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Identification of Two Th1 Cell Epitopes on the *Babesia bovis*-Encoded 77-Kilodalton Merozoite Protein (Bb-1) by Use of Truncated Recombinant Fusion Proteins

WENDY C. BROWN,[1]* SHUMIN ZHAO,[1] VIVIENNE M. WOODS,[1] CYNTHIA A. TRIPP,[2]†
CHRISTINE L. TETZLAFF,[1] VOLKER T. HEUSSLER,[3] DIRK A. E. DOBBELAERE,[3]
AND ALLISON C. RICE-FICHT[2]

Department of Veterinary Pathobiology[1] and Department of Medical Biochemistry and Genetics,[2] Texas A&M University, College Station, Texas 77843, and Institute for Animal Pathology, University of Bern, Bern, Switzerland[3]

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Previous studies have demonstrated the serologic and T-cell immunogenicity for cattle of a recombinant form of the apical complex-associated 77-kDa merozite protein of *Babesia bovis*, designated Bb-1. The present study characterizes the immunogenic epitopes of the Bb-1 protein. A series of recombinant truncated fusion proteins spanning the majority of the Bb-1 protein were expressed in *Escherichia coli*, and their reactivities with bovine peripheral blood mononuclear cells and T-cell clones derived from *B. bovis*-immune cattle and with rabbit antibodies were determined. Lymphocytes from two immune cattle were preferentially stimulated by the N-terminal half of the Bb-1 protein (amino acids 23 to 266, termed Bb-1A), localizing the T-cell epitopes to the Bb-1A portion of the molecule. $CD4^+$ T-cell clones derived by stimulation with the intact Bb-1 fusion protein were used to identify two T-cell epitopes in the Bb-1A protein, consisting of amino acids SVVLLSAFSGN VWANEAEVSQVVK and FSDVDKTKSTEKT (residues 23 to 46 and 82 to 94). In contrast, rabbit antiserum raised against the intact fusion protein reacted only with the C-terminal half of the protein (amino acids 267 to 499, termed Bb-1B), which contained 28 tandem repeats of the tetrapeptide PAEK or PAET. Biological assays and Northern (RNA) blot analyses for cytokines revealed that following activation with concanavalin A, T-cell clones reactive against the two Bb-1A epitopes produced interleukin-2, gamma interferon, and tumor necrosis factors beta and alpha, but not interleukin-4, suggesting that the Bb-1 antigen preferentially stimulates the Th1 subset of $CD4^+$ T cells in cattle. The studies described here report for the first time the characterization, by cytokine production, of the Th1 subset of bovine T cells and show that, as in mice, protozoal antigens can induce Th1 cells in ruminants. This first demonstration of *B. bovis*-encoded Th1 cell epitopes provides a rationale for incorporation of all or part of the Bb-1 protein into a recombinant vaccine.

Hemoparasitic diseases are endemic in half of the world's livestock production areas and constitute an important obstacle to the improvement of meat and milk production in developing nations. It is estimated that over 500 million cattle are at risk for babesiosis alone (25), and despite decades of research, there is still no effective, practical, and safe means of immunizing livestock against this disease. *Babesia bovis* can cause a virulent form of babesiosis which shares many features of human malaria in individuals infected with the related parasite *Plasmodium falciparum*, including anemia, toxemia, and adherence of parasitized erythrocytes to endothelial cells lining the brain and lung microcapillaries (45). The feasibility of developing a successful babesial vaccine is based on the observations that recovery from acute *B. bovis* and *B. bigemina* infection is associated with development of protective immunity (23) and that immunization with nonliving parasite extracts can induce partial protection against subsequent challenge (reviewed in reference 28). An optimal vaccine would include proteins with both T- and B-cell epitopes that induce anamnestic cellular and humoral immunity upon natural exposure to the parasite (15). However, very little is known about the nature of either protective babesial antigens or the immune responses in cattle that they evoke.

Antibody is undoubtedly important in immunity directed against extracellular organisms, in which the infectivity of babesial sporozoites or merozoites might be neutralized by serum antibodies (18). However, T cells would more probably play an important role as effector cells in the immune response directed against the intracellular parasite. Studies on experimental models of malaria and babesia infections in mice have shown that $CD4^+$ T cells are required for protective immunity directed against the intraerythrocytic stage of infection (2, 8, 9, 19, 31). Although the helper T-cell (Th) subsets that are involved in protective immunity to these parasites have not been conclusively identified, it has been suggested that gamma interferon (IFN-γ) and lymphotoxin, or tumor necrosis factor beta (TNF-β), produced by both Th1 and $CD8^+$ T cells (13, 26) are important for the resolution of hemoparasite infections. These cytokines inhibit parasites by direct toxic effects on the intraerythrocytic parasite (8), by inhibition of the intrahepatocytic development of exoerythrocytic stages (34, 36), and by the activation of macrophages (1) and neutrophils (20), resulting in enhanced phagocytosis of parasitized erythrocytes and the production of TNF-α, which itself has antiparasitic properties in vivo (40, 41). It should be noted, however, that in malaria, TNF-α also mediates the cerebral sequestration of

* Corresponding author.
† Present address: Department of Pathology, Colorado State University, Fort Collins, CO 80526.

parasitized erythrocytes (45), exemplifying the intricate balance between protective immunity and immunopathology.

The central role of T cells in the immune response against hemoprotozoan parasites, both as helper cells for T-dependent antibody production and as effector cells acting directly or indirectly on intracellular parasites through the elaboration of cytokines, has prompted an investigation of the bovine cellular immune response against *B. bovis* antigens. In a previous report, we described the induction of $CD8^+$ and $CD4^+$ T cells from *B. bovis*-immune cattle by a recombinant form of the 77-kDa merozoite protein, Bb-1 (42), which was selected for studies on T-cell immunogenicity for several reasons. The Bb-1 gene is conserved among New World and Australian parasites (43) and was predominant during immunoselection with bovine sera obtained from cattle naturally infected with *B. bovis* (44). Affinity-purified bovine antibody reacted with a 77-kDa merozoite protein on immunoblots and with the apical end of the merozoites by immunofluorescence staining (44). Together, these results suggested a functional importance for the Bb-1 protein and a logical target for immune intervention and showed that the Bb-1 protein is immunogenic for naturally infected animals, i.e., upon natural exposure it could presumably boost memory T-cell and B-cell responses in cattle vaccinated with this antigen. In the present study, we have used truncated recombinant fusion proteins to further characterize the antibody- and T-cell-reactive regions of the Bb-1 protein. Our results show that the antibody-reactive region is in the C-terminal half of Bb-1, which contains 28 tandem repeats of a tetrapeptide, PAEK or PAET, whereas at least two T-cell epitopes reside in the N-terminal part of the protein. Two Th clones reactive with these epitopes express the Th1 profile of cytokines (26): interleukin-2 (IL-2), IFN-γ, TNF-β, and TNF-α, but not IL-4, suggesting the preferential induction of Th1 cells by the Bb-1 protein. Furthermore, these studies have demonstrated for the first time the presence of the Th1 subset of T cells in cattle.

MATERIALS AND METHODS

Experimental cattle. The details of experimental infection of two cattle with the Mexican isolate of *B. bovis* were described in a previous publication (5). Briefly, 2-year-old crossbred cow C15 was inoculated intravenously with *B. bovis* merozoites derived from autologous, infected erythrocyte cultures three times over the course of 3 years. Nine-month-old crossbred cow C97 was infected by infestation with *Boophilus microplus* tick larvae infected with the same Mexican isolate of *B. bovis*. This animal recovered from clinical babesiosis after treatment with diaminizine aceturate and was solidly immune to challenge infection 3 months later with an intramuscular inoculation of ground-up tick stabilate. Peripheral blood mononuclear cells (PBMC) were obtained from immune cattle approximately 1.5 years following the last challenge infection for proliferation assays and for establishing T-cell clones. PBMC from animals C15 and C97 expressed the BoLA major histocompatibility complex (MHC) class I phenotypes w11/w12 and w5/w6, respectively, and apparently expressed different MHC class II alleles as well, since they were incapable of presenting antigen to T cells from the reciprocal donor (4).

Construction, expression, and purification of recombinant Bb-1–GST and truncated Bb-1–GST fusion proteins. Subcloning and expression of the 1,650-bp Bb-1 clone in the pGEX expression vector was described in a previous publication (42). Briefly, the Bb-1 gene was excised from recombinant λgt11 and subcloned into pGEX1N (Glutagene; Amrad Corp. Ltd., Kew, Australia) in frame with the glutathione *S*-transferase (GST) gene (39). The recombinant plasmid was used to transform *Escherichia coli* DH5α. Polymerase chain reaction (PCR) was used to produce recombinant proteins representing the N-terminal (Bb-1A) and C-terminal (Bb-1B) halves of Bb-1 and to produce a nested set of fusion proteins truncated from the amino-terminal end of Bb-1A (Bb-1C, Bb-1D, and Bb-1E). To accomplish this, primers based on the nucleotide sequence (43) were designed so that the DNA encoding the Bb-1 mutant proteins could be cloned as *Eco*RI fragments in the fusion vector pGEX2T (Pharmacia, Piscataway, N.J.) in frame with the gene encoding GST. PCRs were carried out by using a thermocycler and kit (Perkin-Elmer Corp., Norwalk, Conn.) with 100-μl reaction volumes (containing 1.25 mM nucleotide mix, 2.5 U of *Taq* DNA polymerase, 20 pM of each primer, 20 ng of Bb-1 template DNA, and PCR buffer containing 2 mM Mg^{2+}) for 35 cycles with a denaturation temperature of 94°C for 1 min, an annealing temperature of 60°C for 1 min, and an extension temperature of 72°C for 1 min with a 15-s autoextension setting. The amplified gene products were electrophoresed on a 1% agarose gel, electroeluted, purified on a NACS 52 PREPAC column (GIBCO BRL, Gaithersburg, Md.), and ligated into the pGEX2T fusion vector for expression in *E. coli*.

To obtain a nested set of deletion mutants of the Bb-1A fragment, which were truncated from the carboxy end of the protein, exonuclease III and mung bean nuclease digestions proceeding from the 3′ ends of the cDNAs were performed by using the Erase-a-Base kit (Promega, Madison, Wis.). We selected a series of truncated cDNAs which were expressed as fusion proteins designated Bb-1A–GST clone 10, clone 6C, clone 2D, and clone 2B. The resulting deletion mutants were then ligated into pGEX2T, and the DNA was transfected into *E. coli* DH5α. Recombinant fusion proteins were induced for 90 min with 5 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma Chemical Co., St. Louis, Mo.). Cells were centrifuged for 15 min at 5,000 × *g*; resuspended in 1/100 the starting volume in phosphate-buffered saline (PBS; pH 7.2) containing 1 mM phenylmethylsulfonyl fluoride, 50 mM EDTA, and 1% Triton X-100; and lysed by sonication. To affinity purify the fusion proteins, we centrifuged the lysates for 5 min at 11,000 × *g* and incubated the supernatants for 1 h at room temperature with a 50% suspension of glutathione-agarose beads (Sigma) prepared as specified by the manufacturer. Bound fusion protein was eluted from the beads with 25 mM reduced glutathione (Sigma) in 50 mM Tris-HCl buffer (pH 8.5) and concentrated with a Centriprep-10 concentrator (Amicon, Beverly, Mass.). GST was prepared in the same manner from *E. coli* transformed with pGEX2T.

Sequence analysis. Peptide sequences encoded by truncated Bb-1 genes were determined from the DNA sequence of each clone. Nucleotide sequence analysis of the 3′ ends of truncated Bb-1 genes was performed by directly sequencing duplex DNA in the pGEX2T vector by the dideoxy sequencing method of Sanger et al. (32). Sequenase enzyme (U.S. Biochemical Corp., Cleveland, Ohio) and a primer complementary to residues 1212 to 1233 of the pGEX2T vector were used in the reactions. The amino acid sequence of the *B. bovis* 77-kDa Bb-1 protein was analyzed for potential T-cell epitopes by using the computer program TSites (11), kindly provided by Vidal de la Cruz, MedImmune, Inc., Gaithersburg, Md. This program predicts amphipathic regions of the sequence characteristic of structures that might form stable

α-helical configurations, using the AMPHI algorithm with overlapping blocks of 11 amino acids (24), and simultaneously identifies the structural T-cell motif described by Rothbard and Taylor (29) as well as I-A^d- and I-E^d-binding motifs identified by Sette et al. (38).

SDS-PAGE and immunoblot analysis of recombinant Bb-1–GST fusion proteins. Intact Bb-1–GST (20 μg per lane), GST (10 μg per lane), and deletion fusion proteins Bb-1A (10 μg per lane), clone 10 (20 μg per lane), clone 6C (15 μg per lane), Bb-1C (10 μg per lane), Bb-1D (10 μg per lane), and Bb-1E (10 μg per lane) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% polyacrylamide) and stained with Coomassie blue. Low-molecular-mass standards (Bio-Rad, Richmond, Calif.) were included. To analyze the serologic reactivity of a monospecific rabbit antiserum raised against Bb-1–GST (42) for the Bb-1 fusion proteins, 10 μg of Bb-1B–GST, Bb-1A–GST, intact Bb-1–GST, or GST proteins were electrophoresed in a 12% polyacrylamide gel and transferred to nitrocellulose. Hyperimmune rabbit antiserum was preabsorbed with *E. coli* lysate and GST-agarose beads, diluted 1:560 in PBS, and used to develop the immunoblot as described previously (42) with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G heavy and light chains (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) and chromogenic substrates Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma).

T-cell clones. Three $CD4^+$ T-cell clones were established previously (42) from animal C97 by stimulating PBMC in vitro with recombinant Bb-1–GST and limited-dilution cloning of antigen-activated T cells in the presence of bovine T-cell growth factor (TCGF), Bb-1–GST, and irradiated (3,000 rad) PBMC as a source of antigen-presenting cells (APC). The clones, designated C97.1B10, C97.1C8, and C97.1D1, proliferated in a dose-dependent manner to Bb-1–GST but not to GST (42). The T-cell clones were maintained for several months in 1.5-ml cultures in 24-well plates (Costar, Cambridge, Mass.) by weekly stimulation with 25 μg of Bb-1–GST per ml, APC, and 10% TCGF. Cells were also cryopreserved in a mixture of 10% dimethyl sulfoxide in fetal bovine serum.

Lymphocyte proliferation assays. PBMC were obtained from *B. bovis*-immune animals C15 and C97 for proliferation assays, which were performed in triplicate wells of 96-well half-area plates (Costar) for 6 days as described previously (42) with the following modifications. Briefly, 2×10^5 PBMC were cultured in a total volume of 100 μl of complete RPMI 1640 medium (5) containing 5 to 25 μg of recombinant Bb-1–GST, Bb-1A–GST, Bb-1B–GST, or GST per ml. The cells were radiolabeled for the last 4 h of culture with [^{125}I]iododeoxyuridine (ICN Radiochemicals, Inc., Costa Mesa, Calif.) and harvested onto glass filters, and radioactivity was counted in a gamma counter. T-cell clones were assayed 6 to 7 days after the last stimulation with antigen and APC for proliferation against recombinant fusion proteins in duplicate wells of 96-well half-area plates essentially as described previously (42). Briefly, 3×10^4 T cells were cultured for 3 days in a total volume of 100 μl in complete medium containing 0.4 to 50 μg of antigen per ml and 2×10^5 irradiated autologous or MHC-mismatched APC. The cells were radiolabeled during the last 4 h of culture with [^{125}I]iododeoxyuridine, harvested, and counted. In one experiment, a monoclonal antibody, IL-A21 (kindly provided by John Ellis, University of Wyoming), which reacts with bovine MHC class II antigens was added at a final concentration of 10 μg/ml to the T cells in the presence of either antigen or IL-2. Results are presented as the mean cpm and one standard deviation of replicate cultures.

Stimulation of T-cell clones and biological assays for cytokine production. T cells obtained 6 or 7 days after the last stimulation with antigen plus APC were washed in complete medium and cultured for 17 h at a concentration of 2×10^6 cells per ml of complete medium containing 5 μg of concanavalin A (ConA; Sigma) per ml in the absence of APC. Supernatants were harvested by centrifugation and stored at −80°C. IFN activity in culture supernatants was measured in a microtiter cytopathic effect assay with vesicular stomatitis virus and Madin-Darby bovine kidney (MDBK) cells as described previously (6). MDBK cell survival was evaluated by the 4-h uptake of MTT dye in the cells (16). IFN titers were compared with a human recombinant IFN-α_2 reference reagent (no. Gxa01-901-535; National Institutes of Health, Bethesda, Md.) with a reported activity of 3.95 $\log_{10}$ IU/ml, which in our assay had a titer of 3.35 ± 0.18 $\log_{10}$ IU/ml. ConA alone had no IFN activity.

To measure TNF-α and TNF-β produced by activated T cells, a TNF-sensitive WEHI-164 subline from Edward Lattime, Memorial Sloan-Kettering, New York, N.Y., was incubated with culture supernatants in a 48-h assay (30). Cytotoxicity was determined by the MTT dye reduction assay. TNF titers in the supernatants were compared with a standard human recombinant TNF-α (Upstate Biotechnology Inc., Lake Placid, N.Y.), which had a reported activity of 6 $\log_{10}$ U/ml and in our assay had a titer of 5.92 ± 0.15 $\log_{10}$ U/ml. ConA alone had no TNF activity.

A $CD8^+$, cloned, IL-2-dependent bovine T-cell line, designated G1.G3, was used to measure IL-2/IL-4 activity in culture supernatants. Residual ConA was removed from supernatants harvested from the T-cell cultures by addition of 20 μg of methyl-α-D-mannopyranoside (Sigma) per ml and subsequent centrifugation and filter sterilization. These and a control sample consisting of complete medium containing 5 μg of ConA per ml treated in the same manner with methyl-α-D-mannopyranoside were diluted 1/2 to 1/256 in complete medium and added to duplicate wells of G1.G3 cells which had been distributed at a density of 3×10^4 cells per well in 96-well half-area microtiter plates. In all assays, recombinant human IL-2 (Boehringer-Mannheim Biochemicals, Indianapolis, Ind.) was serially diluted from 100 to <1 U/ml for use as a reference standard. The cells were cultured for 48 to 72 h and were radiolabeled with 25 μCi of [^{125}I]iododeoxyuridine during the last 4 h. A semiquantitative estimate of IL-2/IL-4 activity in the culture supernatants was obtained by comparison with a standard curve of human recombinant IL-2.

Northern blot analysis. Total RNA was obtained from unstimulated MDBK cells or from PBMC and T-cell clones that had been cultured for 8 or 18 h with ConA by using 2 ml of RNAzol B (Biotecx Laboratories, Inc., Houston, Tex.) per 10^7 cells as specified by the manufacturer. RNA (30 μg) was subjected to electrophoresis in a formaldehyde–morpholinepropanesulfonic acid (MOPS)–1.6% agarose gel, transferred to GeneScreen nylon filters (DuPont NEN, Boston, Mass.), and hybridized with the following cDNA probes. The IL-2 cDNA probe consisted of a 0.79-kb *Eco*RI fragment of the bovine IL-2 cDNA (27) and was a generous gift from Raymond Reeves, Washington State University. The IL-4 cDNA probe consisted of a 0.33-kb *Hin*dIII-*Xba*I fragment of bovine IL-4 cDNA (17). The actin cDNA probe consisted of a 1.0-kb *Eco*RI fragment of bovine actin cDNA (10) and was kindly provided by Angelika Ehrfeld, Max Planck Institute for Immunology, Freiburg, Germany. The

TNF-α cDNA probe consisted of a 0.6-kb *Eco*RI-*Nsi*I fragment of bovine TNF-α cDNA (14). The IFN-γ probe consisted of a 0.5-kb *Xba*I-*Dra*I fragment of bovine IFN-γ cDNA. Both TNF-α and IFN-γ probes were a generous gift from Arjun Singh, Genentech, South San Francisco, Calif. The TNF-β cDNA probe consisted of a 0.71-kb *Kpn*I-*Hin*cII fragment of the murine TNF-β cDNA (21) and was a generous gift from Nancy Ruddle, Yale University Medical School. The cDNAs were labeled with ^{32}P by the random primer method with a kit from Boehringer-Mannheim, yielding probes with a specific activity of 1×10^9 to 2×10^9 cpm/μg. Filters were prehybridized for 24 h at 42°C in a solution of 50% formamide in hybridization buffer (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 1× Denhardt's reagent, 0.5% SDS) and hybridized with the radiolabeled probes (5×10^6 cpm of probe per ml of hybridization buffer) for 48 h under the same conditions. The filters were washed twice at room temperature for 5 min with 2× SSC–0.5% SDS and then either twice at 50°C for 30 min with 0.2× SSC–0.5% SDS when using IFN-γ, TNF-β, TNF-α, and actin probes or once at 50°C for 15 to 30 min with 0.5× SSC–0.5% SDS when using the IL-2 and IL-4 probes. The filters were then exposed to Hyperfilm (Amersham Corp., Arlington Heights, Ill.) at −80°C. The approximate sizes of the transcripts in kilobases were determined from nucleic acid molecular size standards (0.24- to 9.5-kb RNA ladder; GIBCO BRL) that were electrophoresed, transferred to nylon, and stained with methylene blue.

Nucleotide sequence accession number. The nucleotide and amino acid sequences reported in this paper have been submitted to the GenBank data base (accession number M99575).

RESULTS

Expression of Bb-1–GST deletion fusion proteins. The 1,650-bp Bb-1 gene fragment was previously subcloned and expressed by using the pGEX expression vector (42). This approach permits the production of large amounts of recombinant fusion protein fused to GST and the ready purification of the recombinant protein through the affinity of GST for its substrate (glutathione-coated beads). The cloned gene encodes 59 kDa of the 77-kDa native protein observed by SDS-PAGE (42, 44). The nucleotide sequence of the Bb-1 gene will be reported elsewhere (43). The predicted amino acid sequence of the Bb-1 gene is shown in Fig. 1A. The sequence was found to be unique, with the exception of 28 tandem repeats of a tetrapeptide PAEK or PAET in the carboxy-terminal half of the protein (residues 316 to 427). A second distinctive characteristic of the protein is the presence of a polyserine tract (residues 288 to 300) flanked by negatively charged residues. To define the location of antibody- and T-cell-reactive regions, the Bb-1 gene was truncated and nested sets of recombinant proteins were produced for proliferation and immunoblot assays (Fig. 1B). The relative sizes of the intact Bb-1–GST and Bb-1A–GST and truncated Bb-1A–GST fusion proteins were determined by SDS-PAGE and are shown in Fig. 2. As noted previously (42), the intact Bb-1 fusion protein migrates as a faintly visible band of approximately 72 kDa and degradation products of approximately 28 to 30 kDa (lane A), whereas fusion proteins Bb-1A (lane B), Bb-1C (lane C), Bb-1D (lane D), and Bb-1E (lane E), ranging in apparent molecular mass from 54 to 46 kDa, are relatively stable. Bb-1A clone 2B has an apparent molecular mass of 50 kDa (not shown), clone 2D has an apparent molecular mass of 43 kDa (not shown), clone 6C (lane F) has an apparent molecular mass of 38 kDa, and clone 10 (lane G) has an apparent molecular mass of 33 kDa. The last two fusion proteins contain smaller degradation products. Recombinant GST (lane H) is also shown.

Antibody-reactive epitopes on Bb-1. Hyperimmune rabbit antiserum raised against Bb-1–GST was previously shown to react on immunoblots with both recombinant Bb-1–GST and the native 77-kDa protein in crude extracts of *B. bovis* merozoites (42). To more precisely define the antibody-binding epitopes, we reacted hyperimmune rabbit antiserum on immunoblots with the two halves of the Bb-1 protein, Bb-1A and Bb-1B (Fig. 3), following extensive absorption of GST reactivity with GST-coated agarose beads. Interestingly, the antiserum recognized only fusion proteins containing sequences in the carboxy-terminal half of the protein, revealing an intense reaction with Bb-1B–GST (lane A), a fainter reaction with Bb-1–GST (lane C), and no binding to either Bb-1A–GST (lane B) or GST (lane D).

Proliferative responses of PBMC from *B. bovis*-immune cattle to Bb-1A and Bb-1B fusion proteins. Previous experiments had documented that PBMC from two immune cattle (C15 and C97) but not a normal control animal proliferated in response to Bb-1–GST, with little or no response to recombinant GST (42). To localize the T-cell-reactive epitopes in the Bb-1 protein, we repeated the proliferation assay with recombinant Bb-1A and Bb-1B fusion proteins in addition to those already tested (Fig. 4). The results confirmed our previous finding and further showed that *B. bovis*-immune PBMC from animals C97 (Fig. 4A) and C15 (Fig. 4B) responded at least as well to Bb-1A–GST as to intact Bb-1–GST, whereas the response to Bb-1B–GST was less than or equal to the response to the control GST protein. PBMC from two nonimmune cattle were not stimulated by either of these fusion proteins or GST (data not shown).

T-cell epitope mapping with Bb-1-specific T-cell clones. Three Bb-1-specific $CD4^+$ T-cell clones derived from animal C97 were obtained by limited-dilution cloning in the presence of intact Bb-1–GST (42). The results of assays with two of the Th clones (C97.1C8 and C97.1B10) are presented, since the third clone (C97.1D1) had the same pattern of reactivity as clone C97.1B10. To further define the epitopes recognized by these Th clones, we measured proliferation against the Bb-1A and Bb-1B fusion proteins. Both clones responded to Bb-1A–GST in a dose-dependent manner but not to either Bb-1B–GST or GST, as shown in Fig. 5. The response to Bb-1A–GST was shown to be MHC restricted, since APC from MHC-mismatched animal C15 could not present antigen to the two clones, whereas the proliferation induced by TCGF in the presence of mismatched APC was not abolished (Fig. 6). A monoclonal antibody, IL-A21, that reacts specifically with bovine MHC class II determinants completely blocked the proliferation to antigen but did not inhibit the response to IL-2 (data not presented), demonstrating that the Th clones react to antigen in an MHC class-II restricted manner.

A set of nested deletion fusion mutants of Bb-1A were produced by exonuclease III and mung bean nuclease digestion from the 3′ end of the DNA, and some of these mutants were sequenced (Fig. 1B). Expressed fusion proteins of the Bb-1A subclones were then assayed for the ability to stimulate the Th clones. Interestingly, the two T-cell clones responded in a differential manner to expressed proteins of Bb-1A clones 2B, 2D, 6C, and 10 (Table 1). T-cell clone C97.1C8 responded in a dose-dependent manner to all fusion proteins except for the smallest (clone 10), against which no proliferation was observed at any concentration of antigen

A.

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   5   10   15   20   25   30   35   40   45   50   55   60   65   70   75
VSGHLVCKSGFGLGKVAKLMLASVVLLSAFSGNVWANEAEVSQVVKPGSVNGWWPTKGEYDVDPEWNEADIVYDD
                        ************************

  80   85   90   95  100  105  110  115  120  125  130  135  140  145  150
YKKYNGFSDVDKTKSTEKTSLGDIFMKLGPKKCMNLRVKDVDVDMQRANRRLATILQHITDHTPKSPMFAVSENI
      *************

 155  160  165  170  175  180  185  190  195  200  205  210  215  220  225
VKALNKQHTIELPKALAQQLCQFDAKAMKTCESRQFKDAFSTFFADFIADVLLGYSPMSEHMGVRCKAFGQIKKH

 230  235  240  245  250  255  260  265  270  275  280  285  290  295  300
VLLSLNGIEDLDKESCVNVTQPAIPTIPEEPVAEEPSDVTVTAPEECEEEIVINPEEENKPDSSSSSSSSSSSS

 305  310  315  320  325  330  335  340  345  350  355  360  365  370  375
ESDSDEEDREPVVEEPAEKPAEKPAEKPAEKPAETPAETPAEKPAEKPAEKPAEKPAEKPAEKPAETPAET

 380  385  390  395  400  405  410  415  420  425  430  435  440  445  450
PAETPAEKPAETPAETPAETPAETPAETPAETPAETPAETPAETPAEKPAEKPASRPCYGGRKGEEVVVLQTTST

 455  460  465  470  475  480  485  490  495  500  505  510  515  520  525
KQTPLKELPVVKGPSTNRLWHENVDIDMPVVQKRLSTILGYVTNYDPKHPFFAVSENIVKVLNKSCDFALPEDLA

 530  535  540  545
VELSSIESGKIFGYEWEVFT
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B.

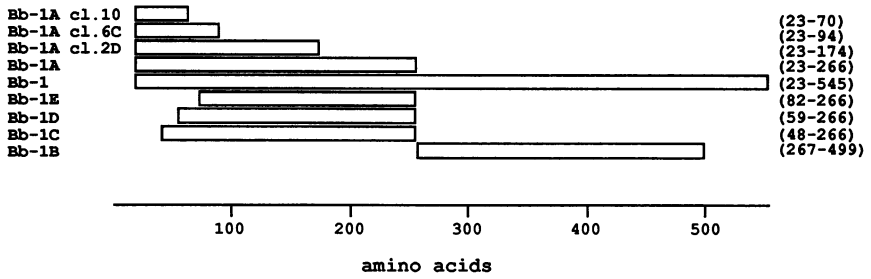

FIG. 1. Analysis of Bb-1 and truncated fusion proteins. (A) Predicted amino acid sequence of the Bb-1 protein encoded by 1,650 bp of the Bb-1 gene. Experimentally identified T-cell epitopes are underlined and indicated by asterisks. (B) Graphic representation of Bb-1 and truncated fusion proteins, encoded by Bb-1 subfragments indicated at the left of the figure. Subclones Bb-1A, Bb-1B, Bb-1C, Bb-1D, and Bb-1E were obtained by PCR amplification of specific residues. Bb-1A subclones 10, 6C, and 2D were obtained by exonuclease III and mung bean nuclease digestion of the Bb-1A DNA from the 3′ end. The amino acid residues included in each subclone are indicated in parentheses on the right of the figure.

ranging from 0.4 to 50 μg of protein per ml. In contrast, T-cell clone C97.1B10 proliferated against all fusion proteins, including the smallest (clone 10). These data show that clone C97.1C8 recognized an epitope within residues 71 to 94, whereas clone C97.1B10 recognized an epitope within residues 23 to 70.

To further define these two epitopes, we used PCR to create additional fusion proteins Bb-1C, Bb-1D, and Bb-1E, which contained sequences within these T-cell-reactive regions (Fig. 1B). Clone C97.1C8 recognized all three of these proteins, whereas clone C97.1B10 did not respond to any of them (Table 2), thus mapping the T-cell epitopes within amino acids 23 to 46 (clone C97.1B10) and 82 to 94 (clone C97.1C8), as depicted in Fig. 1A. Both of these sequences contained regions predicted by the AMPHI algorithm (24) to form amphipathic helices that are antigenic T-cell sites (data not presented).

Analysis of cytokines produced by the Th clones. Biological assays to measure bovine TNF-α (6), TNF-β (6), IFN-γ (6), and IL-2 and IL-4 activities were performed. The $CD8^+$, IL-2-dependent cell line, G1.G3, expressed both IL-2 and IL-4 mRNA upon ConA activation and proliferated in response to delectinated supernatants from ConA-stimulated G1.G3 cells (3). The cell line also responded to recombinant human IL-2. We have been unable to detect stimulation of bovine lymphocytes with recombinant human IL-4, and recombinant bovine IL-4 is unavailable, so the ability of these cells to respond to IL-4 has not been confirmed. These three bioassays showed that supernatants of T-cell clone C97.1C8 produced detectable levels of all three activities

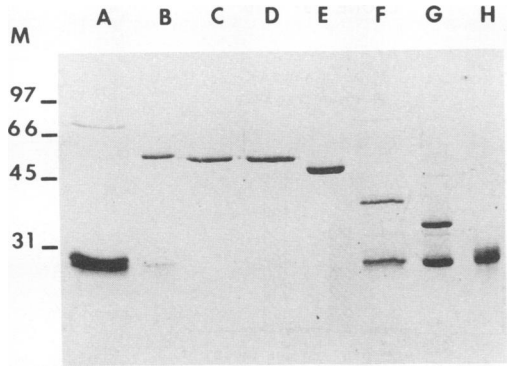

FIG. 2. Analysis of the relative molecular masses of the nested deletion Bb-1 fusion proteins by SDS-PAGE. All recombinant proteins, with the exception of GST, were babesial proteins fused with GST. Lanes: A, Bb-1; B, Bb-1A; C, Bb-1C; D, Bb-1D; E, Bb-1E; F, Bb-1A subclone 10; G, Bb-1A subclone 6C; H, GST. The relative mobilities (M) of the molecular mass standards are indicated on the left (in kilodaltons).

whereas clone C97.1B10 produced detectable levels of IFN and TNF-α/TNF-β following ConA activation (Table 3). Because the assays do not distinguish IL-2 and IL-4 or TNF-α and TNF-β activities, Northern blot analysis of total RNA extracted from ConA-activated T-cell clones, ConA-stimulated PBMC (positive control), or MDBK cells (negative control) was performed with cDNA probes for bovine IL-2, IL-4, IFN-γ, and TNF-α and murine TNF-β (Fig. 7). The blot probed with IL-4 was stripped and rehybridized with the bovine actin probe as a positive control to semiquantify RNA on the blot. IFN-γ mRNA was present in both clones stimulated for 8 or 18 h with ConA. IL-2 mRNA was detectable only in cells stimulated for 8 h, whereas IL-4 mRNA was never detected in the T-cell clones. Both Th clones expressed TNF-β and TNF-α mRNAs. As expected, the intensities of the mRNA bands corresponded to the relative amounts of cytokines detected in the culture supernatants (Table 3), with clone C97.1C8 having stronger signals on Northern blots and higher levels of all three cytokine activities. In contrast to the results with the Th clones, PBMC activated for 18 h with ConA expressed detectable levels of IL-2, IL-4, IFN-γ, and TNF-β but not TNF-α mRNA and MDBK cells never expressed any of the cytokine mRNAs. The failure to detect IL-4 mRNA in the two

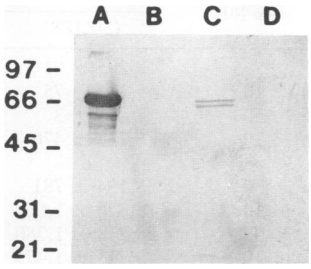

FIG. 3. Antibody-reactive epitopes localized to the carboxy-terminal half of Bb-1 by immunoblot analysis of recombinant Bb-1–GST fusion proteins. Hyperimmune rabbit antiserum extensively adsorbed of GST reactivity was used to probe fusion proteins or GST following electrophoresis of 10 μg of protein per lane and transfer to nitrocellulose. Lanes: A, Bb-1B–GST; B, Bb-1A–GST; C, Bb-1–GST; D, GST. The relative mobilities of the molecular mass standards are indicated on the left (in kilodaltons).

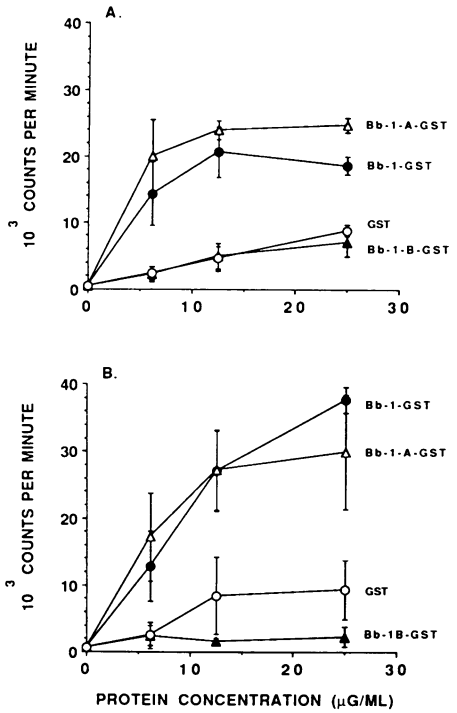

FIG. 4. Proliferative responses of PBMC against recombinant Bb-1 fusion proteins or GST. PBMC from *B. bovis*-immune cattle C97 (A) and C15 (B) were stimulated for 6 days with the indicated concentrations of antigen. Error bars indicate standard deviations.

T-cell clones was not caused by insufficient RNA on the blot, since this blot reprobed with bovine actin revealed intense signals. Furthermore, exposure of blots for up to 10 days failed to reveal any IL-4 mRNA signals in the T-cell clones (data not shown). These results show that the two Bb-1-specific T-cell clones described in this study belong to the Th1 subset of helper T cells.

DISCUSSION

T-cell recognition of protozoal antigens is believed to be associated with protective immunity. In malaria, both $CD4^+$ and $CD8^+$ T cells are important for resolving infection. In

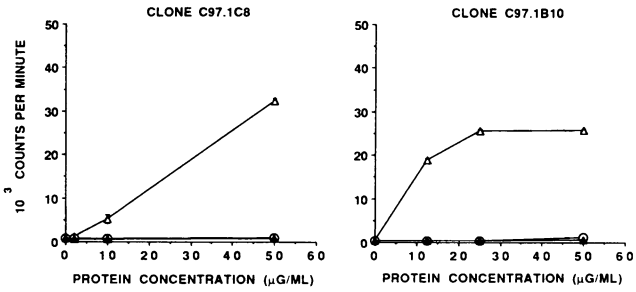

FIG. 5. T-cell-reactive epitopes localized to the amino-terminal half of the Bb-1 protein by analysis of proliferative responses of Bb-1-specific T-cell clones. T cells were stimulated with the indicated concentrations of Bb-1A–GST (△), Bb-1B–GST (○), or GST (▲) in a 3-day proliferation assay. Error bars that indicate standard deviations were included but may not be apparent because of the small values.

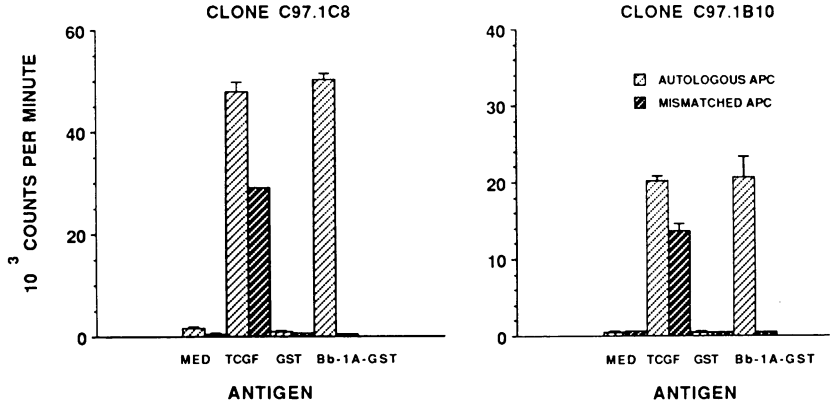

FIG. 6. Requirement for autologous APC by Bb-1A-specific T-cell clones for antigen-induced proliferation. T-cell clones were stimulated for 3 days with bovine TCGF (5%) or antigen (25 μg of either GST or Bb-1A–GST per ml) in the presence of either autologous C97 APC or MHC-mismatched C15 APC. Error bars indicate standard deviations.

experimental leishmania infection, Th1 cells conferred protection whereas Th2 cells, which produce IL-4, exacerbated the disease (37). However, in bovine babesiosis, neither the type of T-cell response nor the protective epitopes of babesial parasite antigens have been identified. Because T cells, through the elaboration of cytokines, are central to both humoral and cell-mediated immune responses and are critical for activating macrophages, we have examined in detail the T-cell response to the *B. bovis*-encoded recombinant Bb-1 protein, a potential vaccine antigen.

In this study, truncated recombinant fusion proteins and T-cell clones were used to identify T-cell-reactive epitopes on Bb-1. Preferential stimulation of *B. bovis*-immune PBMC by Bb-1A suggested the presence of T-cell epitopes on the N-terminal half of the protein. This was confirmed by studies showing that Th clones induced by stimulation with intact Bb-1–GST reacted with Bb-1A but not Bb-1B fusion proteins. In addition, attempts to generate T-cell lines from animals C97 and C15 by repeated stimulation with the Bb-1B fusion protein were unsuccessful, confirming that this part of the molecule is less immunogenic for T cells. Two T-cell epitopes on Bb-1A were localized by examining the proliferation of T-cell clones against fusion proteins truncated from either the amino- or carboxy-terminal end of Bb-1A. The T-cell-reactive regions are located within amino acids 23 to 46 (SVVLLSAFSGNVWANEAEVSQVVK) and 82 to 94 (FSDVDKTKSTEKT). Both regions contain motifs capable of forming α-helical structures predicted by the AMPHI algorithm (24), but none of the other algorithms were useful in predicting T-cell epitopes.

Both Bb-1-specific T-cell clones appear to belong to the Th1 subset, since they expressed IL-2, IFN-γ, and TNF-β but not IL-4 mRNA (26). Importantly, the clones also secreted these cytokines, as determined by biological assays and the observation that the clones proliferated in response to antigen in the absence of exogenous IL-2 or IL-4. To our knowledge, this is the first report of bovine T-cell clones that can be classified as Th1 cells. Others have reported the expression of both IL-2 and IL-4 mRNA in bovine T-cell clones specific for *Oesophagostosum radiatum* (7), and we have observed that the majority of $CD4^+$ T-cell clones reactive with undefined *B. bovis* merozoite antigens (6) expressed IL-2, IL-4, and IFN-γ mRNA (3), indicating the induction of the Th0 (12) subset of T cells by these different cattle parasites. It has been suggested that the conditions under which T cells are selected in vitro before cloning can

TABLE 1. Proliferative responses of Bb-1-specific Th clones against Bb-1A fusion proteins truncated from the C-terminal end

Fusion protein (approx mol wt)[a]	Radioactivity (mean cpm ± SD) incorporated by Th clone[b]:	
	C97.1C8	C97.1B10
Medium	363 ± 2	385 ± 12
GST (26,000)	386 ± 25	470 ± 24
Bb-1A–GST (54,000)	20,401 ± 1,639	25,626 ± 448
Clone 2B (50,000)	63,071 ± 4,709	21,895 ± 946
Clone 2D (43,000)	27,347 ± 4,718	9,244 ± 673
Clone 6C (38,000)	84,130 ± 1,830	27,471 ± 379
Clone 10 (33,000)	492 ± 1	26,830 ± 386

[a] A final concentration of 25 μg of protein per ml was used. The estimated molecular weights of the fusion proteins analyzed by SDS-PAGE are indicated in parentheses.

[b] Results are presented as the mean cpm ± standard deviation (SD) of duplicate cultures of T cells stimulated with antigen for 3 days.

TABLE 2. Fine-specificity mapping of Th epitopes on Bb-1A by induction of proliferative responses of Bb-1-specific Th clones against Bb-1A fusion proteins truncated from the N-terminal end

Fusion protein[a]	Concn (μg/ml)	Radioactivity (mean cpm ± SD) incorporated by Th clone[b]:	
		C97.1C8	C97.1B10
Medium		295 ± 91	896 ± 66
GST	10	321 ± 75	880 ± 156
	50	251 ± 3	1,188 ± 272
Bb-1A–GST, clone 6C	10	8,138 ± 476	5,571 ± 917
	50	38,134 ± 781	9,182 ± 34
Bb-1C–GST	10	18,826 ± 1,068	1,821 ± 139
	50	23,258 ± 1,280	650 ± 55
Bb-1D–GST	10	9,713 ± 1,479	1,448 ± 460
	50	30,731 ± 156	820 ± 173
Bb-1E–GST	10	7,579 ± 135	1,315 ± 24
	50	23,189 ± 303	2,143 ± 67

[a] Recombinant GST and GST fusion proteins Bb-1A clone 6C, Bb-1C, Bb-1D, and Bb-1E were tested at final concentrations of 10 and 50 μg of protein per ml for the capacity to stimulate two Th clones.

[b] Results are presented as the mean cpm ± standard deviation (SD) of duplicate cultures of T cells stimulated with antigen for 3 days.

TABLE 3. Production of cytokines by Bb-1-specific Th clones stimulated with ConA[a]

Th clone	Cytokine activity (U/ml)		
	IL-2/IL-4	IFN	TNF
C97.1C8	8–24	45–57	32
C97.1B10	<2	9	6

[a] T cells were stimulated with 5 μg of ConA per ml for 17 h, and the supernatants were tested for the indicated cytokines.

bias the cytokines produced. Maggi et al. (22) recently demonstrated that when human purified protein derivative-specific T-cell lines were cultured with IL-2 and antigen, T cells cloned from the cell lines produced the Th1 pattern of cytokines: IL-2 and IFN-γ but not IL-4 or IL-5. However, addition of IL-4 to purified protein derivative-specific cell lines resulted in the development of purified protein derivative-specific T-cell clones able to produce an unrestricted or Th0 pattern of cytokines: IL-2, IFN-γ, IL-4, and IL-5. In our experiments, T cells were stimulated with Bb-1 antigen in the absence of exogenous growth factors prior to cloning and were cloned in the presence of bovine TCGF that contains both IL-2 and IL-4. The absence of IL-4 expression by the Bb-1-specific T-cell clones therefore indicates that the Bb-1 protein preferentially induced the proliferation of Th1 cells in the uncloned T-cell lines. The production of IL-2, IFN-γ, TNF-β, and TNF-α by Bb-1-specific T cells provides a rational basis for hypothesizing that this antigen may evoke a protective immune response against *Babesia* infection in cattle, since these cytokines and Th1 cells have been shown to be associated with the resolution of related intracellular protozoal infections.

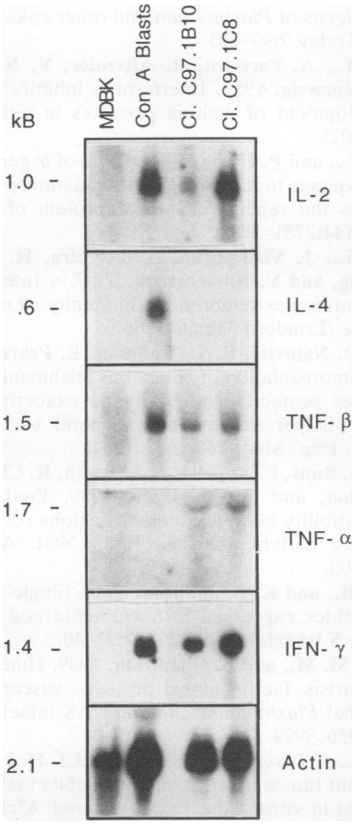

FIG. 7. Identification of cytokine mRNA expression in Bb-1A-specific helper T-cell clones by Northern blot analysis. RNA was obtained from the following cells: PBMC activated for 18 h with ConA (ConA blasts), MDBK cells, and T-cell clones C97.1C8 and C97.1B10 activated for 8 h with ConA. Total cellular RNA (30 μg) derived from the indicated cells was electrophoresed in a 1.6% agarose gel, transferred to nylon filters, and probed with the radiolabeled cDNA probes (see Materials and Methods) indicated on the right. Filters were exposed for the following periods: IL-2, 7 days; IL-4, 4 days; TNF-β, 7 days; TNF-α, 8 h; IFN-γ, 4 to 5 h; actin, 1 h. The approximate sizes (in kilobases) of the indicated cytokine transcripts are shown on the left.

Although bovine B-cell epitope-mapping studies have not yet been performed, results with rabbit anti-Bb-1 serum suggest that conformation-independent antibody-binding epitopes reside on the Bb-1B half of the protein, which contains 28 tandem repeats of tetrapeptide PAEK or PAET. On the basis of the immunogenicity of this type of repeating element in other proteins of protozoal parasites (33), the tetrapeptide repeats probably constitute the antibody-binding region. It has been proposed that in malaria infections, the repeat regions function to stimulate nonprotective T-independent antibody responses (immunological smokescreen), which are inferior to T-dependent ones since they do not usually undergo affinity maturation or involve T- and B-cell memory (33, 35). Furthermore, the repeating epitopes may suppress the development of antibody responses to adjacent (and possibly protective) regions of the protein (33), as suggested by our data showing a selective antibody response to Bb-1B in Bb-1-immunized rabbits. Recombinant proteins either containing or lacking the repetitive sequences will be used in epitope-mapping studies to determine whether the tetrapeptide is a target of the antibody response.

In conclusion, the Bb-1A region of the 77-kDa merozoite protein has properties consistent with the induction of a protective helper T-cell response important for the design of a subunit protozoal vaccine. Further experiments are needed to define how restricted the response is with respect to MHC class II expression, to map B-cell epitopes in cattle, to identify the isotype of the antibody induced by the Th cell clones, and to determine whether anti-Bb-1 antibody neutralizes parasite infectivity.

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