb-1) o a la proteína del merozoito mayor 42kDa (MSA-1). Estos experimentos biológicos para medir el IL-2/IL-4, IFN-γ y TNF-α /TNF-β y el analisis por "Northern blot" para detectar el ARNm que codifica la IL-2, IL-4, IFN-γ, TNF-β y TNF-α, demostraron la diferencia en cuanto a la producción de citoquinas por parte de los clones con diferentes especificidades antigénicas. Dos clones celulares Bb-1 específicos para células T, produjeron citoquinas del patron Th1: IL-2, IFN-γ ,TNF-β y TNF-α, pero no IL-4. Los clones específicos para la proteína 42 kDa produjeron niveles no detectables de todas la citoquinas, pero mostraron un patrón sin restricción o patrón Th0 de citoquina de ARNm: IL-2, IL-4, IFN-γ y TNF-α. Finalmente, la mayoría de los clones Th reaccionaron con antigenos indefinidos del merozoito, expresando el patron Th0 de citoquinas. Varios clones fueron del fenotipo Th1, mientras que ninguno de ellos mostró un perfil Th2 de citoquinas. Bb-1 es el candidato lógico para una vacuna contra *B. bovis*, debido a que en otras infecciones por protozoarios las células Th1, IFN-γ, TNF-α y TNF-β, pero no IL-4, se asocian con el desarrollo de la inmunidad protectora.

Palabras claves : *Babesia bovis* - Célula T - Clon - Antígeno - Inmunidad - Respuesta inmunologica.