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Babesia bovis Rhoptry-Associated Protein 1 Is Immunodominant for T Helper Cells of Immune Cattle and Contains T-Cell Epitopes Conserved among Geographically Distant *B. bovis* Strains

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The ability of rhoptry-associated protein 1 (RAP-1) of *Babesia bovis* and *Babesia bigemina* to confer partial protective immunity in cattle has stimulated interest in characterizing both B-cell and T-cell epitopes of these proteins. It was previously shown that *B. bovis* RAP-1 associates with the merozoite surface as well as rhoptries and expresses B-cell epitopes conserved among otherwise antigenically different *B. bovis* strains. An amino-terminal 307-amino-acid domain of the molecule that is highly conserved in the *B. bigemina* RAP-1 homolog did not contain cross-reactive B-cell epitopes. The studies reported here demonstrate that *B. bovis* RAP-1 is strongly immunogenic for T helper (Th) cells from *B. bovis*-immune cattle and that like B-cell epitopes, Th-cell epitopes are conserved in different *B. bovis* strains but not in *B. bigemina* RAP-1. Lymphocytes from cattle immune to challenge with the Mexico strain of *B. bovis* proliferated against recombinant *B. bovis* RAP-1 protein derived from the Mexico strain. T-cell lines established by stimulating lymphocytes with recombinant RAP-1 protein responded against *B. bovis*, but not *B. bigemina*, merozoites. T-cell lines established by repeated stimulation of lymphocytes with *B. bovis* membrane antigen proliferated strongly against RAP-1, demonstrating the immunodominant nature of this protein. RAP-1-specific $CD4^+$ T cell clones recognized Mexico, Texas, Australia, and Israel strains of *B. bovis* but neither *B. bigemina* merozoites nor recombinant *B. bigemina* RAP-1. Analysis of cytokine mRNA in RAP-1-specific Th cell clones revealed strong expression of gamma interferon but little or no expression of interleukin-2 (IL-2), IL-4, or IL-10. Gamma interferon production was confirmed by enzyme-linked immunosorbent assay. These results indicate the potential to use selected *B. bovis* RAP-1 peptides as immunogens to prime for strong, anamnestic, strain-cross-reactive type 1 immune responses upon exposure to *B. bovis*.

Babesia bovis is an economically important hemoprotozoan parasite of cattle that is prevalent in many tropical and subtropical regions of the world. Vaccination of cattle with attenuated *B. bovis* merozoites derived from either infected cattle or in vitro cultures results in protective immunity against challenge infection with both homologous and heterologous strains (15). The feasibility of developing a successful subunit babesial vaccine is based on the observation that immunization with nonliving parasite extracts or highly purified proteins can induce partial protection against subsequent challenge (7, 31, 40, 51).

Proteins derived from apical complex organelles, including micronemes, spherical bodies, dense granules, and rhoptries, are candidates for recombinant vaccines of both babesial and related malarial parasites (16, 26, 35, 37). The existence of these proteins as homologs in different genera of apicomplexan parasites implies their functional relevance (35, 37, 44, 51). These proteins, which are often soluble and secreted by the parasite, are believed to play a major role in host erythrocyte invasion or possibly egression (24, 37). Several apical complex proteins of *B. bovis* have been described. Among these are 225-kDa (27) and 77- to 80-kDa (24, 50) spherical body proteins that are also associated with the inner surface of the infected erythrocyte membrane and the 60-kDa rhoptry-associated protein 1 (RAP-1), which is also present on the surface of a proportion of merozoites (20, 45). *B. bovis* RAP-1, also designated T21B4 by Commins et al. and Wright and coworkers (14, 51), was reported to be present in minute amounts in the merozoite and associated with a parasite fraction containing protease activity. *B. bovis* RAP-1 is a member of a multigene family of 58- to 60-kDa RAP-1 proteins also identified in *Babesia bigemina*, *Babesia bovis*, and *Babesia canis* parasites (17, 31, 33, 34, 43–46). Australia and cloned Mexico strains of *B. bovis* express two tandem gene copies of RAP-1 (17, 43), which in the Australia strain appear to have similar sequences and to have arisen by gene duplication (17). Four different gene copies of *B. bigemina rap-1* were identified in a cloned Mexico strain of *B. bigemina* (33, 34). The *B. bovis rap-1* gene derived from the Mexico strain encodes an open reading frame of 565 amino acids (46). Residues 317 to 477 of *B. bovis* RAP-1 consist of seven tandem repeats of a degenerate 23-amino-acid

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sequence (44, 46). The carboxy-terminal 163 amino acids are highly conserved (93% identity) between the Mexico and Australia strains (17). Residues 1 to 307 of *B. bovis* RAP-1 are highly conserved (45% identity) with residues 1 to 319 of *B. bigemina* RAP-1 and include a strictly conserved 14-amino-acid sequence (44).

Analysis of RAP-1-specific serological responses from cattle naturally or experimentally infected with *B. bovis* has shown that merozoite surface epitopes defined by monoclonal antibodies (MAbs) are conserved among the Mexico, Texas, Brazil, Argentina, Australia, and Israel strains of *B. bovis* but not with *B. bigemina* (29, 36, 43). Furthermore, surface B-cell epitopes of RAP-1 are distinct from sequences conserved between *B. bovis* and *B. bigemina* that are predicted to constitute functional domains, including the species-conserved 14-amino-acid sequence (45).

The ability of rhoptry-associated proteins to induce partial protective immunity against parasite challenge was shown in studies with *Plasmodium falciparum* using native (42) or recombinant proteins (38), with *B. bovis* using partially purified native RAP-1 protein or a recombinant glutathione *S*-transferase fusion protein consisting of a fragment of the *B. bovis* RAP-1 protein (51), and with affinity-purified native *B. bigemina* RAP-1 protein (31). Interestingly, in all of these studies, the titer of specific antibody to RAP-1 did not correlate with the degree of protective immunity, thus underscoring the importance of characterizing $CD4^+$ T cells specific for RAP-1 and other babesial apical complex proteins as potential effector and helper cells relevant to the development of epitope-based vaccines for *B. bovis.*

An optimal vaccine would include peptides that contain T- and B-cell epitopes capable of stimulating anamnestic cellular and humoral immunity upon natural exposure to the parasite (21). The nature of acquired resistance to challenge infection with *B. bovis* is poorly defined, and little is known about either the phenotype of the T cells responding to *B. bovis* or the antigens that evoke protective T-cell responses in cattle. Because the replication of *B. bovis* occurs selectively within bovine erythrocytes, which do not express major histocompatibility complex (MHC) antigens and therefore cannot serve as targets of MHC-restricted cytolytic activity, we have focused our research on characterizing antigen-specific $CD4^+$ T helper (Th) cell responses in immune cattle against different *B. bovis* antigens (7). The present study examines the memory Th cell response against the 60-kDa *B. bovis* RAP-1 protein in cattle immune to challenge infection. We demonstrate that RAP-1 is an immunodominant antigen for Th cells from immune cattle, that T-cell epitopes are conserved among diverse strains of *B. bovis*, and that RAP-1-specific Th cell clones express type 1 cytokine profiles.

MATERIALS AND METHODS

Babesial parasite strains and cultivation. The Mexico strain of *B. bovis* was originally isolated in 1978 from cattle infected with natural, tick-derived isolates from an area of Mexico where *Babesia* parasites are endemic (4). The Texas strain of *B. bovis* originated from an infected animal in southern Texas in 1978 (36) and was provided by Will Goff (USDA Agricultural Research Service, Pullman, Wash.). The Australia L strain of *B. bovis* originated from an infected animal in New South Wales, Australia, in 1965 (30) and was kindly provided by Ian Wright (Commonwealth Scientific and Industrial Research Organisation, Brisbane, Australia). The Israel strain of *B. bovis* was obtained from the 14th passage of a vaccine strain. The Mexico strain of *B. bigemina* was obtained from an infected animal in northeastern Mexico in 1982 (4). In vitro microaerophilous cultures of all babesial strains were maintained in bovine erythrocytes obtained from an uninfected donor animal (4). For use as antigen in proliferation assays, control uninfected erythrocytes (URBC) were similarly maintained in vitro. The percentage of parasitized erythrocytes was determined by counting giemsa-stained cultured blood smears.

Experimental cattle. Cattle were infected with the Mexico strain of *B. bovis* as described in detail elsewhere (4). Briefly, animal C15 was inoculated intravenously four times over the course of 6 years with cultured, autologous infected erythrocytes. The final inoculation of 10^9 parasitized erythrocytes did not evoke a fever or a reduction in packed erythrocyte cell volume. Animal C97 was infected by infestation with *Boophilus microplus* tick larvae infected with the same strain of *B. bovis*. The animal recovered from clinical babesiosis following treatment with 3 mg of diaminazine aceturate per kg of body weight and was solidly immune to challenge infection 3 months later with an intramuscular inoculation of a virulent, infected tick stabilate. A *Babesia*-naive control animal used to assess the virulence of the stabilate became clinically ill, experiencing a 57% reduction in packed erythrocyte cell volume on day 14 postchallenge (4). Following infection, animals C15 and C97 were serologically positive for *B. bovis* (4). T cell lines specific for *B. bovis* (Mexico) crude membrane (CM) antigen were established from animals C15 and C97 2 and 5 years, respectively, following the last parasite challenge inoculation. T cell lines derived from these cattle and stimulated with recombinant *B. bovis* RAP-1 antigen were established 3 and 6 years, respectively, following the last parasite challenge.

Parasite antigens. Crude parasite antigens were prepared by homogenization of culture-derived merozoites with a French pressure cell (SLM Instruments, Inc., Urbana, Ill.) and ultracentrifugation to yield a fraction enriched in CMs as described previously (4). URBC were similarly fractionated for use as control antigens. These antigens were stored at −70°C.

Recombinant *B. bovis* and *B. bigemina* RAP-1s were prepared as maltose-binding fusion proteins as follows. The region encoding *B. bovis* RAP-1 was obtained from plasmid pBv60 (44) by PCR amplification with the GeneAmp kit (Perkin-Elmer Cetus, Norwalk, Conn.) and primers P1 and P2. Primer P1 contains sequences located in the 5′ end of the open reading frame of *B. bovis* RAP-1 (5′-ATGAGGATCCTTAGCGGCGTTGTCGGT-3′), modified to include a *Bam*HI restriction site. Primer P2 is based on a sequence present in the multicloning site of the pBluescript vector (Stratagene, La Jolla, Calif.) (5′-TT AAGCTATAGTTCGAATAGCTAT-3′) and includes a *Hin*dIII restriction site. The pMAL-2c vector (New England Biolabs, Beverly, Mass.) and PCR products were double digested with *Bam*HI-*Hin*dIII and ligated as described in the manufacturer's protocol. Expression of *B. bovis* RAP-1 by recombinant plasmid pMAL-60.1 was verified by Western blotting (immunoblotting) with MAb BABB75A4 (45). Plasmid pBbg58 encoding one of the four *B. bigemina rap-1* genes (Bbg-13) of the JG-29 biological clone of the Mexico strain of *B. bigemina* (33, 34) was similarly amplified by PCR. 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. Forward primer P3 consisted of 5′-GCGCTCTAGAATGAGGAGCTTCT TGGGTGTG-3′, and reverse primer P4 consisted of 5′-CGCGTCTAGATTAC GCATCTGAATCATCTG-3′. The PCR products were digested with *Bam*HI and subcloned into the pMAL-2c vector as described in the manufacturer's protocol. *Escherichia coli* XL-1 Blue (Stratagene) containing the recombinant plasmids encoding the maltose-binding protein (MBP) or MBP fused to *B. bovis* RAP-1 or *B. bigemina* RAP-1 were 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 (IPTG) 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 submitted 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) as described in the manufacturer's protocol. The purified fusion proteins were dialyzed overnight against phosphate-buffered saline (PBS) and then stored at −70°C.

The protein concentrations in the parasite CM fractions and recombinant proteins were determined as described previously (4).

***B. bovis*- and RAP-1-specific T cell lines.** T cell lines reactive with *B. bovis* merozoite antigens were established from animals C15 and C97 as described previously (3). Briefly, 4×10^6 peripheral blood mononuclear cells (PBMC) were cultured per well in 24-well plates (Costar, Cambridge, Mass.) in a volume of 1.5 ml of complete RPMI 1640 medium (4) with 25 μg of *B. bovis* CM antigen per ml. After 7 days, the cells were subcultured to a density of 5×10^5 cells per ml and cultured with antigen and 2×10^6 fresh irradiated (4,000 rads) autologous PBMC as a source of antigen-presenting cells (APC). T cell lines were maintained for a total of 7 to 10 weeks by weekly stimulation with antigen and APC and periodically cryopreserved in liquid nitrogen in a solution of 10% (vol/vol) dimethyl sulfoxide in fetal bovine serum. Six weeks after initiation, the T cell line from animal C97 was stimulated for an additional 4 weeks with 12.5 μg of recombinant *B. bovis* RAP-1 per ml. Cells were assayed for antigen-dependent proliferation 7 days following the last antigenic stimulation. Alternatively, PBMC from these *B. bovis*-immune cattle were stimulated with 12.5 μg of recombinant *B. bovis* RAP-1 per ml and weekly thereafter with RAP-1 for 2 to 6 weeks and then tested for specific proliferation.

RAP-1-specific T cell clones. T cell clones were derived from a C97 T cell line that was sequentially stimulated for 6 weeks with *B. bovis* CM antigen and for 2 weeks with recombinant *B. bovis* RAP-1 fusion protein. This cell line proliferated to *B. bovis* CM and recombinant RAP-1 protein in a dose-dependent manner. Cells were cloned by limiting dilution as described previously (6, 10) in 96-well

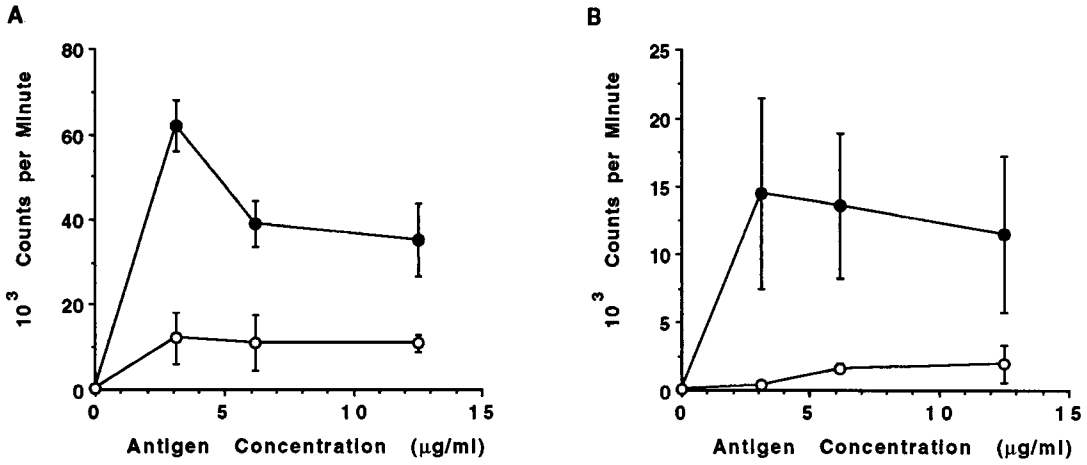

FIG. 1. Dose-dependent proliferative responses of PBMC from *B. bovis*-immune cattle C15 and C97 against recombinant *B. bovis* RAP-1 antigen. PBMC (2×10^5) from animals C15 (A) and C97 (B) were stimulated for 6 days with medium or 3 to 12.5 μg of antigen per ml consisting of recombinant protein *B. bovis* RAP-1 (closed circles) or MBP (open circles). Cells were radiolabeled for 4 h, harvested, and counted. Results are presented as the mean ± SD of triplicate cultures.

round-bottom plates (Costar). Briefly, T cells were diluted to a statistical average of 1.0 or 0.3 cells per well and stimulated with either 5 μg of recombinant RAP-1 fusion protein per ml or 25 μg of *B. bovis* CM antigen per ml in medium containing 10% bovine T-cell growth factor and 5×10^4 autologous APC. Proliferating cells were transferred successively to 48- and 24-well plates and tested for *B. bovis* RAP-1 antigen-specific proliferation. The cloning frequencies for T cells plated at 1.0 or 0.3 cells per well in the presence of RAP-1 were 23 and 10%, respectively, whereas the cloning frequencies for cells plated at 1.0 or 0.3 cells per well in the presence of *B. bovis* CM antigen were 9 and 5%, respectively.

Lymphocyte proliferation assays. Proliferation assays were carried out in replicate wells of half-area 96-well plates (Costar) for 6 days when PBMC were used and for 3 days when T cell lines and clones were used, essentially as described previously (3, 4, 6). Briefly, 2×10^5 PBMC were cultured in triplicate wells with antigen in a total volume of 100 μl of complete medium. T cell lines and clones were assayed 7 days after the last stimulation with antigen and APC. T cells (3×10^4) were cultured in duplicate wells in a total volume of 100 μl of complete medium containing 2×10^5 autologous or MHC-mismatched APC and antigen. Antigens consisted of 0.2 to 25 μg of the following per ml: CM prepared from different geographical strains of *B. bovis*, *B. bigemina* Mexico, or URBC; recombinant *B. bovis* RAP-1 fusion protein, *B. bigemina* RAP-1 fusion protein, or control MBP; and freshly harvested uninfected or *B. bovis* Mexico-parasitized erythrocytes. Where indicated, 2 U of human recombinant interleukin-2 (IL-2; Boehringer Mannheim, Indianapolis, Ind.) per ml was added with antigen in the proliferation assay. Bovine T-cell growth factor (10% vol/vol) was used as a positive control to induce proliferation of PBMC or T cells. Irradiated, heterologous PBMC from MHC-mismatched animal C15 or G3 were used to verify MHC restriction of the Th cell clones. To measure proliferation, cells were radiolabeled for the last 4 to 8 h of culture with 0.25 μCi of [^{125}I]iododeoxyuridine (ICN Radiochemicals, Costa Mesa, Calif.), and radiolabeled nucleic acids were harvested onto glass filters and counted in a gamma counter. Results are presented as the counts per minute (cpm) of replicate cultures (mean ± standard deviation [SD]) or as the stimulation index, which represents the mean cpm of replicate cultures of cells plus antigen divided by the mean cpm 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 (3) by using MAbs specific for bovine CD4 (MAb IL-A12), CD8 (MAb IL-A51), CD2 (MAb IL-A26), and WC1 (MAb IL-A29) obtained from the International Laboratory for Research on Animal Diseases, Nairobi, Kenya.

Stimulation of cells and detection of cytokine mRNAs and gamma interferon (IFN-γ) protein. T cell clones were obtained 7 days after the last stimulation with antigen and APC, washed twice in complete medium, and counted. On the basis of parallel 7-day cultures of APC, residual viable APC constitute <2% of the starting number and <2% of the total number of viable cells obtained from the cultured Th cell clones, which usually undergo two cycles of replication. To prepare RNA, T cells were stimulated for 8 h at a concentration of 2×10^6 cells per ml with 2.5 μg of concanavalin A (ConA; Sigma Chemical Co., St. Louis, Mo.) per ml. Total cellular RNA was prepared from the cells following lysis with guanidinium isothiocyanate (13). As a positive control for cytokine mRNA expression, RNA was prepared from freshly isolated bovine PBMC stimulated for 18 h with ConA. For negative controls, RNA was prepared from cultures of unstimulated murine WEHI-164 fibroblasts or from APC cultured for 7 days and stimulated for 8 h with ConA. To confirm the integrity of RNA in each sample, RNA was size fractionated in 1% agarose gels, and ethidium bromide-stained gels were visualized under UV light.

To evaluate cytokine mRNA expression, a reverse transcription-PCR (RT-PCR) assay was employed as described earlier (13, 39) 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 cpm in ^{32}P-radiolabeled PCR products (13). Those RNA concentrations and cycle numbers that fell on the linear portion of the plotted curve were then selected for experimental use. Cytokine and actin cDNAs were prepared from 0.5 μg of total RNA with a GeneAmp RNA PCR kit (Perkin-Elmer Cetus) and as described in 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-γ-specific primer sequences were provided by Heng-Fong Seow, Commonwealth Scientific and Industrial Research Organisation, Parkville, Australia. Primer sequences specific for bovine β-actin, IL-2, and IL-10 were selected from the published sequences by use of the MacVector DNA analysis software (IBI, New Haven, Conn.). GenBank accession numbers for the bovine cytokine or actin sequences are as follows: IL-2, M12791 (11); IL-4, M77120 (23); IL-10, U00799 (22); IFN-γ, M29867 (12); β-actin, K00622 (18). 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 with reverse transcriptase and reverse primers (20 to 34 pmol) for IL-2 (5′-GAGAGGCACTTAGTGATC-3′; nucleotides [nt] 484 to 501), IL-4 (5′-GTCTTTCAGCGTACTTGT-3′; nt 406 to 423), IFN-γ (5′-GCTCTCCGG CCTCGAAAGAGATT-3′; nt 562 to 584), IL-10 (5′-TATGTAGTTGATGAA GATGTC-3′; nt 559 to 579), and β-actin (5′-ACGTAGCAGAGCTTCTCCTT GATG-3′; nt 414 to 437), with an initial 15-min incubation at 48°C followed by a 5-min incubation at 99°C. Forward primers (20 to 32 pmol) for IL-2 (5′-ACA TTTGACTTTTACGTGCCCAAGGT-3′; nt 198 to 223), IL-4 (5′-TGCATTGT TAGCGTCTCCT-3′; nt 1 to 19), IFN-γ (5′-GGGCCTCTCTTCTCAGAAATT TTG-3′; nt 238 to 261), IL-10 (5′-GTTGCCTGGTCTTCCTGGCTG-3′; nt 98 to 118), and β-actin (5′-CCTTTTACAACGAGCTGCGTGTG-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 35 cycles under the following conditions: 95°C, 1 min; 50°C, 1 min; 72°C, 1 min. Each PCR product (25 μl) 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 potential contribution of cytokine mRNA by residual APC in the T cell cultures is negligible, since <2% of the total viable cells were derived from APC. 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.

Supernatants from T cells stimulated as described above for 23 h were harvested by centrifugation and stored frozen at −70°C to assay for IFN-γ. The bovine IFN-γ assay was performed with a commercial enzyme-linked immunosorbent assay (ELISA) kit (IDEXX Laboratories, Westbrook, Maine) as described in the manufacturer's protocol. The IFN-γ 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-γ per ml (previously determined by the neutralization of vesicular

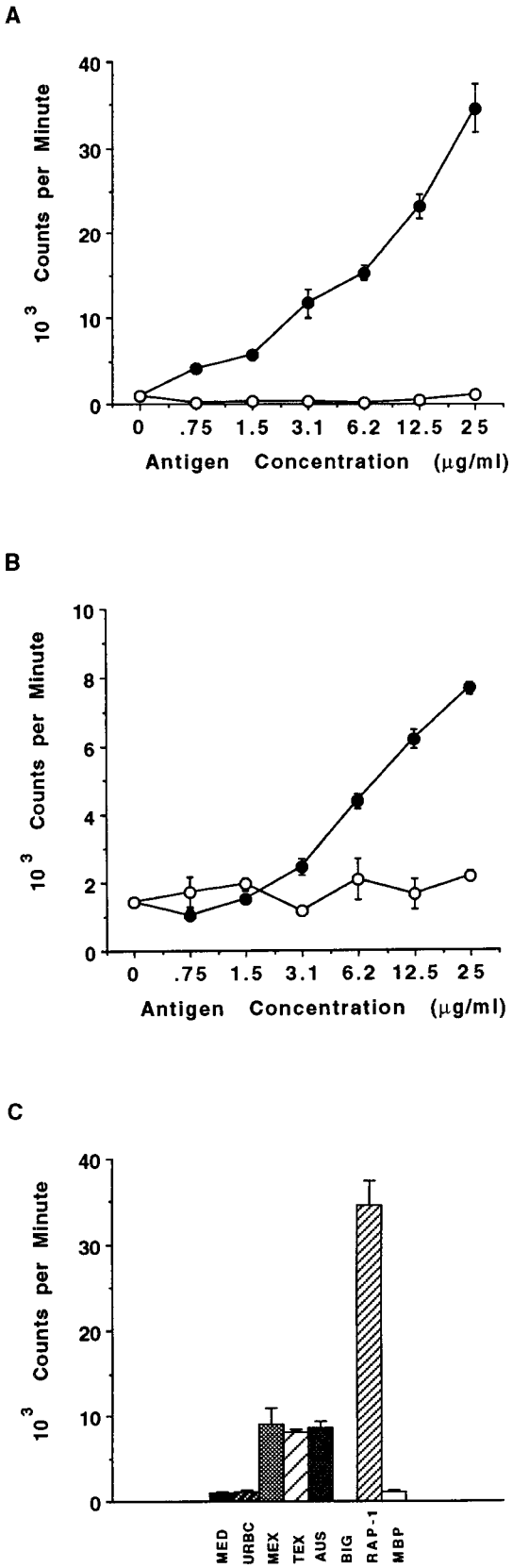

FIG. 2. Dose-dependent proliferative responses of RAP-1-stimulated T cell lines derived from *B. bovis*-immune cattle against recombinant RAP-1 protein or babesial merozoite antigens. T cell lines from animals C15 (A and C) and C97 (B) were stimulated with RAP-1 for 3 (A and C) or 2 (B) weeks. (A and B) T cells (3×10^4) were cultured for 3 days with autologous APC and medium or antigen consisting of 0.75 to 25 μg of recombinant *B. bovis* RAP-1 (closed circles) or MBP (open circles) per ml; (C) 3×10^4 T cells were cultured for 3 days with APC and medium (MED) or a 25-μg/ml concentration of CM prepared from URBC, the Mexico (MEX), Texas (TEX), or Australia L (AUS) strain of *B. bovis*, a Mexico strain of *B. bigemina* (BIG), recombinant RAP-1, or MBP. Cells were radiolabeled for 4 h, harvested, and counted. Results are presented as the mean ± SD of duplicate cultures.

stomatitis virus [9]). Positive control supernatants were obtained from PBMC stimulated for 24 h with ConA, and negative control supernatants were obtained from APC either cultured immediately with ConA for 24 h or cultured for 7 days in complete medium and then cultured for an additional 24 h with ConA.

RESULTS

Proliferative responses of PBMC from *B. bovis*-immune cattle to recombinant RAP-1 protein. Previous studies had demonstrated that PBMC obtained from *B. bovis*-immune cattle C15 and C97 responded reproducibly in proliferation assays against unfractionated and fractionated membrane and soluble forms of *B. bovis* merozoite antigens (4) as well as against recombinant *B. bovis* proteins Bb-1 and MSA-1 (6, 10, 49). To determine the immunogenic nature of the 60-kDa RAP-1 protein for T cells, PBMC from these immune cattle and four uninfected control cattle were assessed for the ability to proliferate against recombinant *B. bovis* RAP-1 expressed as a maltose-binding fusion protein. PBMC from both immune cattle responded in a dose-dependent manner to recombinant *B. bovis* RAP-1 antigen, and optimal proliferation was achieved with 3.1 to 25 μg of protein per ml (Fig. 1). Control MBP antigen elicited a relatively weak response. PBMC from four control cattle displayed weak proliferative responses against both *B. bovis* RAP-1 fusion protein and MBP, with no detectable differences in the response against the two antigens (data not presented).

Proliferative responses of T cell lines initiated and stimulated with recombinant *B. bovis* RAP-1. When PBMC were grown in bulk culture with RAP-1 antigen for 2 or 3 weeks and the T cell lines were assayed for proliferation, specific proliferation against RAP-1 was again observed, with maximal responses achieved with the highest dose of antigen tested (Fig. 2A and B). These cell lines also responded to *B. bovis* merozoite CM antigen (Mexico strain), where 25 μg of protein per ml induced responses comparable to those stimulated with 3.1 μg of recombinant *B. bovis* RAP-1 per ml (data not shown). In addition, the C15 T cell line responded to merozoite CM antigen derived from the Texas and Australia isolates of *B. bovis* but did not proliferate against CM antigen prepared from *B. bigemina* parasites (Fig. 2C), indicating that the RAP-1 T-cell epitopes recognized by animal C15 are conserved among geographically diverse *B. bovis* isolates but not in the Mexico isolate of *B. bigemina.*

To determine the type of T cell proliferating to RAP-1, cells were phenotyped by flow cytometry with MAbs against bovine surface markers. As observed in previous experiments with T cell lines derived from animal C15 (2), by 8 weeks of culture, approximately 50% of the C15 T cells expressed surface CD2 and CD4, fewer than 5% of the cells expressed CD8, and the remaining cells expressed the WC1 antigen found on γ/δ T cells. These results indicated that RAP-1 preferentially stimulated proliferation of $CD4^+$ α/β T cells and γ/δ T cells in this animal. The surface phenotype of the C97 line was not determined.

RAP-1 is an immunodominant antigen for *B. bovis*-immune T cells. It was evident from the preceding experiments that T

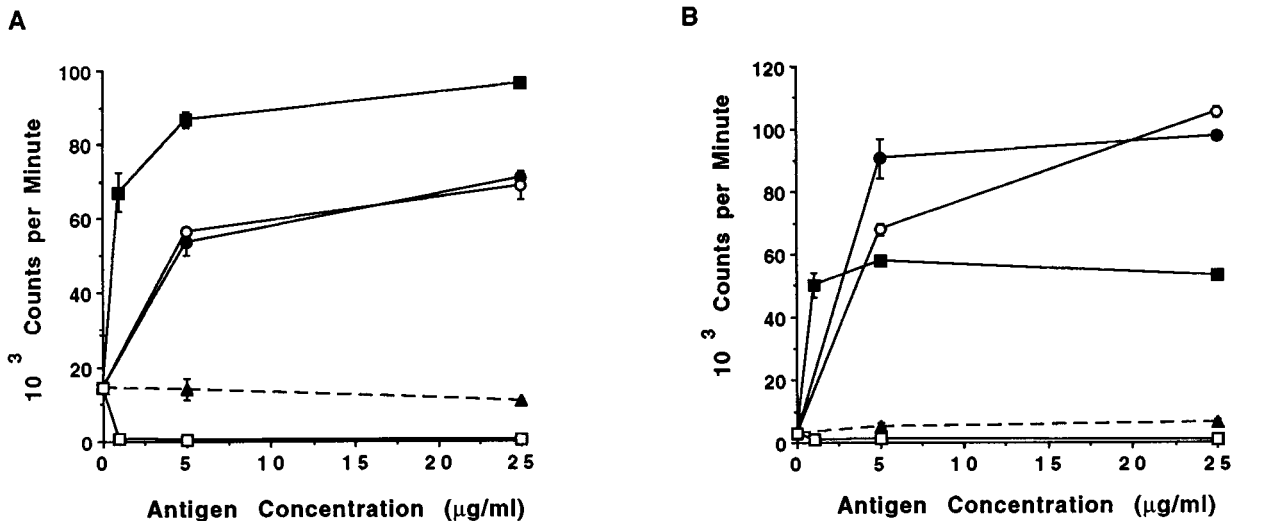

FIG. 3. Dose-dependent proliferative responses of *B. bovis* Mexico CM-stimulated T cell lines derived from *B. bovis*-immune cattle against recombinant RAP-1 proteins or babesial merozoite antigens. T cell lines from animals C15 (A) and C97 (B) were stimulated with *B. bovis* Mexico CM for 5 (A) or 6 (B) weeks. T cells (3×10^4) were cultured with autologous APC and medium or 1 to 25 μg of antigen per ml, which consisted of CM prepared from URBC (closed triangles), the Mexico strain of *B. bovis* (closed circles), or the Mexico strain of *B. bigemina* (open circles) or recombinant protein *B. bovis* RAP-1 (closed squares) or *B. bigemina* RAP-1 (open squares). Cells were radiolabeled for 4 h, harvested, and counted. Results are presented as the mean ± SD of duplicate cultures.

cells from *B. bovis*-infected and immune cattle expressed strong anamnestic proliferative responses when stimulated ex vivo with recombinant *B. bovis* RAP-1 protein. To further determine the immunodominant nature of RAP-1 for T cells from immune cattle, T cell lines from bovines C97 and C15, which had been cultured in vitro for 2 to 7 weeks with *B. bovis* merozoite CM antigen, were tested for the ability to respond to RAP-1 antigen. In these cell lines, 94 to 99% of the cells were $CD4^+$ T cells, and the remaining cells were $CD8^+$ T cells. We found that at all times tested, including 7 weeks following the initiation of culture, the T cell lines derived from both immune animals by repeated stimulation with CM antigen recognized recombinant *B. bovis* RAP-1 but neither control *B. bigemina* RAP-1 nor MBP. However, CM prepared from both *B. bovis* and *B. bigemina* merozoites induced vigorous proliferation of the T cell lines, indicating that T cells with specificities against additional antigens were also present in these cell lines, as observed previously (3). Representative data from $CD4^+$ T cell lines cultured for 5 and 6 weeks are presented in Fig. 3.

Proliferative responses of RAP-1-specific Th cell clones. To further characterize the memory T-cell response against RAP-1 in *B. bovis*-immune cattle, RAP-1-specific T cell clones were obtained by limited dilution cloning of the RAP-1-responsive C97 T cell line that was stimulated with *B. bovis* Mexico CM for 6 weeks and recombinant *B. bovis* RAP-1 protein for an additional 2 weeks prior to cloning. A panel of antigen-specific clones was selected by screening for proliferation against recombinant *B. bovis* RAP-1, MBP, *B. bovis* CM, and URBC. Twenty-seven RAP-1-specific clones were identified. All of the clones were classified as Th cells, since they expressed CD2 and CD4 but neither CD8 nor WC1 surface antigen. All of the clones responded in a dose-dependent manner to both recombinant RAP-1 fusion protein and *B. bovis* CM, and none responded to either MBP or *B. bigemina* RAP-1 fusion protein. Furthermore, unlike the Th cell line from which the clones were derived (Fig. 3B), none of the clones responded to *B. bigemina* CM antigen. An example of the antigen-driven proliferative responses with clone C97.3D2 is illustrated in Fig. 4. Typically, the response to recombinant RAP-1 was stronger than the response to the same concentration of *B. bovis* CM (25 μg/ml), which is consistent with the observation that RAP-1 is not an abundant molecule (51). No specific proliferation against control antigens, including *B. bigemina* CM or recombinant *B. bigemina* RAP-1, was observed. Together with data on T cell lines, these results clearly demonstrate that *B. bovis* RAP-1 T-cell epitopes are not conserved in *B. bigemina*. All of the clones were MHC restricted, since none responded to antigen in the presence of allogeneic APC derived from animal C15 or G3 (data not shown). These control studies also rule out the possibility that the RAP-1 fusion

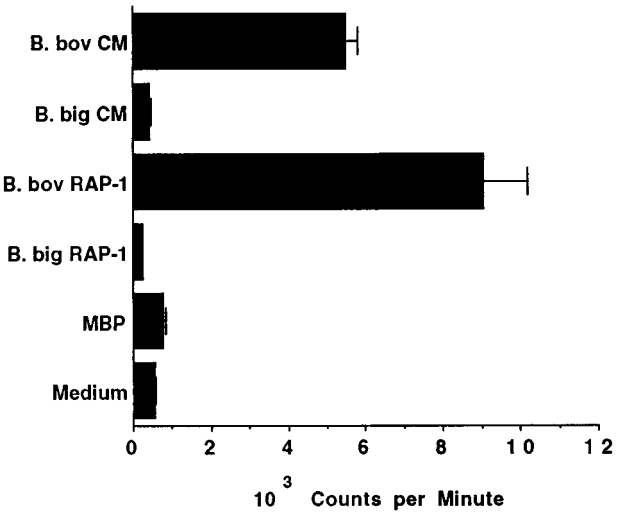

FIG. 4. Proliferative response of RAP-1-specific Th cell clone C97.3D2 against recombinant RAP-1 proteins or babesial merozoite antigens. T cells (3×10^4) were cultured for 3 days with autologous APC and medium or 25 μg of antigen per ml, which consisted of CM prepared from the Mexico strain of *B. bovis*, the Mexico strain of *B. bigemina*, or recombinant protein *B. bovis* RAP-1, *B. bigemina* RAP-1, or control MBP. Cells were radiolabeled for 4 h, harvested, and counted. Results are presented as the mean ± SD of duplicate cultures.

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

Th cell clone	Proliferation (SI) of clone stimulated with *B. bovis* strain[a] (% response to the Mexico strain)[b]				
	URBC	Mexico	Texas	Australia	Israel
Expt 1					
C97.1C10	0.5	79.5	73.8 (93)	22.9 (29)	ND[c]
C97.1E7	1.1	76.5	68.4 (89)	19.2 (25)	ND
C97.2H2	1.1	117.4	149.1 (127)	16.3 (14)	ND
C97.3D2	1.0	12.4	13.3 (107)	8.7 (70)	ND
C97.4E7	1.6	21.0	28.3 (134)	16.5 (79)	ND
Expt 2					
C97.1C10	1.0	21.2	ND	ND	23.8 (112)
C97.1E7	1.0	23.0	ND	ND	12.2 (53)
C97.2H2	1.5	42.8	ND	ND	51.7 (121)
C97.3D2	0.5	2.5	ND	ND	3.0 (120)
C97.4E7	0.8	12.7	ND	ND	11.4 (90)

[a] T cell clones (3×10^4) were stimulated for 3 days with 25 μg of CM per ml prepared from URBC or the indicated parasite strains and 2×10^5 APC. The results are presented as the stimulation index (SI): 100 × (mean cpm [^{125}I] iododeoxyuridine incorporation by T cells cultured with CM antigen/mean cpm of [^{125}I]iododeoxyuridine incorporation by T cells cultured with medium alone). Proliferative responses of the T cell clones cultured with parasite antigen were shown to be significantly different from the proliferative responses of T cell clones cultured with medium alone or URBC CM ($P < 0.05$) by the Student one-tailed *t* test.

[b] Results in parentheses indicate the percentage of the response of an individual Th cell clone to the Mexico strain.

[c] ND, not determined.

protein has superantigenic properties for the $CD4^+$ T cell clones.

Conservation of RAP-1 Th cell epitopes among geographically different strains of *B. bovis*. When the panel of Th cell clones was tested for proliferation against geographically distinct strains of *B. bovis*, it was found that stimulation with either 5 (data not shown) or 25 μg of CM per ml prepared from merozoites of strain Mexico, Texas, Australia, or Israel parasites induced proliferation of all clones tested (Table 1). These results indicate that the epitope(s) recognized by this panel of T cell clones is conserved among the four strains tested. Although the responses against a 25-μg/ml concentration of CM derived from the Australia strain were consistently lower than those against CM derived from the Mexico strain of *B. bovis*, 5 μg of Australia CM and Mexico CM per ml induced comparable levels of proliferation by four of five clones (data not shown). When comparing the responses against the Mexico and Israel strains of *B. bovis*, two patterns of response were observed. Eight (30%) of 27 clones tested expressed proliferative responses against the Israel strain that were approximately 50% as strong as those directed against the Mexico strain, whereas the remaining clones expressed similar levels of proliferation to Israel and Mexico parasites. An example of this dichotomous response is shown in Table 1 (experiment 2), where the response by clone C97.1E7 to Israel parasites was 53% as strong as the response to the Mexico isolate, whereas the responses of the other clones ranged from 90 to 121% of the response to the Mexico isolate.

***B. bovis* Mexico-infected erythrocytes induce proliferation by RAP-1-specific Th cell clones.** The ability of freshly harvested, cultured, parasitized erythrocytes to induce proliferation of three RAP-1-specific Th clones was evaluated. All clones proliferated in a dose-dependent manner in response to erythrocytes infected with the Mexico strain of *B. bovis* but did not respond to uninfected erythrocytes obtained from the same donor. The data for these clones are presented in Fig. 5, where

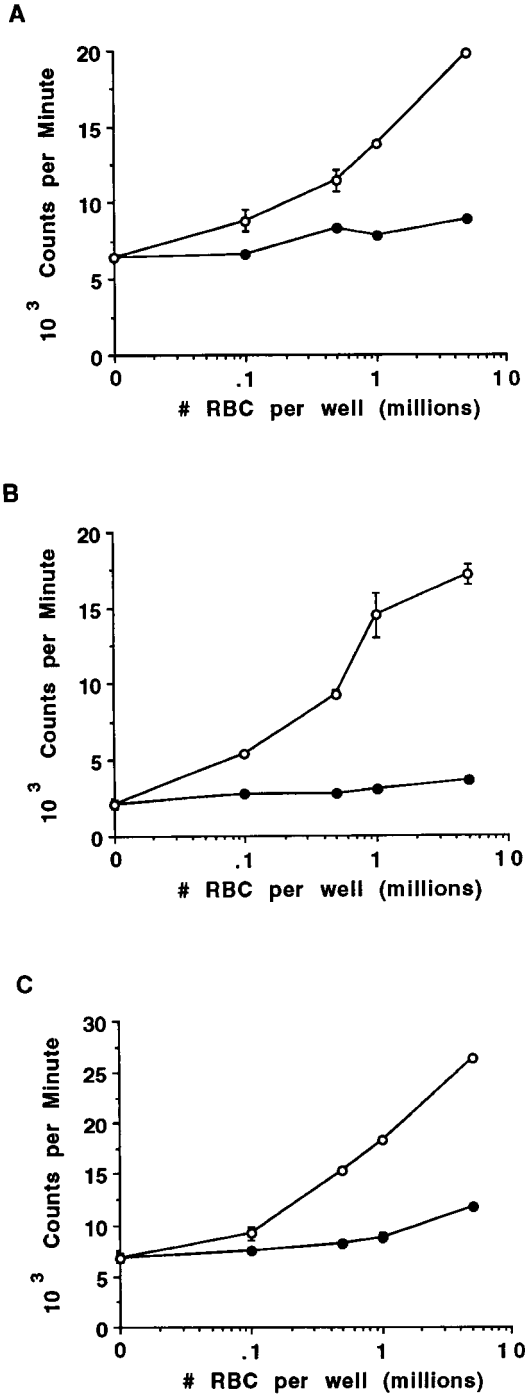

FIG. 5. Dose-dependent proliferative responses of RAP-1-specific Th cell clones against *B. bovis* Mexico-infected erythrocytes. T cells (3×10^4) of clones C97.3D2 (A), C97.1E7 (B), and C97.1C10 (C) were cultured for 3 days with autologous APC, 2 U of human IL-2 per ml, and medium or antigen consisting of 0.1×10^6 to 5×10^6 erythrocytes that were either uninfected (closed circles) or infected with the Mexico strain of *B. bovis* (open circles). Cells were radiolabeled for 4 h, harvested, and counted. Results are presented as the mean ± SD of duplicate cultures.

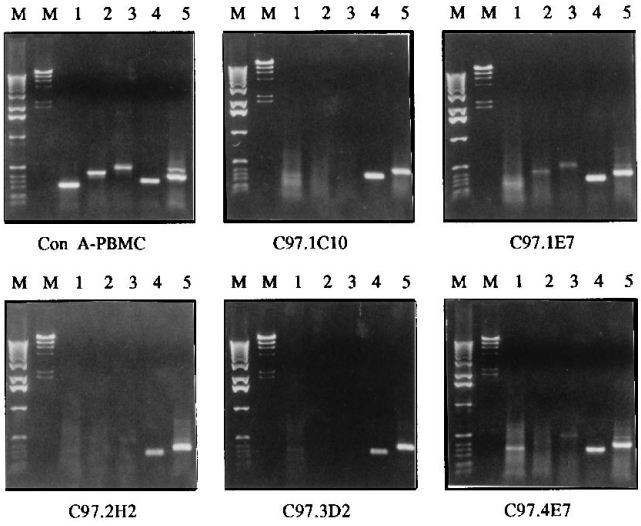

FIG. 6. Analysis of cytokine mRNA expression in *B. bovis* RAP-1-specific Th cell clones by RT-PCR. RT-PCR was performed with total cellular RNA prepared from ConA-stimulated PBMC (positive control) and five Th cell clones as indicated under each gel. Primers specific for bovine IL-2 (lane 1), IL-4 (lane 2), IL-10 (lane 3), IFN-γ (lane 4), and β-actin (lane 5) were used. The amplified PCR products were electrophoresed on agarose gels, stained with ethidium bromide, visualized, and photographed under UV light. The sizes of the amplified PCR products are as follows: IL-2, 304 bp; IL-4, 423 bp; IL-10, 482 bp; IFN-γ, 347 bp; β-actin, 400 bp. Markers (M), consisting of a 1-kb DNA ladder (far left lane) and λ *Hin*dIII (next left lane), were included in each gel.

cells were cultured with antigen in the presence of a low dose of IL-2. Maximal levels of stimulation were achieved with 5×10^6 total erythrocytes per well, which represents approximately 5×10^5 parasites per well. These results demonstrate that T cell clones that recognize both recombinant and native forms of RAP-1 can respond to antigen presented as an infected erythrocyte.

Cytokine expression by RAP-1-specific Th cell clones. Analysis of bovine IL-2, IL-4, IFN-γ, and IL-10 mRNA expression was performed by RT-PCR using RNA prepared from ConA-stimulated T cells. IFN-γ was also measured in the supernatants of cells stimulated with ConA. Cytokine mRNA expression by five clones was compared with that of ConA-stimulated PBMC, with actin as a positive control for the presence of intact RNA (Fig. 6). In comparison with ConA-stimulated PBMC, the RAP-1-specific Th clones expressed undetectable or weak levels of IL-2 (lane 1), IL-4 (lane 2), and IL-10 (lane 3) and strong levels of IFN-γ (lane 4) mRNA. Even though <2% viable APC remained after 7 days of culture, the potential contribution of cytokine mRNA by these cells was definitively ruled out (Fig. 7). APC cultured with ConA expressed actin mRNA but no detectable cytokine mRNA (Fig. 7B). In contrast, simultaneous evaluation of RNA from ConA-stimulated PBMC (Fig. 7A) and ConA-stimulated clone C97.2H2 (Fig. 7C) revealed the same cytokine profiles depicted in Fig. 6. IFN-γ activity in the culture supernatants of ConA-stimulated Th clones ranged from 45 to 158 U/ml (Table 2). Supernatant from ConA-stimulated PBMC contained comparable levels (78 U/ml) of IFN-γ, whereas supernatant from APC cultured with ConA did not contain detectable IFN-γ. Because all clones expressed relatively high levels of IFN-γ and low or undetectable levels of IL-4, they can be classified as either Th0 or Th1 cells or more broadly as type 1 Th cells (1).

DISCUSSION

Rhoptry-associated proteins of several important pathogenic protozoa, including *Plasmodium*, *Toxoplasma*, *Eimeria*, and *Babesia* spp., are the targets of vaccine development (16, 26, 35, 37). The ability of native and recombinant rhoptry-associated proteins of malarial and babesial parasites to confer partial protection against challenge infection (26, 31, 51) has prompted an interest in identifying both B-cell (25, 43) and T-cell (39) epitopes on RAP-1 proteins of *B. bovis* and *B. bigemina* parasites.

Identification of merozoite antigens that induce a Th cell response against *B. bovis* and characterization of the cytokines produced by the Th cells are important considerations for vaccine development for this parasite (7). Studies with related babesial and malarial parasite infections in mice have shown that $CD4^+$ T cells are required for resolution of the erythrocytic stage of infection (7, 47). In addition to providing help for antibody-producing B cells, $CD4^+$ T cells can act directly as effector cells against intracellular protozoa. Type 1 Th cells produce IFN-γ and lymphotoxin that activate macrophages and neutrophils to kill intraerythrocytic parasites via the production of toxic oxygen and nitrogen metabolites (28, 47). In malaria-infected mice, IFN-γ production is dependent on IL-12 produced by activated macrophages (41). Murine Th1 cells can also produce nitric oxide in response to malarial antigen (48). As demonstrated for malaria, we hypothesize that type 1 responses will be required to resolve *B. bovis* parasitemia. Furthermore, proteins that stimulate type 1 recall responses during in vivo exposure to native parasite antigen are predicted to be protective immunogens (7). The results re-

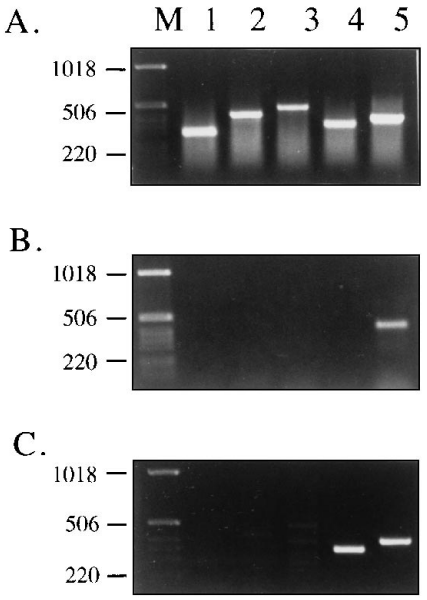

FIG. 7. Analysis of cytokine mRNA expression in ConA-stimulated APC. RT-PCR was performed with total cellular RNA prepared from lymphocytes cultured with ConA. Lymphocytes included freshly obtained PBMC (A), irradiated PBMC as a source of APC cultured for 7 days (B), and clone C97.2H2 cultured for 7 days with *B. bovis* RAP-1 antigen and APC (C). Primers specific for bovine IL-2 (lane 1), IL-4 (lane 2), IL-10 (lane 3), IFN-γ (lane 4), and β-actin (lane 5) were used. The amplified PCR products were electrophoresed on agarose gels, stained with ethidium bromide, visualized, and photographed under UV light. The sizes of the amplified PCR products are as follows: IL-2, 304 bp; IL-4, 423 bp; IL-10, 482 bp; IFN-γ, 347 bp; β-actin, 400 bp. Molecular weight markers (M), consisting of a 1-kb DNA ladder, were included in each gel and are indicated at the left of each figure.

TABLE 2. Production of IFN-γ by *B. bovis* RAP-1-specific Th cell clones

Lymphocytes[a]	IFN-γ (U/ml)[b]
Th cell clones	
C97.1C10	75
C97.1E7	125
C97.2H2	158
C97.3D2	45
C97.4E7	131
PBMC	
Fresh PBMC	78
Fresh APC	0
7-Day APC	0

[a] Th cells were cultured for 23 to 24 h at a density of 2×10^6 cells per ml in complete RPMI 1640 medium with 2.5 μg of ConA per ml. Freshly isolated PBMC or irradiated PBMC (APC) were cultured at 2×10^6 cells per ml with 2.5 μg of ConA per ml either immediately (fresh) or after 7 days of culture.

[b] IFN-γ levels were determined with a bovine IFN-γ-specific ELISA (IDEXX), by use of a standard bovine IFN-γ supernatant containing 400 U/ml.

ported in this paper demonstrate, for the first time, the immunodominant nature of *B. bovis* RAP-1 for $CD4^+$ type 1 Th cells from *B. bovis*-immune cattle and provide a rationale for including RAP-1 Th-cell epitopes in a subunit vaccine for *B. bovis.*

Previous reports from our laboratory have described recall proliferative responses from *B. bovis*-immune animals C15 and C97 against recombinant *B. bovis* spherical body protein Bb-1 and merozoite surface protein MSA-1 (6, 10, 49). In both studies, PBMC responded specifically to these antigens, and T cell lines were established by stimulating T cells with recombinant protein. However, T cell lines derived by stimulation with crude merozoite antigens that were verified by immunoblot analysis to contain MSA-1 and Bb-1 proteins were unresponsive in proliferation assays to MSA-1 or Bb-1 proteins (6, 49). In contrast, recombinant *B. bovis* RAP-1 induced strong proliferative responses by similar merozoite antigen-derived T cell lines established from these animals and cultured for up to 7 weeks, indicating the immunodominant nature of this antigen. Although only two animals were included in this analysis, we know that these cattle do not share MHC class II alleles capable of presenting either RAP-1 or other *B. bovis* antigens (3, 6, 10), showing that this unusual predominant response to RAP-1 is not due to a unique MHC class II allele shared by these particular cattle.

Another important characteristic of the Th cell response to RAP-1 is that memory T cells from cattle exposed to the native form of RAP-1 by infection with *B. bovis* clearly recognize the recombinant antigen. This finding implies that the converse will be true, i.e., that T cells from animals immunized with recombinant RAP-1 protein will recognize the native protein upon in vivo exposure to parasites. This is in fact suggested by one study reported by Wright and colleagues (51), where immunization with a recombinant fragment of *B. bovis* RAP-1 fused to glutathione *S*-transferase conferred partial protection, in the form of reduced parasitemias, upon challenge with virulent *B. bovis*. T-cell responses were not evaluated in this study, and the nature of the protective response was not elucidated. However, it was reported that protection was inversely related to antibody titer, implicating a role of non-antibody immune mechanisms. In addition, we demonstrated that Th cell clones stimulated with recombinant *B. bovis* RAP-1 protein proliferated against soluble and membrane forms of merozoite antigen and cultured, parasitized erythrocytes. These findings show that APC are capable of processing and presenting the relevant peptides from both recombinant and native forms of RAP-1 protein that constitute a T-cell epitope(s). Together, these results argue for the potential use of recombinant RAP-1 protein or peptides as a component in a vaccine that could prime cattle for specific recall responses upon exposure to natural *B. bovis* infection.

The cytokine mRNA profiles of the Th cell clones responding to RAP-1 showed that the cells expressed little IL-4 and strong levels of IFN-γ mRNA following ex vivo stimulation. Consistent with this finding, high levels of IFN-γ were secreted by the T cells. Type 1 cytokine mRNA profiles were also expressed by *B. bovis*-specific Th cell clones reactive with Bb-1 (10), MSA-1 (6), or undefined antigens (8), as well as by *B. bigemina* RAP-1-specific Th cell clones (39). Although it remains to be determined whether immunization with recombinant *B. bovis* RAP-1 in an appropriate adjuvant (such as IL-12) will stimulate type 1 responses, the finding that cloned memory T cells specific for the native protein are type 1 Th cells is predictive of the ability of recombinant protein to induce a similar response.

A final important feature of the T-cell response to RAP-1 is that the immunodominant T-cell epitope(s) is conserved among diverse geographical strains. T cell lines or clones derived from both immune cattle responded to the Texas and Australia L strains and the Israel vaccine strain of *B. bovis* in addition to the immunizing Mexico strain. Several additional Israel strains of *B. bovis* were tested and found to similarly stimulate proliferation of the Th clones (data not shown). The conservation of both B-cell (36) and Th-cell epitopes in RAP-1 proteins from different strains of *B. bovis* is advantageous for developing a vaccine that would provide protection against challenge with heterologous parasite strains. Interestingly, the strictly conserved 14-amino-acid sequence shared by *B. bovis* and *B. bigemina* RAP-1 homologs was not immunogenic for T cells in this study, supporting the contention that conserved epitopes potentially required for parasite function are poorly immunogenic and therefore permissive for parasite survival (32, 35, 45).

Although T-cell epitopes recognized by both of the immune cattle are conserved among the different strains of *B. bovis* examined, reduced proliferation to Australia L and Israel strains by several of the clones may indicate that individual T cell clones recognize different epitopes. In support of this possibility, we previously showed that *B. bovis*-specific T cell clones could be categorized into seven groups on the basis of differential responses to different parasite strains and fractionated proteins. T cell clones from each group recongized different antigens (5). Furthermore, a dichotomous response in the level of proliferation to different strains of *B. bigemina* by *B. bigemina* RAP-1-specific Th clones correlated with the recognition of distinct T-cell epitopes (25, 39). Sequence variation in a T-cell epitope expressed by the two gene copies of *B. bovis* RAP-1 could explain our results, if the copies were differentially expressed in the different strains. Alternatively, limited sequence variation in a T-cell epitope could result in reduced proliferative responses (19). Future experiments will identify the *B. bovis* RAP-1 T-cell epitope(s) and determine the level of expression and possible sequence variation of the T-cell epitopes encoded by the two gene copies of *B. bovis* RAP-1 in the different parasite strains.

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