

## CD4<sup>+</sup> T-Helper Lymphocyte Responses against *Babesia bigemina* Rhopty-Associated Protein 1

SERGIO D. RODRÍGUEZ,<sup>1†</sup> GUY H. PALMER,<sup>2</sup> TERRY F. McELWAIN,<sup>2</sup> TRAVIS C. McGUIRE,<sup>2</sup>  
BARBARA J. RUEF,<sup>1‡</sup> CAROL G. CHITKO-McKOWN,<sup>1</sup> AND WENDY C. BROWN<sup>1,2\*</sup>

Department of Veterinary Pathobiology, Texas A&M University, College Station, Texas 77843,<sup>1</sup> and Department of  
Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington 99164<sup>2</sup>

Received 19 January 1996/Returned for modification 8 March 1996/Accepted 28 March 1996

**A multigene family of 58- to 60-kDa proteins, which are designated rhopty-associated protein 1 (RAP-1) and which come from the parasites *Babesia bigemina* and *Babesia bovis*, is a target for vaccine development. The presence of multiple gene copies and conserved sequences and epitopes of RAP-1 implies that these proteins are functionally important for the survival of these parasites. Furthermore, it was previously shown that *B. bigemina* RAP-1 induced partial protection against challenge infection. However, the lack of correlation between protective immunity to *B. bigemina* infection and antibody titers against a merozoite surface-exposed, neutralization-sensitive epitope of *B. bigemina* RAP-1 indicated the potential importance of RAP-1-specific T helper (Th) cells in the observed protection. To begin to understand the mechanism of RAP-1-induced protective immunity, RAP-1-specific T-cell responses were characterized in cattle. Vigorous and sustained proliferative responses of peripheral blood mononuclear cells from native RAP-1-immunized cattle were observed. The anamnestic response in immunized cattle was specific for *B. bigemina* RAP-1 and predominantly comprised CD4<sup>+</sup> T cells, which upon cloning expressed type 1 cytokine mRNA profiles and high levels of gamma interferon protein. The T cells responded to both native and recombinant forms of RAP-1, indicating the potential to use recombinant protein or epitopes derived therefrom as a vaccine that could evoke specific recall responses after exposure to natural infection. The differential responses of peripheral blood mononuclear cells and seven Th-cell clones derived from RAP-1-immunized cattle to different Central American strains of *B. bigemina* indicated the presence of at least one conserved and one variable Th-cell epitope. The lack of response to *B. bovis* RAP-1 indicated that a strictly conserved 14-amino-acid peptide shared by the two babesial species was not immunogenic for Th cells in these experiments. However, the Th-cell epitope conserved among strains of *B. bigemina* may be a useful component of a RAP-1 subunit vaccine.**

Bovine babesiosis, caused by tick-borne intraerythrocytic parasites from the genus *Babesia*, affects millions of cattle in tropical and temperate regions of the world, greatly reducing milk and meat production. *Babesia bigemina* and *Babesia bovis* are the causative agents of this disease in the Americas, Africa, and Australia, and their presence in endemic grazing regions precludes the movement of highly specialized cattle needed for the improvement of indigenous breeds. Current immunoprophylactic methods are based on the use of live attenuated parasites from intact or splenectomized donor cattle (16) or from continuous erythrocyte cultures (33). The use of these vaccines, however, is limited by problems with standardization (15), contaminating blood-borne pathogens (45), and the selection of non-cross-protective virulent field strains (2), which underscore the need to develop safe and effective vaccines against babesiosis.

Current efforts in our laboratories are aimed at identifying babesial protein antigens that stimulate humoral and cellular immunity, thereby predicting their usefulness as components of a subunit vaccine (10, 40, 57). Of particular interest are

proteins that exist as homologs in different genera of apicomplexan parasites, which therefore implies a functional importance of these proteins for parasite survival (40, 49, 57). Proteins produced by specialized organelles of the merozoite apical complex, including rhoptries, micronemes, spherical bodies, and dense granules, are believed to play a major role in host erythrocyte invasion or possibly egression (42). Many of these apical complex-associated proteins have been targeted for vaccine development against human malarial parasites (32; reviewed in reference 31). Included in the group of babesial antigens is an immunogenic, multigene family of 58- to 60-kDa proteins that are designated rhopty-associated protein 1 (RAP-1) and that are associated with both the merozoite surface and rhoptries of *B. bovis* and *B. bigemina* (20, 21, 35, 36, 49–51). Genes encoding RAP-1 proteins of *Babesia ovis* and *Babesia canis* have also been described (21). Four different gene copies of *B. bigemina* RAP-1 (Bbg-7, Bbg-9, Bbg-13, and Bbg-14) identified in a cloned strain of a Mexico isolate of *B. bigemina* encode variants of RAP-1 that exhibit sequence polymorphisms in the amino- and carboxy-terminal amino acids (37, 38). Three of the genes (all except for Bbg-7) were transcribed. Australian strains of *B. bovis* RAP-1 contain tandem gene copies which appear to have similar sequences (21).

The serological response against RAP-1 proteins has indicated the presence of antigenically conserved and variable epitopes. Cattle naturally or experimentally infected with *B. bovis* or *B. bigemina* develop RAP-1-specific antibodies that recognize merozoite surface-exposed epitopes that are conserved among all geographical strains of *B. bovis* and *B. bigemina*, respectively, but not between these species (27, 35, 36, 41,

\* Corresponding author. Mailing address: Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA 99164-7040. Phone: (509) 335-6067. Fax: (509) 335-8529. Electronic mail address: wbrown@vetmed.wsu.edu.

† Present address: Centro Nacional de Investigaciones Disciplinarias en Parasitología Veterinaria, Instituto Nacional de Investigaciones Forestales y Agropecuarias, Morelos, Mexico.

‡ Present address: Department of Medical Biochemistry and Genetics, Texas A&M University, College Station, TX 77843-1114.

47). A comparison of *B. bovis* and *B. bigemina* RAP-1 sequences revealed broadly conserved 300-amino-acid blocks located at the amino termini that exhibit 45% sequence identity and strictly conserved 14-amino-acid residues (49). However, the sequences conserved between species were different from those of known antigenic B-cell epitopes, and the highly conserved 14-amino-acid peptide was very poorly antigenic for cattle (50). Therefore, the sequences that are conserved among RAP-1 proteins and that are apparently required for function do not appear to contain conserved B-cell epitopes (40, 50).

The protective properties of RAPs were revealed in several vaccine trials with native and recombinant antigens. Immunization of monkeys with an affinity-purified native *Plasmodium falciparum* RAP conferred partial protection, which did not correlate with the antibody titer (48), and immunization with a recombinant RAP was also protective (43). Fractions containing the native form of *B. bovis* RAP-1 induced partial protection, determined as reduced parasitemia and increased survival rates in response to homologous *B. bovis* challenge in calves, as did a recombinant glutathione *S*-transferase fusion protein consisting of a fragment of the *B. bovis* RAP-1 protein (reviewed in reference 57). Although *B. bovis* RAP-1 is not a serologically immunodominant protein (57), recent studies in our laboratory have shown that this protein is very immunodominant for T helper (Th) cells from *B. bovis*-infected and -immune cattle (8). Immunization of calves with the native form of the 58-kDa RAP-1 protein of *B. bigemina* similarly resulted in partial protection, as demonstrated by a substantial decrease in parasitemia in young spleen-intact calves (36). In addition, a *B. bigemina* RAP-1-specific monoclonal antibody (MAb) that is designated C2F3G3 and that recognizes a merozoite surface-exposed epitope inhibited the *in vitro* growth of *B. bigemina* (26). Interestingly, there was little correlation between protection in immunized calves and the level of antibody specific for the neutralization-sensitive B-cell epitope defined by MAbs C2F3G3 and 14/16.1.7 (55), pointing to the need to examine additional effector mechanisms. Together, these studies indicate the importance of characterizing Th cells specific for RAP-1 and other babesial apical complex proteins of interest as both helper cells and potential effector cells for the development of RAP-1 epitope-based vaccines for *B. bovis* and *B. bigemina*.

Considerable evidence in favor of a requirement for type 1 CD4<sup>+</sup> T-cell responses in the induction of protective immunity against intracellular protozoan parasites, including *Babesia* spp., has accumulated (10). Because the immunogenicity of *B. bigemina* RAP-1 proteins for T cells and the nature of the Th-cell response against these antigens have not been previously determined, studies described herein were undertaken to characterize Th-cell responses against *B. bigemina* RAP-1 protein in cattle immunized with native RAP-1 protein. Th-cell clones derived from RAP-1-immunized cattle were found to express type 1 cytokine profiles and will be used to identify RAP-1 Th-cell epitopes for potential inclusion in a subunit vaccine.

#### MATERIALS AND METHODS

**Parasite strains.** Cultured Mexico strains of *B. bigemina* and *B. bovis* were originally isolated from cattle infected with tick-derived isolates from northeastern Mexico (6). These two strains were maintained as uncloned populations in stationary phase cultures (44, 56). Cryopreserved blood stabilates of the Texcoco, St. Croix, and Puerto Rico strains of *B. bigemina* were described previously (26, 34, 36).

**Parasite antigens.** Antigens from the cultured Mexico strains of *B. bigemina* and *B. bovis* were prepared as described previously (6). Parasites from the cryopreserved blood of cattle infected with the Texcoco, St. Croix, and Puerto Rico strains were prepared as follows. Blood stabilates were thawed, washed five

times in phosphate-buffered saline (PBS [pH 7.2]) by centrifugation at 30,000 × *g*, and resuspended in PBS. The infected erythrocyte ghosts were then mixed with protease inhibitors phenylmethylsulfonyl fluoride (5 mM), E-64 (25 μg/ml), and antipain (25 μg/ml) and homogenized with a French pressure cell (SLM Instruments, Inc., Urbana, Ill.) as described previously (6). The resulting crude membrane (CM) pellet was resuspended in PBS containing 50 μg of gentamicin sulfate (Sigma Chemical Co., St. Louis, Mo.) per ml and stored at -70°C.

Native *B. bigemina* RAP-1 protein was purified by affinity chromatography with MAb 14.16.1.7 from parasitized erythrocytes obtained from a calf infected with the Mexico strain (36). This MAb binds to two (Bbg-13 and Bbg-7) of the four potentially expressed gene products (30, 37). This affinity-purified protein was used for cattle immunization.

Recombinant *B. bigemina* RAP-1 protein was prepared as a maltose-binding fusion protein. Briefly, plasmid pBbg58 (37, 38) encoding one of the four *rap-1* genes (Bbg-13) of the JG-29 biological clone of *B. bigemina* was amplified by PCR with the GeneAmp kit (Perkin-Elmer Cetus, Norwalk, Conn.). Forward and reverse primer sequences P3 and P4 are located at the 5' and 3' ends of the open reading frame of *B. bigemina* RAP-1 and were modified to include a *Bam*HI restriction site at the 5' end. The forward primer P3 sequence was 5'-GCGCTCTA GAATGAGGAGCTTCTGGGTGTG-3', and the reverse primer P4 sequence was 5'-CGCGTCTAGATTACGATCTGAATCATCTG-3'. The PCR products were digested with *Bam*HI and subcloned into the pMAL-2c vector (New England Biolabs, Beverly, Mass.) according to the manufacturer's protocol. *Escherichia coli* XL-1 Blue (Stratagene, La Jolla, Calif.) containing the recombinant plasmids encoding the maltose-binding protein (MBP) or MBP fused to RAP-1 was grown for 4 h at 37°C in Luria broth containing 0.2% (wt/vol) glucose under constant agitation. Protein expression was induced with 300 mM isopropyl-β-D-thiogalactopyranoside for 2 h at 37°C, and the cells were then harvested by centrifugation at 2,000 × *g*. Pelleted bacteria were resuspended in lysis buffer (200 mM NaCl, 1 mM EDTA, 20 mM Tris [pH 7.4]) containing 0.5% Nonidet P-40 and were subjected to one cycle of freeze-thawing and sonication. Lysates were centrifuged for 10 min at 15,000 × *g*, and the supernatants were harvested. The recombinant proteins were further purified by affinity chromatography on amylose resin columns (New England Biolabs) by following the manufacturer's protocol. The purified fusion proteins were dialyzed overnight against PBS and then stored at -70°C. Recombinant *B. bovis* RAP-1 protein expressed as maltose-binding fusion protein was kindly provided by Carlos Suarez, Washington State University. Recombinant, and not native, RAP-1 proteins were used for all experiments performed *in vitro*.

Protein concentrations in native and recombinant antigens were determined as described previously (6).

**Cattle used in this study.** Four 10-month-old crossbred heifers (2216, 2234, 2238, and 2242) that were seronegative for *Babesia* spp. were selected for use. The animals were inoculated subcutaneously in the left side of the neck three times at 3- or 4-week intervals with 20 μg of affinity-purified native RAP-1 protein diluted in 0.5 ml of PBS and mixed with an equal volume of RIBI adjuvant (catalog number R-730; RIBI Immunochem Research, Inc., Hamilton, Mont.) consisting of monophosphoryl lipid A plus trehalose dimycolate plus cell wall skeleton. The animals were bled weekly from the jugular vein to obtain peripheral blood mononuclear cells (PBMC) for T-cell proliferation assays. Eight months later, two animals (2216 and 2234) were given boosters of 20 μg of protein as described above. All inoculated animals simultaneously received control injections of PBS diluted in RIBI adjuvant in the right side of the neck.

**SDS-PAGE and immunoblotting.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed essentially as described previously (53), with 7.5% acrylamide gels and 20 μg of *B. bigemina* CM or control uninfected erythrocyte (URBC) CM protein per lane being used. Low-molecular-weight standards (Bio-Rad Laboratories, Richmond, Calif.) were included in each gel. Immunoblotting onto nitrocellulose was performed for 1 h at 100 V with the Transblot (Bio-Rad) apparatus. Blots were reacted with pre- and post-RAP-1 immunization bovine sera diluted 1:50 in Tris-buffered saline (10 mM Tris, 150 mM NaCl [pH 7.4]) containing 2% skim milk powder, and serologically reactive proteins were detected with a 1:500 dilution of alkaline phosphatase-conjugated goat anti-bovine immunoglobulin G (IgG) (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) and substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma) per the manufacturers' instructions.

***B. bigemina* RAP-1-specific ELISA.** Enzyme-linked immunosorbent assay (ELISA) plates (Immulon 2; Dynatech Laboratories, Inc., Chantilly, Va.) were coated with 100 μl of 10-μg/ml recombinant *B. bigemina* RAP-1 protein or control MBP diluted in PBS and were incubated overnight at 4°C. After the plates were washed, preimmunization sera and sera obtained 2 weeks following the fourth immunization with native *B. bigemina* RAP-1 protein were serially diluted (to 1:10,000) in PBS and incubated in the plates for 1 h at 37°C. Serially diluted (to 100 ng/ml) purified bovine IgG1 and IgG2 (Chemicon International, Inc., Temecula, Calif.) were used to directly coat the plates to verify the specificity of the anti-subclass antibodies. After being washed, the plates were incubated for 1 h at 37°C with biotinylated sheep anti-bovine IgG1 or IgG2 (The Binding Site, Birmingham, United Kingdom) diluted to 10 μg/ml with PBS, and this incubation was followed by washing and incubation at 37°C with alkaline phosphatase avidin D (Vector Laboratories, Burlingame, Calif.) diluted to 0.2 U/ml with PBS. Following the washing, the reaction was developed with *p*-

nitrophenyl phosphate (Kirkegaard & Perry) per the manufacturer's instructions. The optical density at 410 nm (OD<sub>410</sub>) was determined with an ELISA plate reader (Dynatech). The mean OD<sub>410</sub> of the preinoculation sera was considered the baseline. Titers of the experimental samples were then calculated by linear regression, with the OD<sub>410</sub> values of the serially diluted samples being plotted against the log of the dilution. The titer is expressed as the reciprocal of the serum dilution at which the titration curve intersects the baseline OD<sub>410</sub> value.

**Bovine RAP-1-specific T-cell lines.** RAP-1-specific T-cell lines were established from PBMC of RAP-1-immunized animals 10 weeks after the initial antigen inoculation as described previously (5). Briefly,  $4 \times 10^6$  PBMC per well were cultured in 24-well plates (Costar, Cambridge, Mass.) in a 1.5-ml volume of complete RPMI 1640 medium (6) with 25  $\mu$ g of *B. bigemina* CM antigen per ml. After 7 days, the cells were subcultured to a density of  $5 \times 10^5$  cells per well and cultured with antigen and  $2 \times 10^6$  fresh irradiated (4,000 rads) autologous PBMC as a source of antigen-presenting cells (APC). Alternatively, recombinant *B. bigemina* RAP-1 fusion protein (5  $\mu$ g/ml) was used to stimulate PBMC from immunized cattle and the resultant T-cell lines. T-cell lines were maintained for 11 to 12 weeks by weekly stimulation with antigen and APC, and the cells were assayed for antigen-dependent proliferation 6 to 8 days following the last antigen stimulation.

**RAP-1-specific T-cell clones.** T-cell clones were derived from T-cell lines established from animals 2216 and 2234. T-cell line 2234 was sequentially stimulated for 4 weeks with *B. bigemina* CM antigen and for 4 weeks with recombinant RAP-1 fusion protein, and T-cell line 2216 was stimulated with recombinant RAP-1 fusion protein for 2 weeks. These cell lines were shown to proliferate to *B. bigemina* CM and recombinant RAP-1 protein in a dose-dependent manner. Cells were cloned by limiting dilution as described previously (9, 12, 13) in 96-well round-bottomed plates (Costar). Briefly, T cells were diluted to a statistical average of 1 or 0.3 cells per well and stimulated with 5  $\mu$ g of recombinant RAP-1 fusion protein per ml in medium containing 10% bovine T-cell growth factor and  $5 \times 10^4$  autologous APC. Proliferating cells were transferred successively to 48- and 24-well plates and tested for *B. bigemina* RAP-1 antigen-specific proliferation.

**Lymphocyte proliferation assays.** Lymphocyte proliferation assays were carried out in replicate wells of half-area 96-well plates (Costar) for 6 days with PBMC and for 3 days with T-cell lines and clones, essentially as described previously (5, 6, 9, 14, 53). Briefly,  $2 \times 10^5$  PBMC were cultured in triplicate wells with antigen in a total volume of 100  $\mu$ l of complete medium. T-cell lines and clones were assayed 7 days after the last stimulation with antigen and APC. Briefly,  $3 \times 10^4$  cells were cultured in duplicate wells in a total volume of 100  $\mu$ l of complete medium containing  $2 \times 10^5$  autologous or major histocompatibility complex (MHC)-mismatched APC and antigen. Antigens consisted of 0.2 to 50  $\mu$ g of the following per ml: CM prepared from different geographical strains of *B. bigemina*, *B. bovis* (Mexico), or URBC; recombinant *B. bigemina* RAP-1 fusion protein; *B. bovis* RAP-1 fusion protein; or control MBP. Concanavalin A (ConA; 2  $\mu$ g/ml; Sigma) or 10% (vol/vol) bovine T-cell growth factor was used as a positive control for the proliferation of PBMC or T cells. In some experiments, T-cell clones were assayed with antigen in the presence of 2 U of recombinant human interleukin 2 (IL-2; Boehringer Mannheim, Indianapolis, Ind.) per ml. Irradiated heterologous PBMC from MHC-mismatched animal C15 were used to verify MHC restriction. To determine proliferation, cells were radiolabeled for the last 4 to 8 h of culture with 0.25  $\mu$ Ci of [<sup>125</sup>I]iododeoxyuridine (ICN Radiochemicals, Costa Mesa, Calif.), and radiolabeled nucleic acids were harvested onto glass filters and counted as described previously (5, 6). The results are presented as the mean counts per minute of replicate cultures  $\pm$  1 standard deviation or as the stimulation index, which is the mean counts per minute of replicate cultures of cells plus antigen divided by the mean counts per minute of replicate cultures of cells plus medium.

The one-tailed Student *t* test was used to determine the levels of significance between control and experimental cultures.

**Cell surface phenotypic analysis.** Cell surface phenotypes of T-cell lines and clones were determined by indirect immunofluorescence and analyzed by flow cytometry as described previously (5), with MAbs specific for bovine CD4 (MAb IL-A12), CD8 (MAb IL-A51), CD2 (MAb IL-A26), and WC1 (MAb IL-A29), which were obtained from the International Laboratory for Research on Animal Diseases, Nairobi, Kenya, and for bovine CD3 (MAb MM1A), which was kindly provided by William C. Davis, Washington State University, Pullman, Wash., being used.

**Stimulation of cells and detection of cytokine mRNAs and gamma interferon (IFN- $\gamma$ ) protein.** T-cell clones were obtained 7 days after the last stimulation with antigen and APC, washed twice in complete medium, and cultured at a concentration of  $2 \times 10^6$  cells per ml in the presence of an equal number of autologous APC and 25  $\mu$ g of *B. bigemina* CM antigen per ml. After 14 h of culture, total cellular RNA was prepared from the cells following lysis with guanidinium isothiocyanate (19). As a positive control for cytokine mRNA expression, RNA was prepared from bovine PBMC stimulated at a concentration of  $2 \times 10^6$  cells per ml with ConA (Sigma) for 18 h. For negative controls, RNA was prepared from cultures of either irradiated APC, antigen, and 10 U of IL-2 per ml or unstimulated murine WEHI-164 fibroblasts. To confirm the integrity of RNA in each sample, RNA was size fractionated in 1% agarose gels and the ethidium bromide-stained gels were visualized with UV light.

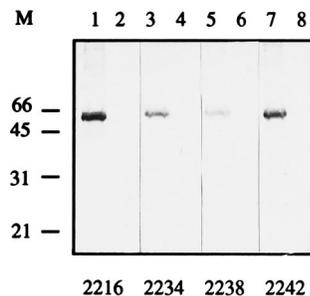


FIG. 1. Reactivity of sera from RAP-1-immunized cattle with *B. bigemina* CM antigen. Immunoblotting was performed with postimmunization sera from RAP-1-immunized cattle 2216 (lanes 1 and 2), 2234 (lanes 3 and 4), 2238 (lanes 5 and 6), and 2242 (lanes 7 and 8) and 20  $\mu$ g of antigen per lane that consisted of CM prepared from either *B. bigemina* merozoites (lanes 1, 3, 5, and 7) or URBC (lanes 2, 4, 6, and 8). Molecular size standards (M) are indicated on the left.

To evaluate cytokine mRNA expression, a reverse transcription PCR (RT-PCR) assay was employed as described previously (19) with the following modifications. PCR conditions were first optimized for each cytokine by varying the concentration of total input RNA and the number of cycles and plotting these against the counts per minute in <sup>32</sup>P-radiolabeled PCR products (19). Those RNA concentrations and cycle numbers that fell on the linear portion of the plotted curve were then selected for experimental use. Cytokine cDNA was prepared from 0.5  $\mu$ g of total RNA and  $\beta$ -actin cDNA was prepared from 0.25  $\mu$ g of total RNA with a GeneAmp RNA PCR kit (Perkin-Elmer Cetus) by following the manufacturer's protocol. The bovine IL-4-specific primer sequences were provided by Dirk Dobbelaere, University of Bern, Bern, Switzerland, and the bovine IFN- $\gamma$ -specific primer sequences were provided by Heng-Fong Seow, Commonwealth Scientific and Industrial Research Organisation, Parkville, Australia. Primer sequences specific for bovine IL-2, IL-2, and IL-10 were selected from the published sequences with MacVector DNA analysis software (IBI, New Haven, Conn.). The GenBank accession numbers for the bovine cytokine and actin sequences are as follows: IL-2, M12791 (17); IL-4, M77120 (29); IL-10, U00799 (28); IFN- $\gamma$ , M29867 (18); and  $\beta$ -actin, K00622 (22). The primers were synthesized by the Gene Technologies Laboratory, Texas A&M University, College Station, Tex. First-strand cDNA synthesis was performed in a thermal cycler employing reverse transcriptase and reverse primers (20 to 34 pmol) for IL-2 (5'-CCCTGTAGTTCACAAAACGATTCTC-3' [nucleotides (nt) 353 to 376]), IL-4 (5'-GTCCTTCAGCGTACTTGT-3' [nt 406 to 423]), IFN- $\gamma$  (5'-GCTCTCCGGCCTCGAAAGAGATT-3' [nt 562 to 584]), IL-10 (5'-TATGTAGTTGATGAAGATGTC-3' [nt 559 to 579]), and  $\beta$ -actin (5'-ACGTAGCAGAGCTTCTCTTGTATG-3' [nt 414 to 437]), with an initial 15-min incubation at 48°C being followed by a 5-min incubation at 99°C. Forward primers (20 to 32 pmol) for IL-2 (5'-TGCACCTACTTCAAGCTCTACGG-3' [nt 77 to 99]), IL-4 (5'-TGCATTGTTAGCGTCTCT-3' [nt 1 to 19]), IFN- $\gamma$  (5'-GGCCCTCTTCTCAGAAATTTTG-3' [nt 238 to 261]), IL-10 (5'-GTTGCTGTCTCTCTGGCTG-3' [nt 98 to 118]), and  $\beta$ -actin (5'-CCTTTTACAA CGAGCTGCGTGTG-3' [nt 38 to 60]) were then added to the reaction mixtures, which were incubated at 95°C for 4 min. *Taq* polymerase was added, and the mixtures were further amplified for 25 cycles ( $\beta$ -actin), 35 cycles (IL-2, IL-4, and IFN- $\gamma$ ), or 40 cycles (IL-10) under the following conditions: 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min. Eighteen microliters of each PCR product was then electrophoresed on a 1% agarose gel and stained with ethidium bromide. PCR products were visualized by exposure to UV light and photographed. The presence of specific cytokine mRNA was determined by the presence of a band with the correct product size. The specificity of the primers and identity of the product bands were previously verified by Southern blotting with RT-PCR products obtained from ConA-stimulated PBMC and radiolabeled cytokine cDNA probes. Antigen-stimulated irradiated PBMC expressed only  $\beta$ -actin and not cytokine mRNA, as determined by visualization of the ethidium bromide-stained gels and Cerenkov radiation (19).

Supernatants from T cells stimulated as described above for 24 h were harvested by centrifugation and stored frozen at -70°C to assay for IFN- $\gamma$ . The bovine IFN- $\gamma$  assay was performed with a commercial ELISA kit (IDEXX Laboratories, Westbrook, Maine) according to the manufacturer's protocol. The IFN- $\gamma$  activity in culture supernatants diluted 1:10 or 1:50 was determined by comparison with a standard curve obtained with a supernatant from a *B. bovis*-specific Th-cell clone that contained 400 U of IFN- $\gamma$  per ml (previously determined by the neutralization of vesicular stomatitis virus [13]).

## RESULTS

**Serologic responses of *B. bigemina* RAP-1-immunized cattle.** To verify that native RAP-1 protein was antigenic, immunoblot

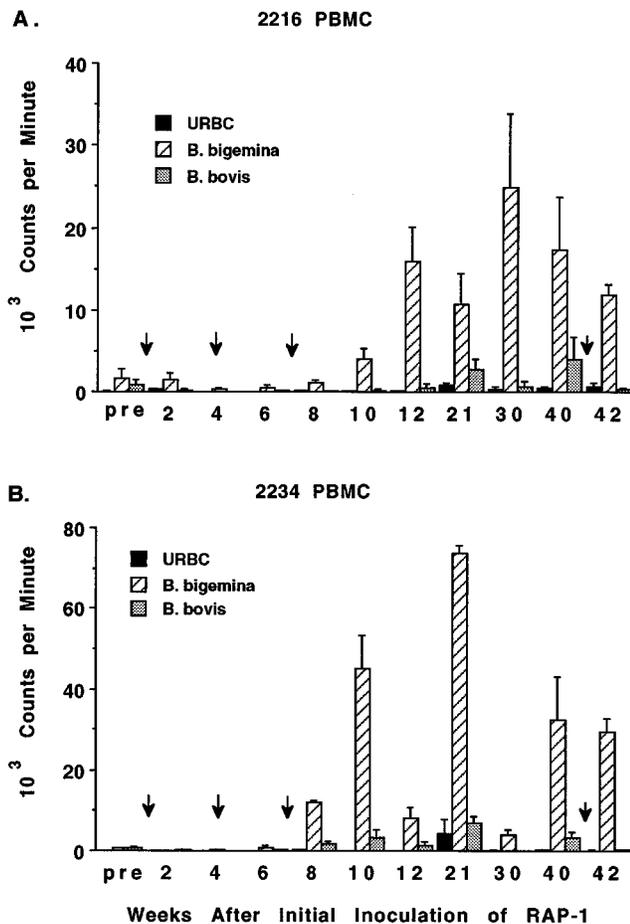


FIG. 2. Time courses of the proliferative responses of PBMC from RAP-1-immunized cattle. PBMC ( $2 \times 10^5$ ) from bovine 2216 (A) and bovine 2234 (B) were assessed for proliferative responses against CM prepared from URBC (solid bars), *B. bigemina* (hatched bars), and *B. bovis* (stippled bars) before (pre) and at various weeks after the initial inoculation with native *B. bigemina* RAP-1 protein, as indicated on the x axis. The arrows indicate the times of antigen inoculation, which were weeks 0, 4, 7, and 41. Cells were cultured with antigen for 6 days, radiolabeled for 4 h, harvested, and counted as described in the text. The data are shown for 25  $\mu$ g of antigen per ml and are presented as the means  $\pm$  1 standard deviation of triplicate cultures.

assays were performed with preinfection sera and sera obtained 2 weeks following the third antigen inoculation. Sera from all four cattle contained a specific antibody against a 58-kDa antigen, which was shown after the sera were reacted against *B. bigemina* (Mexico) CM antigen (Fig. 1, lanes 1, 3, 5, and 7). Reactivity against control URBC CM antigen (Fig. 1, lanes 2, 4, 6, and 8) or either soluble or CM antigens from *B. bovis* (data not shown) was not detected, and preinoculation sera failed to detect any bands in similar assays (data not shown). The same 58-kDa band was observed in other *B. bigemina* parasite extracts, including soluble cytosolic antigen and merozoite membrane-enriched preparations (data not shown).

Hyperimmune sera from animals 2216 and 2234 were tested for RAP-1-specific IgG1 and IgG2 activity by ELISA, and there was little difference in the subclass-specific antibody titers. For these two animals, the IgG1 titers were 1,012 and 922 and the IgG2 titers were 1,354 and 1,383, respectively. There was no antibody response against control MBP.

**Lymphocyte proliferative responses of RAP-1-immunized cattle.** PBMC from immunized cattle were monitored for proliferative responses to *B. bigemina*, *B. bovis*, and URBC CM antigens prior to immunization, at biweekly intervals for 3 months following immunization, and periodically thereafter. PBMC from all four immunized cattle responded specifically to *B. bigemina* and not to URBC antigen. *B. bigemina*-induced proliferation was first observed at between 4 and 8 weeks following the first immunization and was present, although variable, throughout the duration of the study (42 weeks), as is shown for animals 2216 and 2234 in Fig. 2. The responses of animal 2238 were generally somewhat lower than those of animals 2216 and 2234, but they were nevertheless specific for *B. bigemina* CM (data not shown). The responses of animal 2242 were generally as high as those of animals 2216 and 2234, but occasionally nonspecific proliferation against *B. bovis* CM antigen was observed (data not shown). The finding that PBMC from animals 2216, 2234, and 2238 did not respond to *B. bovis* antigen indicates that the short amino acid sequences conserved between RAP-1 homologs of *B. bigemina* and *B. bovis* are not immunogenic for T cells from these cattle. Because strong and consistent *B. bigemina*-specific proliferative responses were obtained with animals 2216 and 2234, these cattle were used for subsequent experiments and for establishing T-cell lines and clones.

To verify that the proliferative response in PBMC from RAP-1-immunized animals stimulated with *B. bigemina* CM antigen was directed against RAP-1, assays with recombinant

TABLE 1. Proliferative responses of PBMC from *B. bigemina* RAP-1-immunized cattle to different geographical strains of *B. bigemina*

Antigen or parasite strain <sup>a</sup>	Mean cpm $\pm$ SD incorporated by PBMC (stimulation index) <sup>b</sup>	
	Bovine 2216	Bovine 2234
Medium	370 $\pm$ 300	157 $\pm$ 37
MBP	1,269 $\pm$ 270 (3.4)	181 $\pm$ 48 (1.2)
<i>B. bigemina</i> RAP-1	16,815 $\pm$ 103 (45.4)	33,926 $\pm$ 4,554 (216.0)
URBC	293 $\pm$ 59 (0.8)	151 $\pm$ 45 (1.0)
<i>B. bovis</i> Mexico (culture)	250 $\pm$ 224 (0.7)	303 $\pm$ 56 (1.9)
<i>B. bigemina</i> Mexico (culture)	11,923 $\pm$ 1,193 (32.2)	29,303 $\pm$ 3,418 (186.6)
<i>B. bigemina</i> Texcoco (blood)	3,023 $\pm$ 1,998 (8.2)	20,544 $\pm$ 3,864 (130.8)
<i>B. bigemina</i> St. Croix (blood)	3,279 $\pm$ 943 (8.9)	11,975 $\pm$ 3,139 (76.3)
<i>B. bigemina</i> Puerto Rico (blood)	1,599 $\pm$ 50 (4.3)	9,426 $\pm$ 1,640 (60.0)

<sup>a</sup> Antigens consisted of 5  $\mu$ g of recombinant MBP or *B. bigemina* RAP-1 fusion protein per ml or 25  $\mu$ g of CM derived from URBC per ml or each of the indicated parasites isolated from culture or infected blood.

<sup>b</sup> PBMC ( $2 \times 10^5$ ) were cultured in half-area 96-well plates with antigen for 6 days, radiolabeled for 4 h with [<sup>125</sup>I]iododeoxyuridine, harvested, and counted as described in the text. The results are expressed as the means  $\pm$  1 standard deviation and stimulation indices of triplicate cultures.

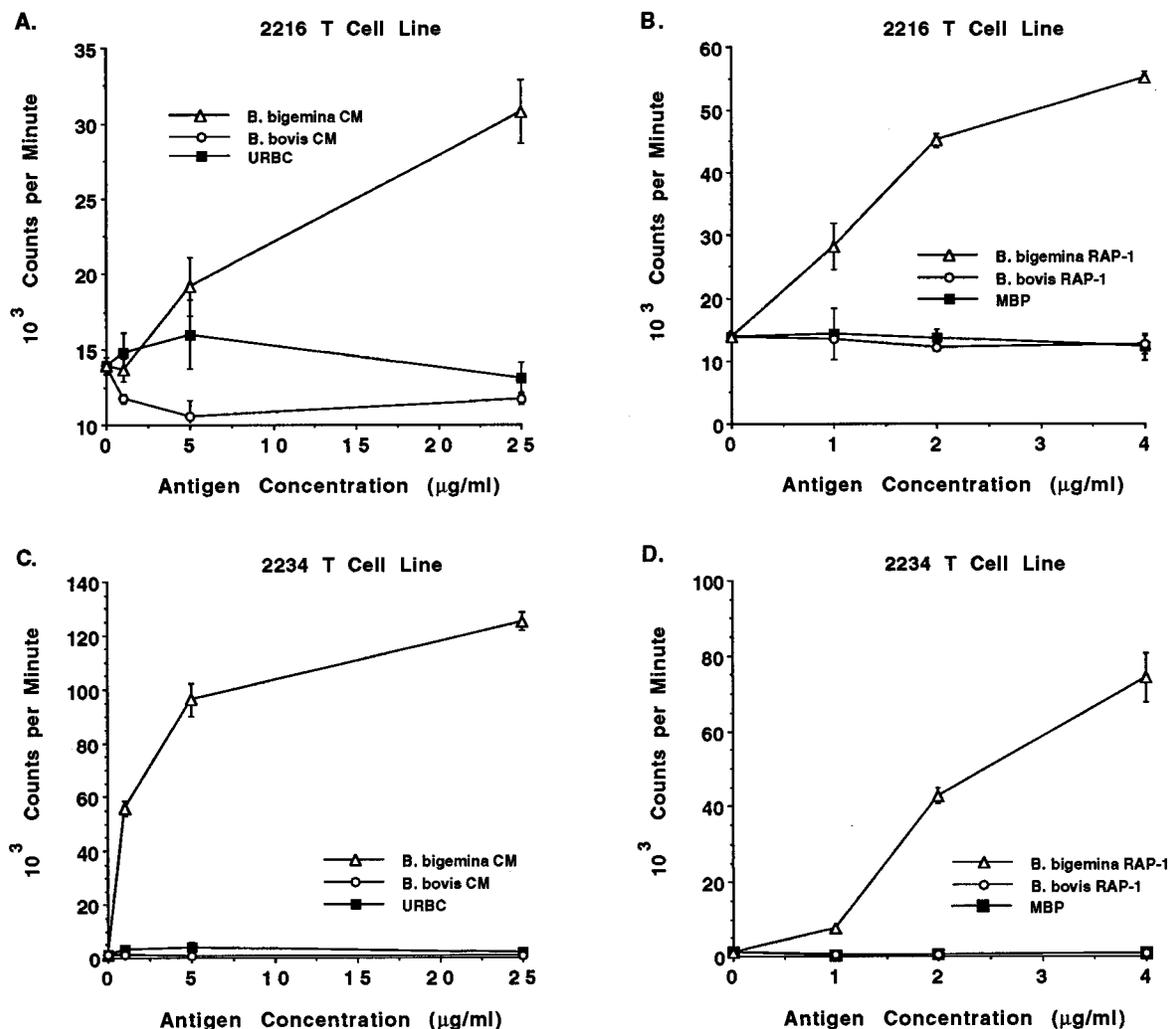


FIG. 3. Dose-dependent proliferative responses of T-cell lines derived from RAP-1-immunized cattle against recombinant RAP-1 protein. T cells ( $3 \times 10^4$ ) from RAP-1-stimulated T-cell lines obtained from bovines 2216 (A and B) and 2234 (C and D) were cultured for 3 days with 1 to 25  $\mu\text{g}$  of antigen per ml consisting of CM derived from *B. bigemina* (open triangles), *B. bovis* (open circles), and URBC (closed squares) (A and C) or 1 to 4  $\mu\text{g}$  of antigen per ml consisting of recombinant proteins *B. bigemina* RAP-1 (open triangles), *B. bovis* RAP-1 (open circles), and MBP (closed squares) (B and D). Cells were radiolabeled for 4 h, harvested, and counted. The results are presented as the means  $\pm$  1 standard deviation of duplicate cultures.

RAP-1 protein were performed. Table 1 shows that PBMC from immune animals 2216 and 2234 proliferated specifically to *B. bigemina* RAP-1 fusion protein but not to control MBP. Responses were antigen dose dependent, and optimal proliferation was generally achieved with 5  $\mu\text{g}$  of protein per ml. Additional experiments showed that PBMC from these cattle did not proliferate against *B. bovis* RAP-1 fusion protein (data not shown), again indicating the lack of cross-reactivity of T-cell epitopes in these protein homologs.

To determine the conservation of RAP-1 T-cell epitopes in different geographic strains of *B. bigemina*, PBMC from animals 2216 and 2234 were stimulated with homogenized parasite antigens derived from other blood-derived strains of *B. bigemina*, which included an additional isolate from Mexico (Texcoco strain) as well as strains from Puerto Rico and St. Croix. Although PBMC from both animals did respond to the CM antigen prepared from the different Central American strains, the responses were variable and consistently lower than those to CM antigen derived from *B. bigemina* (Mexico) parasites or recombinant RAP-1 fusion protein (Table 1). As is often observed with outbred animals, responses

varied among cattle, as exemplified by the higher levels of antigen-specific proliferation of PBMC from animal 2234 compared with those of PBMC from animal 2216 (Fig. 2 and Table 1).

***B. bigemina* RAP-1-specific T-cell lines.** One T-cell line derived from animal 2216 and stimulated with recombinant *B. bigemina* RAP-1 and a second T-cell line derived from animal 2234 and stimulated sequentially with *B. bigemina* CM antigen and recombinant RAP-1 protein were tested repeatedly for RAP-1-specific proliferative responses. Both cell lines proliferated vigorously in a dose-dependent manner to *B. bigemina* CM and RAP-1 protein but not to URBC CM, *B. bovis* CM, *B. bovis* RAP-1, or control MBP (Fig. 3).

Surface phenotypic analysis of these cell lines showed that at 5 and 7 weeks, respectively, the majority of T cells in the cultures were CD4<sup>+</sup> helper cells (Table 2), and in line 2234, CD4<sup>+</sup> T cells still predominated after 11 weeks of culture. In contrast, WC1<sup>+</sup>  $\gamma/\delta$  T cells eventually overgrew CD4<sup>+</sup> T cells in line 2216, as was previously observed for certain bovine T-cell lines stimulated with either *B. bovis* CM antigen (3) or recombinant Bb-1 protein (53).

TABLE 2. Cell surface phenotypic analysis of *B. bigemina* RAP-1-specific T-cell lines

T-cell line and weeks in culture <sup>a</sup>	Percentage of cells stained by MAb <sup>b</sup> :				
	MM1A (CD3)	IL-A26 (CD2)	IL-A12 (CD4)	IL-A51 (CD8)	IL-A29 (WC1)
Line 2216					
4	90	76	66	3	21
5	96	87	79	11	11
12	85	9	3	4	89
Line 2234					
7	71	72	66	4	ND
10	88	94	95	9	2
11	97	95	98	10	1

<sup>a</sup> PBMC obtained from bovine 2234 were stimulated with 25  $\mu$ g of *B. bigemina* (Mexico) CM per ml for 4 weeks and then with 5  $\mu$ g of recombinant *B. bigemina* RAP-1 protein per ml for an additional 7 weeks. PBMC obtained from bovine 2216 were stimulated with 5  $\mu$ g of *B. bigemina* RAP-1 protein per ml for 12 weeks. The cells were analyzed by indirect immunofluorescence and flow cytometry with MAbs directed against surface antigens.

<sup>b</sup> MAbs MM1A, IL-A26, IL-A12, IL-A51, and IL-A29 were directed against surface antigens CD3, CD2, CD4, CD8, and WC1, respectively. ND, not determined.

**Proliferative responses of *B. bigemina* RAP-1-specific T-cell clones.** Limiting dilution cloning of T-cell lines 2216 and 2234 resulted in cloning frequencies of 21 and 7%, respectively, indicating the probability that the clones were derived from a single precursor (23). Nine antigen-specific T-cell clones were obtained. All clones were Th cells that expressed the surface phenotype CD2<sup>+</sup> CD3<sup>+</sup> CD4<sup>+</sup> CD8<sup>-</sup> WC1<sup>-</sup> (data not shown). All clones responded specifically to *B. bigemina* RAP-1 and did not proliferate against *B. bovis* RAP-1 or control MBP (Table 3). All clones responded to CM antigen prepared from *B. bigemina* (Mexican strain) but did not respond to CM antigen from *B. bovis* (Table 4) or URBC (data not shown). A comparison of the proliferative responses of seven Th clones to CM antigen prepared from cultured *B. bigemina* (Mexican strain) parasites or from merozoites of different geographical isolates of *B. bigemina* in infected blood revealed differential responses of the T-cell clones (Table 4). One pattern of response was characterized by Th-cell clones that responded very poorly (i.e., <1 to 20% of the response to the Mexican strain) to the heterologous parasite strains (group I), whereas a second pattern of response was represented by two Th-cell clones that responded relatively better (i.e., 39 to 60% of the response to the Mexican strain) to the heterologous strains (group II).

Control CD4<sup>+</sup> clone C15.2G10 that was derived from a *B. bovis*-infected and immune cow and that was previously shown to respond to an antigen shared by *B. bovis* and *B. bigemina* parasites that is distinct from RAP-1 (7, 12) proliferated vigorously (i.e., 84 to 119% of the response to the Mexican strain) to all three heterologous strains of *B. bigemina*. This result indicates that the CM preparations derived from the heterologous strains are potentially strongly immunogenic for CD4<sup>+</sup> cells and not impaired in the ability to stimulate the T cells. Therefore, the differential patterns of response of the RAP-1-specific T-cell clones to heterologous parasite strains indicate the recognition of at least two different T-cell epitopes by this panel of T-cell clones.

The responses of all Th clones were shown to be MHC restricted and therefore not due to parasite superantigens, since APC from MHC-mismatched animal C15 were unable to present *B. bigemina* CM antigen to these clones (data not shown) but were effective at presenting antigen to autologous clone C15.2G10 (Table 4).

**Cytokine expression by RAP-1-specific Th-cell clones.** RT-PCR analysis of mRNA for IL-2, IL-4, IL-10, and IFN- $\gamma$  revealed that all clones expressed relatively high levels of IFN- $\gamma$  mRNA, negligible to high levels of IL-10 mRNA, but low or undetectable levels of IL-2 and IL-4 mRNA compared with levels of  $\beta$ -actin (Fig. 4A). The levels of IL-2 and IL-4 expression were obviously less than those observed with ConA-stimulated PBMC (Fig. 4B). The presence of inappropriately sized bands in the IL-2 and IL-4 PCR products from several of the Th clones is indicative of a nonspecific reaction caused by low levels of specific cytokine mRNA, as previously observed with some bovine Th-cell clones and negative control murine WEHI 164 cells (11). Following stimulation of RAP-1-specific Th-cell clones with *B. bigemina* RAP-1 antigen, all clones produced IFN- $\gamma$  protein in concentrations ranging from 41 to 98 U/ml. Although we cannot measure bovine IL-4 protein, the relatively low levels of IL-4 mRNA and high levels of IFN- $\gamma$  protein and mRNA classify these clones as either Th0 or Th1 cells, more broadly defined as type 1 T cells (1). These cytokine profiles, which do not include those characteristic of Th2 cells, are typical of those described previously for *B. bovis*-specific Th-cell clones derived from *B. bovis*-infected and -immune cattle (9–12, 14) and are also similar to those of *B. bovis* RAP-1-specific T-cell clones (8). These cytokine profiles differ from those of certain Th clones obtained from *Fasciola hepatica*-infected cattle, which were characterized by relatively high levels of IL-4 mRNA and low levels of IFN- $\gamma$  mRNA expression (4).

TABLE 3. Specificity of proliferative responses of *B. bigemina* RAP-1-specific Th-cell clones

Th-cell clone	Mean cpm $\pm$ SD incorporated by Th clones (stimulation index) <sup>a</sup> :		
	<i>B. bigemina</i> RAP-1	<i>B. bovis</i> RAP-1	MBP
2216.1E4	46,888 $\pm$ 1,920 (73.8)	617 $\pm$ 90 (1.0)	390 $\pm$ 13 (0.6)
2216.1G8	5,385 $\pm$ 590 (28.5)	ND	105 $\pm$ 63 (0.5)
2216.1H4	80,049 $\pm$ 576 (10.3)	3,361 $\pm$ 7 (0.4)	4,953 $\pm$ 170 (0.6)
2216.2B2	81,654 $\pm$ 2,119 (90.5)	452 $\pm$ 2 (0.5)	905 $\pm$ 1 (1.0)
2216.2C2	79,706 $\pm$ 1,149 (140.6)	363 $\pm$ 0 (0.6)	354 $\pm$ 170 (0.6)
2216.2C6	1,766 $\pm$ 609 (34.0)	ND	24 $\pm$ 0 (0.5)
2234.1E3	111,751 $\pm$ 5,324 (82.3)	425 $\pm$ 80 (0.3)	382 $\pm$ 121 (0.3)
2234.1F3	65,909 $\pm$ 697 (115.0)	85 $\pm$ 20 (0.2)	81 $\pm$ 16 (0.1)
2234.1G3	52,297 $\pm$ 2,163 (106.9)	303 $\pm$ 175 (0.6)	166 $\pm$ 3 (0.3)

<sup>a</sup> Th-cell clones ( $3 \times 10^4$  cells) were cultured for 3 days with  $2 \times 10^5$  APC and antigen, which consisted of the indicated recombinant proteins used at 4  $\mu$ g/ml, except for those for clones 2216.1G8 and 2216.2C2, for which the proteins were used at 5  $\mu$ g/ml. The proliferative responses of all Th clones cultured with *B. bigemina* RAP-1 antigen were shown to be significantly different from the proliferative responses of Th clones cultured with medium alone, *B. bovis* RAP-1, or MBP ( $P < 0.05$ ) by the Student one-tailed  $t$  test. The stimulation index is the mean counts per minute for Th cells cultured with antigen divided by the mean counts per minute for Th cells cultured with medium. ND, not determined in this assay.

TABLE 4. Proliferative responses of *B. bigemina* RAP-1-specific Th clones against different geographical strains of *B. bigemina* or *B. bovis*

Th-cell group and clone	Mean cpm $\pm$ SD incorporated by Th clones stimulated with CM (% response to <i>B. bigemina</i> Mexico) <sup>a</sup>				
	Cultured parasites		Blood-derived <i>B. bigemina</i> parasites		
	<i>B. bigemina</i> (Mexico)	<i>B. bovis</i> (Mexico)	Texcoco	St. Croix	Puerto Rico
<b>Group I</b>					
2216.1G8	42,367 $\pm$ 1,205	3 $\pm$ 1	2,629 $\pm$ 137 (6)	1,769 $\pm$ 22 (4)	841 $\pm$ 0 (2)
2216.2B2	77,369 $\pm$ 4,205	6 $\pm$ 5	9,183 $\pm$ 1,215 (12)	4,462 $\pm$ 166 (6)	9,228 $\pm$ 655 (12)
2216.2C2	62,634 $\pm$ 1,987	7 $\pm$ 7	2,531 $\pm$ 150 (4)	771 $\pm$ 107 (1)	2,628 $\pm$ 369 (4)
2234.1E3	92,993 $\pm$ 567	20 $\pm$ 0	18,887 $\pm$ 1,126 (20)	7,749 $\pm$ 277 (8)	14,220 $\pm$ 2,730 (15)
2234.1F3	34,378 $\pm$ 2,337	13 $\pm$ 9	1,457 $\pm$ 164 (4)	432 $\pm$ 32 (1)	28 $\pm$ 10 (<1)
<b>Group II</b>					
2216.1H4	57,269 $\pm$ 871	151 $\pm$ 28	25,593 $\pm$ 470 (45)	22,407 $\pm$ 161 (39)	25,567 $\pm$ 391 (46)
2216.2C6	5,636 $\pm$ 69	455 $\pm$ 162	3,185 $\pm$ 321 (57)	3,362 $\pm$ 39 (60)	3,351 $\pm$ 107 (60)
<b>Control</b>					
C15.2G10	11,672 $\pm$ 114	10,327 $\pm$ 128	13,949 $\pm$ 137 (119)	9,839 $\pm$ 209 (84)	13,921 $\pm$ 416 (119)

<sup>a</sup> The CM fraction was prepared from merozoites derived from either cultured parasites or infected bovine blood. Protein was assayed at a concentration of 25  $\mu$ g/ml.

## DISCUSSION

RAPs of malarial and babesial parasites are promising candidates for subunit vaccine development (20, 31, 32, 40). These antigens contain sequences and/or antigenic epitopes that are highly conserved among geographically different parasite strains, are usually soluble, and in native and recombinant form have been shown to afford animals partial protection against challenge infection (20, 31, 36, 57). However, there was little correlation between antibody titers and protective immunity in animals immunized with the native RAPs of these parasites or animals that had recovered from infection (36, 48, 55, 57). These observations underscore the importance of Th cells in protective immunity against bovine babesiosis (10), and the studies reported here with *B. bigemina* RAP-1 characterize, for the first time, RAP-1-specific Th-cell responses in RAP-1-immune cattle.

In this study, immunization of four calves with native *B. bigemina* RAP-1 protein in RIBI adjuvant led to strong anamnestic responses of PBMC from all animals against antigen presented as CM from *B. bigemina* merozoites. The induction of this response occurred after the second or third antigen inoculation and was sustained throughout the 42-week observation period. T-cell proliferative responses have been reported previously for *B. bovis*-inoculated animals (5–14, 53, 54), but this is the first study to examine specific T-cell responses in cattle immunized with a *B. bigemina* protein. Furthermore, studies with a single *B. bigemina*-infected cow showed detection of RAP-1-specific proliferation up to 11 months postinfection (data not shown). This observation is consistent with our finding of the highly Th-cell-immunodominant nature of *B. bovis* RAP-1, which was still recognized by T-cell lines derived from *B. bovis*-infected cattle after 7 weeks of culture with unfractionated merozoite CM antigen (8).

Immunization with *B. bigemina* RAP-1 protein in RIBI adjuvant induced a predominant CD4<sup>+</sup> T-cell response, as predicted for an exogenous soluble protein antigen, that was characterized by type 1 cytokine expression. At the clonal level, RAP-1-specific T cells all produced high levels of IFN- $\gamma$  following stimulation with parasite antigen. Although these IFN- $\gamma$  titers (which ranged from 41 to 98 U/ml) are lower than those observed for the majority of *B. bovis*-specific Th-cell clones stimulated with ConA (i.e., >125 U/ml), they are higher than those of the same *B. bovis*-specific clones stimulated with specific antigen and APC (i.e., <16 U/ml) (12). RT-PCR analysis of cytokine mRNA revealed consistently high levels of IFN- $\gamma$  and relatively low levels of IL-2 and IL-4, showing that

the majority of T-cell clones expressed Th0 cytokine profiles, characterized by the expression of IL-2, IL-4, and IFN- $\gamma$  or IL-4 and IFN- $\gamma$ . Two clones could be classified as Th1 cells which expressed IL-2 and IFN- $\gamma$  mRNA. IL-10 was expressed by all of the Th-cell clones. The expression of IL-10 mRNA by different subsets of Th cells has been previously reported and is not diagnostic of a Th2 response in cattle (11). The diversity of cytokine mRNA expression patterns among the different T-cell clones and lack of a strict dichotomy of either Th1 or Th2 responses against RAP-1 have also been previously observed with Th-cell clones from *B. bovis*-infected and -immune cattle directed against a variety of different antigens (7–10, 12). These findings likely reflect the complexity of the Th-cell cytokine response, which in our view and that of Kelso (34) constitutes a spectrum characterized by Th1 clones on one end and Th2 clones on the other.

In studies to be reported elsewhere (46), analysis of cytokine mRNA in the draining lymph nodes of immunized animals 2216 and 2234 showed that IFN- $\gamma$  mRNA expression was up-regulated whereas that of IL-4 was not. Upon secondary stim-

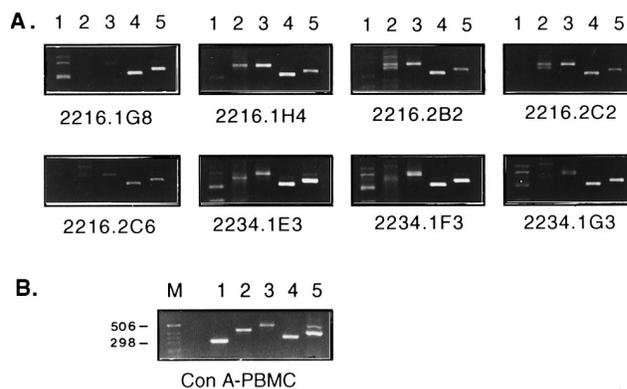


FIG. 4. Analysis of cytokine mRNA expression in *B. bigemina* RAP-1-specific Th-cell clones by RT-PCR. RT-PCR was performed with total cellular RNA prepared from eight Th-cell clones as indicated under each gel (A) or from ConA-stimulated PBMC (positive control) (B). Primers specific for bovine IL-2 (lane 1), IL-4 (lane 2), IL-10 (lane 3), IFN- $\gamma$  (lane 4), and  $\beta$ -actin (lane 5) were used. The amplified PCR products were electrophoresed on agarose gels, stained with ethidium bromide, and visualized and photographed with UV light. The sizes of the amplified PCR products are as follows: IL-2, 300 bp; IL-4, 423 bp; IL-10, 482 bp; IFN- $\gamma$ , 347 bp; and  $\beta$ -actin, 400 bp. Molecular weight markers (M), consisting of a 1-kb DNA ladder ranging from 506 to 220 kb (shown for ConA-PBMC), were included in each gel.

ulation of the lymph node cells with antigen, IFN- $\gamma$  protein and mRNA levels increased over several days, whereas IL-4 mRNA levels did not, reflecting what was observed with the peripheral blood-derived T-cell clones. Taking into consideration the overall response to this antigen, which is characterized by relatively high levels of IFN- $\gamma$  and low levels of IL-4 expression, we conclude that RAP-1, administered in RIBI adjuvant, induced a predominant type 1 immune response in the two cattle examined.

In spite of the induction of a type 1 cytokine response by RAP-1 in these cattle, RAP-1-specific serum IgG1 and IgG2 titers were not appreciably different. Since it is known that bovine IFN- $\gamma$  upregulates IgG2 production (24) and IL-4 upregulates IgG1 production (25) by bovine B cells, this finding likely reflects the fact that both cytokines are expressed by RAP-1-immune T cells, albeit at different levels.

On the basis of evidence from related hemoprotozoan malarial parasites, a type 1 immune response against babesiosis is hypothesized to be required for protective immunity (10). It has been demonstrated by the adoptive transfer of T cells in murine models of malaria that CD4<sup>+</sup> T cells are required for protective immunity against the erythrocytic stages of infection and that cloned Th1 and Th2 cells can each confer protection (reviewed in reference 52). Th1 cells are believed to provide protection during the primary parasitemia of *Plasmodium chabaudi chabaudi* infection, when IFN- $\gamma$  levels were shown to correlate with the control of parasitemia. Through their mediators (IFN- $\gamma$  and tumor necrosis factor beta), Th1 cells can activate macrophages to produce antiparasitic molecules, such as nitric oxide, which can reduce parasitemia. Malaria-specific Th1 cells were also shown to directly produce nitric oxide (52). Thus, the finding that native RAP-1 protein can induce an anamnestic type 1 immune response that is activated during in vitro reexposure to either native parasite antigen or recombinant protein argues for the potential use of recombinant protein or epitopes derived therefrom as a vaccine that could prime for specific recall responses upon exposure to natural infection.

Another important finding from this study is that in a manner similar to that of serologically defined B-cell epitopes, some Th-cell epitopes are conserved among the Central American strains of *B. bigemina*. PBMC from cattle immunized with RAP-1 affinity purified from a Mexico strain of *B. bigemina* proliferated against CM prepared from all other strains. However, the responses to the heterologous strains were less strong than those to the homologous Mexico strain. This differential response was more pronounced at the clonal level, at which the majority of Th-cell clones (group I) responded either very poorly or not at all to the heterologous strains whereas two clones (group II) responded with a pattern similar to that of PBMC. The poor stimulation of RAP-1-specific T cells by the heterologous parasites is not due to an inherently poorly antigenic preparation of CM, since a separate Th-cell clone (C15.2G10) which was derived from a *B. bovis*-immune animal and which recognizes a non-RAP-1-specific T-cell epitope that is shared by both parasite species proliferated vigorously to CM derived from all heterologous strains. Thus, the differential responses of PBMC and Th-cell clones to different *B. bigemina* strains indicate that at least one T-cell epitope (recognized by group II clones) is conserved while others may be variant among the different strains. An alternative possibility is that the expressed gene copies (Bbg-13 and possibly Bbg-7) represented in the affinity-purified native protein used for immunization are differentially expressed in the different strains. The lack of cross-reactivity of *B. bigemina* RAP-1-specific T-cells with *B. bovis* RAP-1 indicates that neither the strictly conserved 14-amino-acid peptide nor the additional shorter

peptides shared by RAP-1 homologs of these parasites (49) are immunodominant Th-cell epitopes. The lack of immunogenicity of conserved epitopes for both B cells (50) and Th cells between species implies the functional significance of these residues, which may contribute to the persistence of these parasites. Experiments are in progress to precisely map the T-cell epitopes recognized by the two groups of clones (30) and to characterize the expression of the different gene copies in different parasite strains.

The following conclusions on the Th-cell response against RAP-1 provide a rationale for developing a subunit vaccine for *B. bigemina* that includes Th-cell epitopes as well as neutralizing antibody epitopes of RAP-1. (i) RAP-1 is an immunodominant protein for Th cells, inducing recall responses in *Babesia*-inoculated cattle and strong anamnestic T-cell proliferative responses against recombinant as well as native parasite antigen in immunized cattle. (ii) RAP-1 stimulates IFN- $\gamma$ -producing type 1 CD4<sup>+</sup> T-cell responses in immunized cattle. (iii) Both IgG1 and IgG2 antibodies, which are complement-fixing and opsonizing antibodies in cattle (39), are produced in response to immunization. (iv) At least one immunodominant Th-cell epitope is antigenically conserved among different parasite strains.

#### ACKNOWLEDGMENTS

We thank Beverly Hunter, Eldon Lipstaff, Kathleen Logan, and Carla Robertson for expert technical assistance.

This research was supported by United States Department of Agriculture US-Israel Binational Agricultural Research and Development Fund grants US-1855-90R and US-2496-94C, National Institutes of Health grant AI30136, USDA NRICGP grants 92-37204-8180 and 93-37206-9075, and a Fulbright Research Scholarship awarded to S.D.R.

#### REFERENCES

- Bloom, B. R., P. Salgame, and B. Diamond. 1992. Revisiting and revising suppressor T cells. *Immunol. Today* **13**:131-136.
- Bock, R. E., A. J. de Vos, T. G. Kingston, I. A. Shiels, and R. J. Dalglish. 1992. Investigations of breakdowns in protection provided by living *Babesia bovis* vaccine. *Vet. Parasitol.* **43**:45-56.
- Brown, W. C., W. C. Davis, S. H. Choi, D. A. E. Dobbelaere, and G. A. Splitter. 1994. Functional and phenotypic characterization of WC1<sup>+</sup>  $\gamma\delta$  T cells isolated from *Babesia bovis*-stimulated T cell lines. *Cell. Immunol.* **153**:9-27.
- Brown, W. C., W. C. Davis, D. A. E. Dobbelaere, and A. C. Rice-Ficht. 1994. CD4<sup>+</sup> T-cell clones obtained from cattle chronically infected with *Fasciola hepatica* and specific for adult worm antigen express both unrestricted and Th2 cytokine profiles. *Infect. Immun.* **62**:818-827.
- Brown, W. C., and K. S. Logan. 1992. *Babesia bovis*: bovine helper T cell lines reactive with soluble and membrane antigens of merozoites. *Exp. Parasitol.* **74**:188-199.
- Brown, W. C., K. S. Logan, G. G. Wagner, and C. L. Tetzlaff. 1991. Cell-mediated immune responses to *Babesia bovis* merozoite antigens in cattle following infection with tick-derived or cultured parasites. *Infect. Immun.* **59**:2418-2426.
- Brown, W. C., K. S. Logan, S. Zhao, D. K. Bergman, and A. C. Rice-Ficht. 1995. Identification of *Babesia bovis* merozoite antigens separated by continuous-flow electrophoresis that stimulate proliferation of helper T-cell clones derived from *B. bovis*-immune cattle. *Infect. Immun.* **63**:3106-3116.
- Brown, W. C., T. F. McElwain, B. J. Ruef, C. E. Suarez, V. Shkap, C. G. Chitko-McKown, A. C. Rice-Ficht, and G. H. Palmer. Submitted for publication.
- Brown, W. C., G. H. Palmer, T. F. McElwain, S. A. Hines, and D. A. E. Dobbelaere. 1993. *Babesia bovis*: characterization of the T helper cell response against the 42-kDa merozoite surface antigen (MSA-1) in cattle. *Exp. Parasitol.* **79**:97-110.
- Brown, W. C., and A. C. Rice-Ficht. 1994. Use of helper T cells to identify potentially protective antigens of *Babesia bovis*. *Parasitol. Today* **10**:145-149.
- Brown, W. C., V. M. Woods, C. G. Chitko-McKown, S. M. Hash, and A. C. Rice-Ficht. 1994. Interleukin-10 is expressed by bovine type 1 helper, type 2 helper, and unrestricted parasite-specific T-cell clones and inhibits proliferation of all three subsets in an accessory-cell-dependent manner. *Infect. Immun.* **62**:4697-4708.
- Brown, W. C., V. M. Woods, D. A. E. Dobbelaere, and K. S. Logan. 1993. Heterogeneity in cytokine profiles of *Babesia bovis*-specific bovine CD4<sup>+</sup> T

- cell clones activated in vitro. *Infect. Immun.* **61**:3273–3281.
13. Brown, W. C., S. Zhao, A. C. Rice-Ficht, K. S. Logan, and V. M. Woods. 1992. Bovine helper T cell clones recognize five distinct epitopes on *Babesia bovis* merozoite antigens. *Infect. Immun.* **60**:4364–4372.
  14. Brown, W. C., S. Zhao, V. M. Woods, C. A. Tripp, C. L. Tetzlaff, V. T. Heussler, D. A. E. Dobbelaere, and A. C. Rice-Ficht. 1993. Identification of two Th1 cell epitopes on the *Babesia bovis*-encoded 77-kilodalton merozoite protein (Bb-1) by use of truncated recombinant fusion proteins. *Infect. Immun.* **61**:236–244.
  15. Callow, L. L. 1977. Vaccination against bovine babesiosis, p. 121–149. In L. H. Miller, J. A. Pino, and J. J. McKelvey (ed.), *Immunity to blood parasites of animals and man*. Plenum Press, New York.
  16. Callow, L. L., and L. T. Mellors. 1966. A new vaccine for *Babesia argentina* infection prepared in splenectomized calves. *Aust. Vet. J.* **42**:464–465.
  17. Cerretti, D. P., K. McKereghan, A. Larsen, M. A. Cantrell, D. Anderson, S. Gillis, D. Cosman, and P. E. Baker. 1986. Cloning, sequence, and expression of bovine interleukin 2. *Proc. Natl. Acad. Sci. USA* **83**:3223–3227.
  18. Cerretti, D. P., K. McKereghan, A. Larsen, D. Cosman, S. Gillis, and P. E. Baker. 1986. Cloning, sequence, and expression of bovine interferon-gamma. *J. Immunol.* **136**:4561–4564.
  19. Chitko-McKown, C. G., B. J. Ruef, A. C. Rice-Ficht, and W. C. Brown. 1995. Interleukin-10 downregulates proliferation and expression of interleukin-2 receptor p55 chain and interferon- $\gamma$ , but not interleukin-2 or interleukin-4, by parasite-specific helper T cell clones obtained from cattle chronically infected with *Babesia bovis* or *Fasciola hepatica*. *J. Interferon Cytokine Res.* **15**:915–922.
  20. Dalrymple, B. P. 1993. Molecular variation and diversity in candidate vaccine antigens from *Babesia*. *Acta Trop.* **53**:227–238.
  21. Dalrymple, B. P., R. E. Casu, J. M. Peters, C. M. Dimmock, K. R. Gale, R. Boese, and I. G. Wright. 1993. Characterisation of a family of multi-copy genes encoding rhoptry protein homologues in *Babesia bovis*, *Babesia ovis* and *Babesia canis*. *Mol. Biochem. Parasitol.* **57**:181–192.
  22. Degen, J. L., M. G. Neubauer, S. J. Friezner Degen, C. E. Seyfried, and D. R. Morris. 1983. Regulation of protein synthesis in mitogen-activated bovine lymphocytes. Analysis of actin-specific and total mRNA accumulation and utilization. *J. Biol. Chem.* **258**:12153–12162.
  23. de St. Groth, S. F. 1982. The evaluation of limiting dilution assays. *J. Immunol. Methods* **49**:R11–R223.
  24. Estes, D. M., N. M. Closser, and G. K. Allen. 1994. IFN- $\gamma$  stimulates IgG<sub>2</sub> production from bovine B cells costimulated with anti- $\mu$  and mitogen. *Cell. Immunol.* **154**:287–295.
  25. Estes, D. M., A. Hirano, V. T. Heussler, D. A. E. Dobbelaere, and W. C. Brown. 1995. Expression and biological activities of bovine interleukin 4: effects of recombinant bovine interleukin 4 on T cell proliferation and B cell differentiation and proliferation in vitro. *Cell. Immunol.* **163**:268–279.
  26. Figueroa, J. V., and G. M. Buening. 1991. In vitro inhibition of multiplication of *Babesia bigemina* by using monoclonal antibodies. *J. Clin. Microbiol.* **29**:997–1003.
  27. Goff, W. L., W. C. Davis, G. H. Palmer, T. F. McElwain, W. C. Johnson, J. F. Bailey, and T. C. McGuire. 1988. Identification of *Babesia bovis* merozoite surface antigens by using immune bovine sera and monoclonal antibodies. *Infect. Immun.* **56**:2363–2368.
  28. Hash, S. M., W. C. Brown, and A. C. Rice-Ficht. 1994. Characterization of a cDNA encoding bovine interleukin-10: kinetics of expression in bovine lymphocytes. *Gene* **139**:257–261.
  29. Heussler, V. T., M. Eichorn, and D. A. E. Dobbelaere. 1992. Cloning of a full-length cDNA encoding bovine IL-4 by the polymerase chain reaction. *Gene* **114**:273–278.
  30. Hötzel, I., W. C. Brown, T. F. McElwain, S. D. Rodriguez, and G. H. Palmer. Submitted for publication.
  31. Howard, R. J., and B. L. Pasloske. 1993. Target antigens for asexual malaria vaccine development. *Parasitol. Today* **9**:369–372.
  32. Jakobsen, P. H., L. Hviid, T. G. Theander, E. A. Afare, R. G. Ridley, P. M. H. Heegaard, D. Stuber, K. Dalsgaard, and F. K. Nkrumah. 1993. Specific T-cell recognition of the merozoite proteins rhoptry-associated protein 1 and erythrocyte-binding antigen 1 of *Plasmodium falciparum*. *Infect. Immun.* **61**:268–273.
  33. Jorgensen, W. K., A. J. de Vos, and R. J. Dalgliesh. 1989. Comparison of immunogenicity and virulence between *Babesia bigemina* from continuous culture and from a splenectomized calf. *Aust. Vet. J.* **66**:371–372.
  34. Kelso, A. 1995. Th1 and Th2 subsets: paradigms lost? *Immunol. Today* **16**:374–379.
  35. McElwain, T. F., L. E. Perryman, W. C. Davis, and T. C. McGuire. 1987. Antibodies define multiple proteins with epitopes exposed on the surface of live *Babesia bigemina* merozoites. *J. Immunol.* **138**:2298–2304.
  36. McElwain, T. F., L. E. Perryman, A. J. Musoke, and T. C. McGuire. 1991. Molecular characterization and immunogenicity of neutralization-sensitive *Babesia bigemina* merozoite surface proteins. *Mol. Biochem. Parasitol.* **47**:213–222.
  37. Mishra, V. S., T. F. McElwain, J. B. Dame, and E. B. Stephens. 1992. Isolation, sequence, and differential expression of the p58 gene family of *Babesia bigemina*. *Mol. Biochem. Parasitol.* **53**:149–158.
  38. Mishra, V. S., E. B. Stephens, J. B. Dame, L. E. Perryman, T. C. McGuire, and T. F. McElwain. 1991. Immunogenicity and sequence analysis of recombinant p58: a neutralization-sensitive, antigenically conserved *Babesia bigemina* merozoite surface protein. *Mol. Biochem. Parasitol.* **47**:207–212.
  39. Musoke, A. J., F. R. Rurangirwa, and V. M. Nantulya. 1986. Biological properties of bovine immunoglobulins and systemic antibody responses, p. 391–408. In W. I. Morrison (ed.), *The ruminant immune system in health and disease*. Cambridge University Press, Cambridge.
  40. Palmer, G. H., and T. F. McElwain. 1995. Molecular basis for vaccine development against anaplasmosis and babesiosis. *Vet. Parasitol.* **57**:233–253.
  41. Palmer, G. H., T. F. McElwain, L. E. Perryman, W. C. Davis, D. R. Reduker, D. P. Jasmer, V. Shkap, E. Pipano, W. L. Goff, and T. C. McGuire. 1991. Strain variation of *Babesia bovis* merozoite surface-exposed epitopes. *Infect. Immun.* **59**:3340–3342.
  42. Perkins, M. E. 1992. Rhoptry organelles of apicomplexan parasites. *Parasitol. Today* **8**:28–32.
  43. Ridley, R. G., K. Takacs, H. Etlinger, and J. G. Scaife. 1990. A rhoptry antigen of *Plasmodium falciparum* is protective in *Saimiri* monkeys. *Parasitology* **101**:187–192.
  44. Rodriguez, S. D., G. M. Buening, T. J. Green, and C. A. Carson. 1983. Cloning of *Babesia bovis* by in vitro cultivation. *Infect. Immun.* **42**:15–18.
  45. Rogers, R. J., C. K. Dimmock, A. J. de Vos, and B. J. Rodwell. 1988. Bovine leucosis virus contamination of a vaccine produced *in vivo* against bovine babesiosis and anaplasmosis. *Aust. Vet. J.* **65**:285–287.
  46. Ruef, B. J., S. D. Rodriguez, A. J. Roussel, G. H. Palmer, T. F. McElwain, C. G. Chitko-McKown, A. C. Rice-Ficht, and W. C. Brown. Unpublished data.
  47. Shompole, S., T. F. McElwain, D. P. Jasmer, S. A. Hines, J. Katende, A. J. Musoke, F. R. Rurangirwa, and T. C. McGuire. 1994. Identification of *Babesia bigemina* infected erythrocyte surface antigens containing epitopes conserved among strains. *Parasite Immunol. (Oxford)* **16**:119–127.
  48. Siddiqui, W. A., L. Q. Tam, K. J. Kramer, G. S. N. Hui, S. E. Case, K. M. Yamaga, S. P. Chang, E. B. T. Chan, and S. C. Kan. 1987. Merozoite surface coat precursor protein completely protects *Aotus* monkeys against *Plasmodium falciparum* malaria. *Proc. Natl. Acad. Sci. USA* **84**:3014–3018.
  49. Suarez, C. E., T. F. McElwain, E. B. Stephens, V. S. Mishra, and G. H. Palmer. 1991. Sequence conservation among merozoite apical complex proteins of *Babesia bovis*, *Babesia bigemina* and other apicomplexa. *Mol. Biochem. Parasitol.* **49**:329–332.
  50. Suarez, C. E., G. H. Palmer, S. A. Hines, and T. F. McElwain. 1993. Immunogenic B-cell epitopes of *Babesia bovis* rhoptry-associated protein 1 are distinct from sequences conserved between species. *Infect. Immun.* **61**:3511–3517.
  51. Suarez, C. E., G. H. Palmer, D. P. Jasmer, S. A. Hines, L. E. Perryman, and T. F. McElwain. 1991. Characterization of the gene encoding a 60-kilodalton *Babesia bovis* merozoite protein with conserved and surface exposed epitopes. *Mol. Biochem. Parasitol.* **46**:45–52.
  52. Taylor-Robinson, A. W. 1995. Regulation of immunity to malaria: valuable lessons learned from murine models. *Parasitol. Today* **11**:334–342.
  53. Tetzlaff, C. L., A. C. Rice-Ficht, V. M. Woods, and W. C. Brown. 1992. Induction of proliferative responses of T cells from *Babesia bovis*-immune cattle with a recombinant 77-kilodalton merozoite protein (Bb-1). *Infect. Immun.* **60**:644–652.
  54. Timms, P., N. P. Stewart, B. J. Rodwell, and D. N. Barry. 1984. Immune responses of cattle following vaccination with living and non-living *Babesia bovis* antigens. *Vet. Parasitol.* **16**:243–251.
  55. Ushé, T. C., G. H. Palmer, L. Sotomayor, J. V. Figueroa, G. M. Buening, L. E. Perryman, and T. F. McElwain. 1994. Antibody response to a *Babesia bigemina* rhoptry-associated protein 1 surface-exposed and neutralization-sensitive epitope in immune cattle. *Infect. Immun.* **62**:5698–5701.
  56. Vega, C. A., G. M. Buening, T. J. Green, and C. A. Carson. 1985. *In vitro* cultivation of *Babesia bigemina*. *Am. J. Vet. Res.* **46**:416–420.
  57. Wright, I. G., R. Casu, M. A. Commins, B. P. Dalrymple, K. R. Gale, B. V. Goodger, P. W. Riddles, D. J. Waltisbuhl, I. Abetz, D. A. Berrie, Y. Bowles, C. Dimmock, T. Hayes, H. Kalnins, G. Leatch, R. McCrae, P. E. Montague, I. T. Nisbet, F. Parrodi, J. M. Peters, C. Scheiwe, W. Smith, K. Rode-Bramanis, and M. A. White. 1992. The development of a recombinant *Babesia* vaccine. *Vet. Parasitol.* **44**:3–13.