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Induction of Proliferative Responses of T Cells from *Babesia bovis*-Immune Cattle with a Recombinant 77-Kilodalton Merozoite Protein (Bb-1)

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A major portion of a *Babesia bovis*-specific gene encoding a 77-kDa merozoite protein (Bb-1) produced during natural infection in cattle and in microaerophilous culture was subcloned into the pGEX1N expression vector. Recombinant Bb-1 protein fused to glutathione *S*-transferase (Bb-1-GST) was used to examine cellular immune responses in *B. bovis*-immune cattle. Sera from rabbits immunized with Bb-1-GST reacted with fusion protein and with the native antigen present in crude *B. bovis* but not with *B. bigemina* merozoites. Bb-1-GST but not GST induced strong proliferation of T lymphocytes from these immune cattle, and Bb-1-reactive T-cell lines which consisted of a mixed population of either $CD4^+$ and $CD8^+$ cells or $CD4^+$, $CD8^+$, and "null" ($\gamma\delta$ T) cells were established by in vitro stimulation of peripheral blood mononuclear cells with the recombinant fusion protein. Three $CD4^+$ $CD8^-$ and three $CD4^-$ $CD8^+$ Bb-1-specific T-cell clones were identified after limiting-dilution cloning of the cell lines. The studies described here demonstrate that the 77-kDa protein of *B. bovis* contains T-cell epitopes capable of eliciting proliferation of two types of T cells in immune cattle, an important consideration for the design of a recombinant subunit vaccine.

Bovine babesiosis is a tick-transmitted, blood-borne disease of economic importance to the livestock industry throughout the world (26). Application of vector-directed acaricides has virtually eliminated babesiosis from the southern United States; however, the potential for reemergence exists due to the appearance of acaricide-resistant ticks, and in other semitropical areas it has been impossible to eradicate babesiosis through vector control methods. Therefore, control of this disease will depend upon improved methods of prophylaxis and, ultimately, the identification of parasite antigens that stimulate a protective immune response. An optimal vaccine would include proteins with T- and B-cell epitopes that induce secondary (anamnestic) cellular and humoral immune responses upon natural exposure to the parasite (16).

Numerous *Babesia*-specific antigens and the antibodies which they elicit have been described in the literature. Merozoite antigens of 29 and 44 kDa and an additional 180-kDa protein associated with the apical end of the merozoite were identified by Wright and coworkers (50, 51). Vaccination of cattle with the 44- or 29-kDa antigen resulted in partial protection against challenge with virulent organisms, which was assessed as significant differences in packed cell volume, parasitemia, temperature, and survival rates among vaccinated animals and controls. Merozoite surface antigens with apparent molecular masses of 16, 37, 42, 44, 55, 60, 85, 98, 120, 225, and 250 kDa have been identified by immunoprecipitation with monoclonal antibodies or hyperimmune bovine sera (13, 18, 33). The immunodominant 42-kDa integral membrane glycoprotein was shown to be species specific (27). The 60-kDa protein exhibited punctate, polar staining restricted to a small percentage of viable merozoites (13) and was shown to be conserved between geographically distinct isolates of *Babesia bovis* (42).

While parasite-directed antibodies may play an important role in diagnosis and control of babesial infection, antibody does not always correlate with protection and cannot alone account for all patterns of immunity observed (44). It is increasingly evident that T lymphocytes, the primary effector cells of cell-mediated immunity, are important in controlling diseases caused by many intracellular protozoan parasites (28, 35, 37, 38). Adoptive-transfer studies in mice have revealed that protective immunity to the rodent parasite *Babesia microti* is T-cell mediated (35). Research efforts in our laboratory are directed at defining T-cell epitopes of *B. bovis* merozoite proteins which are important in eliciting a protective immune response in cattle (4, 5).

We selected a previously isolated 77-kDa *B. bovis* merozoite protein (designated Bb-1) to characterize immunogenicity for bovine T-cell subsets. The Bb-1 gene originated from a λgt11 expression library and predominated during antibody screening with polyvalent bovine sera from a natural infection in Mexico (48). The gene encoding Bb-1 is conserved among widely divergent geographic isolates of *B. bovis*, but is not present in *B. bigemina* (47). Bb-1 protein appears to be expressed in the apical region of the intraerythrocytic parasite (48), which stained with an immunofluorescence pattern reminiscent of plasmodial rhoptry proteins (20). In the present study, the Bb-1 gene was subcloned into the plasmid expression vector pGEX1N, resulting in production of a recombinant protein (Bb-1-GST) consisting of the babesial polypeptide fused to glutathione *S*-transferase (GST) from *Schistosoma japonicum* (40). Our results demonstrate that, in addition to representing an immunodominant B-cell antigen, the Bb-1 gene product also possesses epitopes capable of stimulating at least two different subsets of bovine T cells in vitro.

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MATERIALS AND METHODS

Parasites. The *B. bovis* strain used in this study was originally isolated from cattle infected with a natural, tick-derived isolate from a *Babesia*-endemic area of Mexico (5, 48). *B. bovis* merozoites were maintained in vitro in microaerophilous culture (25) as previously described (5).

Experimental cattle. Two Brangus (*Bos indicus* × *Bos taurus*) yearling steers provided peripheral blood mononuclear cells (PBMC) for production of bovine T-cell growth factor (TCGF) as described in a previous publication (3). The details of experimental infection of these and additional cattle with *B. bovis* were described elsewhere (5). Briefly, cattle were immunized by infection with viable *B. bovis* parasites in the following ways. Two-year-old cross-bred 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 cross-bred cow C97 was infected by infestation with *Boophilus microplus* tick larvae infected with the same isolate of *B. bovis*. The animal recovered from clinical babesiosis following treatment with diminazene aceturate and was solidly immune to challenge infection 3 months later with an intramuscular inoculation of ground-up tick stabilate. A *Babesia*-naive animal used as a control to assess the virulence of the parasite stabilate became clinically ill, experiencing a 57% reduction in packed erythrocyte volume on day 14 postchallenge (5). Animal C99, a Brahman-European cross-bred steer, served as an uninfected, age-matched control animal for some experiments. Pre- and postinfection serum samples were obtained. PBMC were obtained approximately 1.5 years following the last challenge infection for use in proliferation assays and for establishing T-cell lines. Both infected animals (C15 and C97) were serologically positive for *B. bovis*, as determined by indirect immunofluorescence staining of cultured parasite smears and by immunoblotting crude merozoite antigens (5).

Bovine PBMC were typed for bovine lymphocyte antigens (BoLA) (43) by using antisera characterized in the Second International BoLA Workshop that define bovine class I major histocompatibility complex (MHC) specificities (32). Animals C15, C97, and C99 had BoLA phenotypes w11/w12, w5/w6, and w6/w32, respectively.

Preparation of recombinant protein antigen Bb-1-GST. The Bb-1 gene was first excised from recombinant λgt11 by using *Eco*RI (12 U/μg; Boehringer Mannheim Biochemicals, Indianapolis, Ind.), gel purified, and subsequently ligated into the *Eco*RI site of the expression vector pGEX1N (Glutagene; Amrad Corp. Ltd., Kew, Victoria, Australia) in frame with the GST gene (40). Ligations (10-μl volumes) were carried out at 12°C for 12 to 16 h in the presence of T4 DNA ligase (0.1 Weiss unit; Boehringer Mannheim) as described by Sambrook et al. (36). The recombinant plasmid was used to transform *Escherichia coli* DH5α. Recombinant fusion protein was 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. The lysate was centrifuged for 5 min at 11,000 × *g*, and the supernatant was incubated for 1 h at 37°C with a 50% suspension of glutathione-agarose beads (Sigma) prepared according to the manufacturer's instructions. Bound fusion protein was eluted from the beads with 25 mM reduced glutathione (Sigma) in PBS and concentrated with a Centriprep-10 concentrator (Amicon, Beverly, Mass.). GST was prepared in the same manner from *E. coli* transformed with pGEX1N.

Production of rabbit antisera. New Zealand White female rabbits were injected with 100 μg of either purified fusion protein (Bb-1-GST) or GST alone as a control. Antigens were prepared as sterile emulsions in RIBI adjuvant (MPL plus TDM plus CWS; RIBI Immunochem Research Inc., Hamilton, Mont.) and administered at six intradermal and two intramuscular sites. Rabbits were boosted at regular intervals with antigen and adjuvant to achieve high-titer antisera. Antisera were absorbed of any cross-reactive *E. coli* antigens by an established protocol (36).

Parasite antigens. *B. bovis* merozoites were collected from infected erythrocyte cultures following CO_2 deprivation for 18 h (5). Although high yields of merozoites can be obtained in this manner, examination of Giemsa-stained smears of purified merozoites revealed the presence of some erythrocyte membranes. For this reason, membranes prepared from cultured, nonparasitized erythrocytes were included as control antigens in assays with merozoite antigens. Subcellular fractions of *B. bovis* merozoites, including a membrane pellet (designated CM) and a soluble high-speed supernatant fraction (designated HSS), were prepared as described previously (5). CM and HSS fractions were also prepared from uninfected erythrocytes (URBC).

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by an established protocol (23) and routinely done with the minigel system (Bio-Rad Laboratories, Richmond, Calif.) and 12% acrylamide gels. Molecular mass standards, obtained from Bio-Rad, included rabbit muscle phosphorylase B (97 kDa), bovine serum albumin (66 kDa), hen egg white ovalbumin (42.7 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and hen egg white lysozyme (14.4 kDa). SDS-PAGE-fractionated proteins were electrophoretically transferred from acrylamide to nitrocellulose filters for 1 h at 100 V (46).

Pre- and postimmunization rabbit sera were used to develop the immunoblots. Nitrocellulose filters to which protein was bound were incubated in a PBS (pH 7.2) blocking solution with 5% nonfat dried milk and 0.02% sodium azide for 1 h at room temperature. Without being washed, the filters were placed directly in a solution of rabbit sera diluted 1:500 in blocking solution and incubated for 1 to 2 h at 4°C. The filters were washed three times in PBS and once in Tris-buffered saline (TBS; 150 mM NaCl, 50 mM Tris [pH 7.5]) and incubated for 1 h at room temperature with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG; heavy and light chains; Kirkegaard & Perry Laboratories, Gaithersburg, Md.) diluted 1:5,000 in blocking solution prepared in TBS. The filters were again washed four times in TBS. The appropriate chromogenic substrates, obtained from Sigma (Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolylphosphate) were used at a concentration of 50 μg/ml in TBS and added to the filters to detect bound antibodies (36).

Generation of antigen-specific bovine T-cell lines. Bovine T-lymphocyte cell lines specific for Bb-1-GST were established with PBMC from animals C15 and C97 as described previously (8) with the following modifications. Four million PBMC were cultured per well in a volume of 1.5 ml in 24-well plates (Costar, Cambridge, Mass.) with 25 μg of Bb-1-GST protein per ml. After 7 days, cells were subcultured to a density of 5×10^5 cells per well and restimulated with antigen and 2×10^6 autologous, irradiated (3,000 rad

from a ^{60}Co source) PBMC as a source of antigen-presenting cells (APC). The cultures were thereafter maintained by weekly stimulation with antigen and APC. Cells were cloned by limiting dilution essentially as described before (6) with the following modifications. A statistical average of 10, 3, 1, or 0.3 cells per well were stimulated with 25 μg of Bb-1-GST per ml, 10% bovine TCGF, and 5×10^4 irradiated autologous PBMC. T-cell lines and clones stimulated with 25 μg of *B. bovis* CM and/or HSS antigen protein per ml were similarly established from these immune cattle (4). Six or seven days after the last stimulation, cultures were tested for antigen-dependent proliferation.

Cell surface phenotypic analysis. T-cell lines and clones were stained by indirect immunofluorescence (24) and analyzed by flow cytometry with a Coulter EPICS 741 flow cytometer. The following monoclonal antibodies obtained from ILRAD were used: IL-A51, specific for $CD8^+$ cells (10); IL-A12, specific for $CD4^+$ cells (2); IL-A26, specific for $CD2^+$ cells (1); and IL-A29, specific for a 210- and 300-kDa protein complex, designated workshop cluster 1 (WC1), on bovine T cells which express the γδ T-cell receptor (9, 19). Goat anti-mouse immunoglobulin [affinity-purified $F(ab')_2$ fragments; Cappel/Organon-Teknika Inc., Malvern, Pa.] was used as a second reagent. To assess whether bovine B cells were present, cells were stained with fluorescein-conjugated rabbit anti-bovine IgG (heavy and light chain specific; Cappel). The percentage of positive cells was determined by the maximum-positive-difference method (30).

Lymphocyte proliferation assays. Proliferation assays were carried out in duplicate or triplicate wells of half-area 96-well plates (Costar) at 37°C, in a humidified atmosphere of 5% CO_2 in air for 3 to 6 days as described before (5, 6). Each well (100-μl total volume) contained complete RPMI-1640 medium (5), responder cells added at a final concentration of either 2×10^6 PBMC per ml or 2×10^5 or 3×10^5 T cells per ml (obtained 6 to 8 days following the last stimulation of the cell line with antigen) and 2×10^6 autologous, irradiated (3,000 rads) PBMC per ml as a source of APC. Antigens, including Bb-1-GST, GST, *B. bovis* CM and HSS, and URBC CM and HSS, were added to the assay mixes at a final concentration of 0.2 to 100 μg of protein per ml of complete medium. As a positive control, TCGF was added at a final concentration of 2.5 or 5%. Proliferation was determined by measuring the incorporation of 0.25 μCi of [^{125}I]iododeoxyuridine ([^{125}I]IUdR; ICN Radiochemicals, Inc., Costa Mesa, Calif.) added during the final 4 h of the assay. The cells were harvested onto glass filters with an automated cell harvestor (Skatron Instruments, Inc., Sterling, Va.), and the radioactivity on the filters was counted in a gamma counter (Packard, Laguna Hills, Calif.). Results are expressed as the mean counts per minute (cpm) and standard deviations of replicate samples or as the stimulation index (mean cpm of replicate cultures of cells plus antigen divided by the mean cpm of replicate cultures of cells plus medium).

Statistical analysis. The one-tailed Student *t* test was used to determine the levels of significance between control and experimental cultures (41).

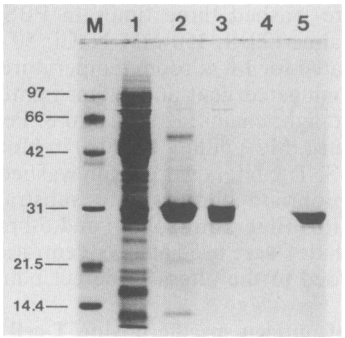

FIG. 1. SDS-PAGE analysis of recombinant Bb-1-GST. Recombinant *E. coli* lysate (approximately 35 μg, lane 1), Bb-1-GST-bound glutathione-agarose beads (approximately 22 μg, lane 2), Bb-1-GST eluted from the beads with free glutathione and concentrated (5 μg, lane 3), filtrate remaining after concentration (no detectable protein, lane 4), and recombinant GST eluted from the beads (5 μg, lane 5) were electrophoresed in 12% acrylamide gels and stained with Coomassie blue. Molecular mass standards were included in lane M (sizes shown in kilodaltons).

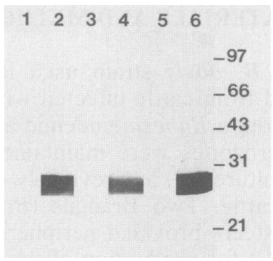

FIG. 2. Immunoblot analysis of recombinant GST. Preimmunization sera from rabbits 42 (lane 1), 43 (lane 3), and 44 (lane 5), serum from GST-immunized rabbit 42 (lane 2), and sera from Bb-1-GST-immunized rabbits 43 (lane 4) and 44 (lane 6) were used to probe GST after electrophoresis of 400 μg of protein in a preparative 12% acrylamide gel and transfer to nitrocellulose. The relative mobilities of the molecular mass standards are indicated on the right (in kilodaltons).

RESULTS

Expression of a *B. bovis*-GST fusion protein. Approximately 77% of the Bb-1 gene, containing 1,650 bp, was inserted into the *Eco*RI site of pGEX1N, and the resulting plasmids were used to transform *E. coli*. *B. bovis*-GST fusion protein was recovered from transformed *E. coli* cell cultures with a yield of approximately 1 to 2 mg of protein per liter of culture. SDS-PAGE and Coomassie blue staining (Fig. 1) of crude recombinant *E. coli* lysate (lane 1), glutathione-agarose bead-bound protein (lane 2), and affinity-purified, eluted Bb-1-GST fusion protein (lane 3) show that

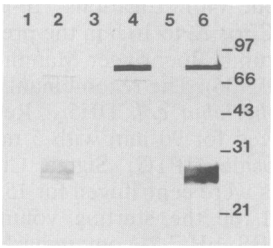

FIG. 3. Immunoblot analysis of recombinant Bb-1-GST. Preimmunization sera from rabbits 42 (lane 1), 43 (lane 3), and 44 (lane 5), serum from GST-immunized rabbit 42 (lane 2), and sera from Bb-1-GST-immunized rabbits 43 (lane 4) and 44 (lane 6) were used to probe the fusion protein after electrophoresis of 400 μg of Bb-1-GST in a preparative 12% acrylamide gel and transfer to nitrocellulose. The relative mobilities of the molecular mass standards are indicated on the right (in kilodaltons).

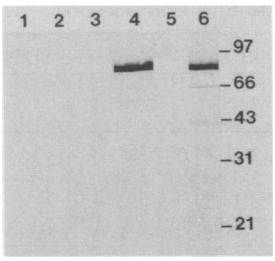

FIG. 4. Immunoblot analysis of *B. bovis* merozoites with anti-Bb-1-GST sera. The same rabbit antisera described in the legends to Fig. 2 and 3 were used to probe native babesial merozoite proteins on an immunoblot. Homogenized *B. bovis* merozoites (300 μg) were electrophoresed in a preparative 12% acrylamide gel and transferred to nitrocellulose. Lanes 1, 3, and 5, preimmunization sera. Lane 2, anti-GST serum. Lanes 4 and 6, anti-Bb-1-GST sera. Sizes are shown in kilodaltons.

the intact fusion protein migrated as a single, faintly visible band of 72 kDa (lane 3); however, a smaller protein, 28 to 30 kDa, was also uniformly present despite the addition of protease inhibitors during purification. Affinity-purified GST migrated at the expected size of 26 kDa (Fig. 1, lane 5). Silver staining revealed additional bands between the bands of 28 to 30 and 72 kDa (not shown).

Serologic responses against Bb-1-GST. All rabbit antisera reacted strongly and with equal intensity with purified GST on immunoblots (Fig. 2). Antisera obtained from two rabbits immunized with the Bb-1-GST protein reacted on immunoblots with the fusion protein and some additional bands, including the 28- to 30-kDa protein (Fig. 3, lanes 4 and 6), whereas antiserum from a rabbit immunized with GST did not recognize the 72-kDa fusion protein, but did react with some smaller proteins (Fig. 3, lane 2). Importantly, rabbit anti-Bb-1-GST sera also recognized the native *B. bovis* merozoite protein in homogenized merozoites, 77 kDa (Fig. 4, lanes 4 and 6), whereas control anti-GST rabbit serum failed to react with any native babesial proteins (Fig. 4, lane 2). Furthermore, rabbit anti-Bb-1-GST sera reacted in a similar way with native parasite antigen in merozoite CM and HSS fractions, but did not detect either URBC proteins or any merozoite-associated proteins of a nonrelated species, *B. bigemina* (data not shown). None of the preimmunization rabbit sera reacted with GST, recombinant Bb-1-GST, or native protein (Fig. 2, 3, and 4, lanes 1, 3, and 5).

Proliferative responses of C99, C97, and C15 PBMC to Bb-1-GST. PBMC from *B. bovis*-immune and control cattle were assessed for stimulation by several antigenic preparations, including Bb-1-GST fusion protein, control GST protein, the membrane fraction of *B. bovis* merozoites (CM), and control URBC. Figure 5 compares the proliferative responses of PBMC from both immune cattle (C97 and C15) and one control animal (C99) at two concentrations of protein. PBMC from control animal C99 did not respond to any parasite-derived antigens. PBMC from immune animal C97 responded vigorously to the merozoite CM antigen in a dose-dependent fashion, whereas the proliferative response of C15 PBMC to this antigen was not different when either 5 or 25 μg of protein per ml was used. There was no response to URBC. In comparison, PBMC from immune cattle responded to affinity-purified Bb-1-GST fusion protein, although the proliferation was not as vigorous as that induced by merozite CM. The greatest proliferation to Bb-1-GST observed was achieved with the highest concentration of antigen (in this case, 25 μg of protein per ml). Purified GST induced only a weak response from C15 PBMC at the highest concentration of protein assayed.

Bb-1-specific T-cell lines. Bovine T cells derived from C97 PBMC were cultured with recombinant Bb-1-GST for several weeks and examined for proliferative responses and for antigenic specificity. Table 1 shows that C97 T cells stimulated for 2 or 3 weeks with fusion protein responded more strongly to Bb-1-GST fusion protein than to recombinant GST. The cell surface phenotypic analysis of these cells revealed that at the time of assay, responder cells comprised a mixture of $CD8^+$ and $CD4^+$ cells (Table 2). No γδ T cells were detected in either culture. Three $CD4^+$ clones, derived from cell lines stimulated with antigen for 2 weeks, proliferated vigorously and specifically to recombinant Bb-1-GST (Table 3). The response of these clones was MHC-restricted, in that the T cells failed to proliferate to antigen in the presence of allogeneic APC obtained from animal C15 (data not shown). These clones did not respond to *B. bovis* CM or HSS antigens (data not shown).

A T-cell line established from PBMC from *B. bovis*-immune animal C15 was evaluated for proliferative responses to the recombinant protein Bb-1-GST (Fig. 6A) and to the CM and HSS fractions of *B. bovis* or URBC (Fig. 6B). In this case, the C15 T-cell line responded vigorously and in

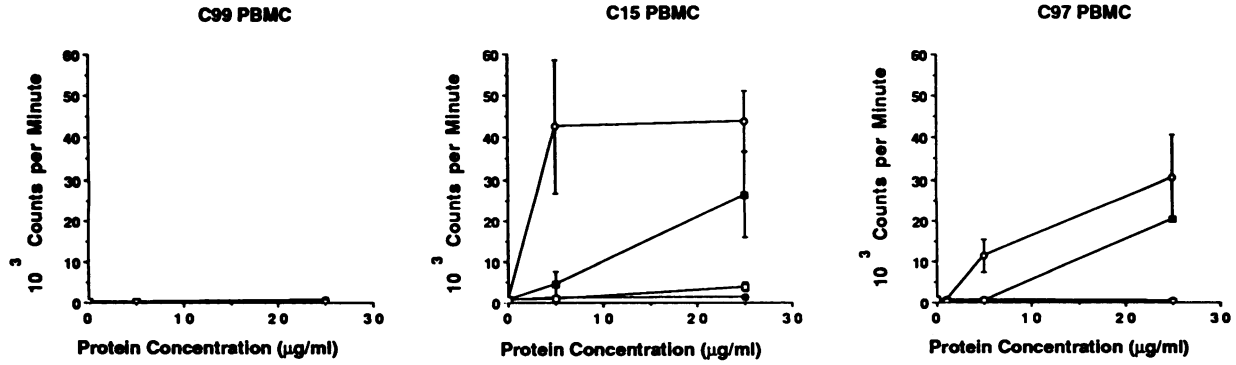

FIG. 5. Proliferative responses of PBMC against *B. bovis* and recombinant Bb-1-GST. PBMC (2×10^5) from one normal (C99) and two immune (C15 and C97) cattle were stimulated for 6 days with a final concentration of 5 or 25 μg of URBC (●), *B. bovis* CM (○), GST (□), or Bb-1-GST (■) protein per ml. Error bars indicate standard deviations.

TABLE 1. Proliferative response of C97 Bb-1-specific T-cell lines to Bb-1-GST and GST

Antigen (concn)	Radioactivity (mean cpm ± SD) incorporated by T-cell lines stimulated with antigen[a]			
	2 wk	*P* vs control	3 wk	*P* vs control
None (medium control)	184 ± 2		2,811 ± 358	
TCGF (2.5%)	ND[b]		42,876 ± 2,232 (15.2)	
Bb-1-GST (25 μg/ml)	420 ± 69 (2.3)	0.1	13,935 ± 922 (4.9)	0.005
GST (25 μg/ml)	124 ± 10 (0.7)	0.5	5,290 ± 457 (1.9)	0.1

[a] 2×10^4 lymphocytes from cultures of PBMC stimulated with Bb-1-GST for 2 or 3 weeks were cultured for 3 days with antigen or TCGF in 96-well plates, radiolabeled with [^{125}I]IUdR for the last 4 h of culture, harvested, and counted. Results are presented as the mean ± standard deviation of duplicate cultures. Stimulation indexes are indicated in parentheses. The Student *t* test was used to determine the levels of significance between cultures stimulated with medium and with an antigen.

[b] ND, not determined.

a dose-dependent manner to Bb-1-GST at protein concentrations ranging from 0.2 to 25 μg/ml. The proliferative response to GST alone was lower than the response to Bb-1-GST at each concentration of protein tested (Fig. 6A), showing that the response was predominantly directed against Bb-1. When this T-cell line was assayed for stimulation by parasite-derived antigenic fractions, weak stimulation was observed only with the HSS fraction (Fig. 6B). Finally, we evaluated the ability of APC from MHC-mismatched animal C97 to present antigen to the C15 Bb-1-specific T-cell line. There was no proliferation to Bb-1-GST when mismatched PBMC were used (Fig. 7), although the response to TCGF was not reduced in the presence of mismatched APC (there were 19,412 ± 612 cpm with autologous APC and 25,314 ± 1,077 cpm with mismatched APC).

Cell surface phenotypic analysis of this C15 T-cell line, performed for the first time after 8 weeks in culture (at the time of the assays depicted in Fig. 6), was surprising in that only 16 to 24% of the cells expressed T-cell surface markers (11.5% $CD4^+$ and 4.7% $CD8^+$; Table 2). None of the cells expressed surface immunoglobulin, suggesting that the majority of the cells were $CD2^-$ $CD8^-$ $CD4^-$ T cells. Two weeks later, the same cell line was examined again, this time with the addition of a monoclonal antibody (IL-A29) specific for a 215- and 300-kDa protein complex (WC1) expressed on the surface of bovine T cells that express the γδ T-cell receptor (9). Surprisingly, 72.6% of the cells expressed the γδ surface marker, whereas only 9 to 12% of the cells expressed the markers found on T cells expressing the αβ T-cell receptor (4.3% $CD4^+$ and 4.7% $CD8^+$). To confirm this unexpected result, an aliquot of the C15 Bb-1-specific cell line frozen after 6 weeks in culture was thawed, cultured for 3 days, and analyzed for cell surface markers. At this time, the cell line had a similar phenotype (Table 2). When the cell line was cloned by limiting dilution after 9 weeks in culture, several $CD8^+$ Bb-1-reactive T-cell clones were obtained (Table 3).

TABLE 2. Cell surface phenotype of T-cell lines which respond to Bb-1-GST

Antibody (cell type)	% of cells stained by antibody after indicated time of culture				
	C15			C97	
	6 wk	8 wk	10 wk	2 wk	3 wk
ILA-26 (CD2)	30.9	24.1	12.1	ND[a]	ND
ILA-12 (CD4)	8.8	11.5	4.3	13.7	19.4
ILA-51 (CD8)	13.1	4.7	4.7	31.9	56.8
ILA-29 (WC1)	60.6	ND	72.6	0.6	0
Rabbit anti-immunoglobulin (B)	ND	0	ND	ND	ND

[a] ND, not determined.

Lastly, we examined the ability of *B. bovis* merozoite-specific T-cell lines and clones, derived from PBMC of animals C97 and C15 stimulated repeatedly with the crude merozoite CM or HSS antigen, to respond to the recombinant Bb-1-GST protein. We never detected Bb-1-specific proliferation with these merozoite-reactive cells. The results of a representative experiment for a *B. bovis*-specific T-cell line from animal C15 stimulated with crude CM and HSS antigen for 7 weeks are presented in Fig. 8. As expected, the C15 cell line responded vigorously to both fractions of *B. bovis* merozoites tested and weakly or not at all to URBC fractions. However, no significant reactivity above that to URBC HSS was observed with either the Bb-1-GST fusion protein or GST alone. Analysis of the cell surface phenotype of this and additional *Babesia*-specific T-cell lines derived from other immune animals after 7 weeks in culture revealed that the majority of cells were of the helper phenotype, and T-cell clones derived from these lines were also $CD4^+$ (4).

DISCUSSION

Although parasite-specific T cells have been implicated in mediating protective immune responses against numerous protozoan infections of humans and experimental animals, few published studies have examined *Babesia*-specific T-lymphocyte responses in cattle immune to *B. bovis* (5, 45). We have recently demonstrated long-lived blastogenic responses directed against crude babesial merozoite antigens by PBMC of cattle infected with either tick-derived or cultured parasites (5), but the nature of the antigens and the subsets of T cells involved in such responses have not been fully characterized.

In the study reported here, we have demonstrated immunogenicity for different T-cell subsets of a recombinant form of the 77-kDa merozoite protein of *B. bovis*. Several interesting features of the native protein make it an attractive choice for studies on T-cell immunogenicity. First, the Bb-1 gene was isolated from a parasite DNA expression library by immunoscreening clones with bovine sera obtained from cattle residing in a *Babesia*-endemic region of Mexico (48). Furthermore, Bb-1-specific antibody, affinity purified from the field sera, reacted with native parasite antigen on Western immunoblots and by indirect immunofluorescence staining of infected erythrocytes (48). Together, these findings show that the Bb-1 protein is immunogenic for naturally

TABLE 3. Proliferative response of Bb-1-specific T-cell clones

Clone[a]	Phenotype	Radioactivity (mean cpm ± SD) incorporated[b] by T cells stimulated with:				
		Medium (control)	GST	*P* vs control	Bb-1-GST	*P* vs control
C97.1C8	$CD4^+$	578 ± 24	585 ± 22	>0.5	27,464 ± 1,117	0.001
C97.1B10	$CD4^+$	532 ± 24	547 ± 57	>0.5	3,049 ± 141	0.0025
C97.1D1	$CD4^+$	450 ± 4	569 ± 41	0.1	1,429 ± 62	0.0025
C15.3E6	$CD8^+$	271 ± 2	298 ± 27	0.2	1,664 ± 317	0.025
C15.1H5	$CD8^+$	6,557 ± 2,178	7,003 ± 1,345	>0.5	16,320 ± 2,613	0.05
C15.2C11	$CD8^+$	3,510 ± 319	6,464 ± 2,439	0.2	14,330 ± 1,957	0.02

[a] Clone C97.1C8 was stimulated with 50 μg of antigen per ml; all other clones were stimulated with 25 μg of antigen per ml. For clones C15.1H5 and 2C11, 5% TCGF was added to all wells. The Student *t* test was used to determine the levels of significance between cultures stimulated with medium and with antigen.
[b] 3×10^4 T cells were cultured with 2×10^5 APC plus medium or antigen for 3 days, radiolabeled with [^{125}]IUdR, harvested, and counted as described in the text.

infected cattle and could presumably boost memory T-cell and B-cell responses upon natural exposure. Second, immunoprecipitation of [^{35}S]methionine-labeled merozoites with rabbit anti-Bb-1 serum revealed that the 77-kDa protein was induced by both nutritional and oxidative stress (52), showing that Bb-1 is a stress-induced protein. Interestingly, the Bb-1 gene predominated in clonal isolation from the expression library screened with immune sera (48), which is a characteristic of stress-induced or heat shock proteins in other protozoa (11). Stress proteins are present in all eucaryotes and procaryotes and have been shown to be immune targets for T cells and B cells in a broad range of infections, including *Plasmodium falciparum* (31). Third, the Bb-1 protein is a logical target for immune intervention because it is associated with the apical end of the merozoite (48) and therefore may play a role in erythrocyte invasion by *Babesia bovis* (21, 34). Finally, conservation of the Bb-1 gene among isolates from the New World and Australia implies a functional importance for the Bb-1 protein in parasite survival, so that the use of this protein or an immunogenic derivative as a vaccine might have worldwide applicability.

The largest band visualized on SDS-PAGE corresponding to the Bb-1-GST fusion protein has a size of 72 kDa. This size is not that predicted from the fusion of *S. japonicum* GST (26 kDa) with that of the Bb-1 subfragment (59 kDa), for which the complete DNA sequence has been determined (47). The size discrepancy could be due to anomalous migration of the fusion protein on SDS-PAGE or to limited proteolysis of the Bb-1-GST fusion protein. Instability of foreign proteins in *E. coli* is routinely observed and results in limited digests or stable intermediates formed in the process of degradation (14, 15). Additional bands were observed upon silver staining and on immunoblots which likely represent degradation products of the intact fusion polypeptide rather than *E. coli* contaminants, since no similar bands were apparent on immunoblots of GST which was purified from *E. coli* lysate in the same way as Bb-1-GST. Furthermore, preimmunization sera did not detect these bands.

Our results show that a recombinant form of the Bb-1 protein can induce T-cell proliferation in PBMC from cattle immune to *B. bovis* but not from normal cattle. The proliferative response of the C15 line was MHC restricted (Fig. 7), and clones C97.1C8, 1B10, and 1D1, derived from C97 cultures stimulated with Bb-1-GST, also responded to Bb-1 in an MHC-restricted manner. These results show that because the responses require autologous APC, the Bb-1

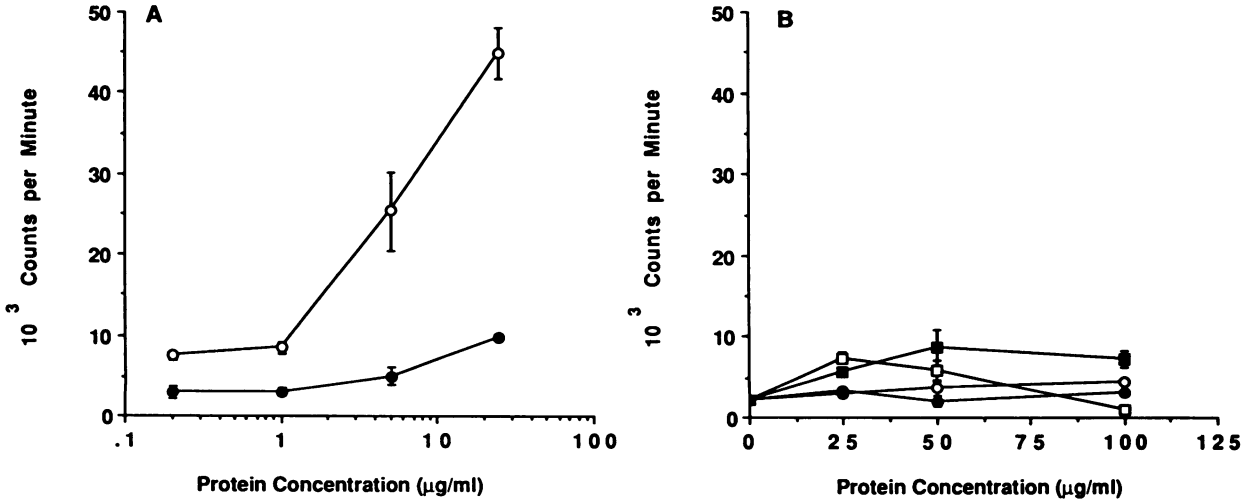

FIG. 6. Proliferative responses of a C15 Bb-1-specific T-cell line to Bb-1-GST and merozoite antigens. T cells (2×10^4) from a cell line cultured with Bb-1-GST for 8 weeks were assayed for proliferation for 3 days with 2×10^5 autologous APC and antigen. (A) Cells were stimulated with a final concentration of 0.2, 1, 5, or 25 μg of GST (●) or Bb-1-GST (○) protein per ml. Background proliferation (medium only) was 2,185 ± 542 cpm. (B) Cells were stimulated with a final concentration of 25, 50, or 100 μg of URBC HSS (□), *B. bovis* HSS (■), URBC CM (○), or *B. bovis* CM (●) protein per ml.

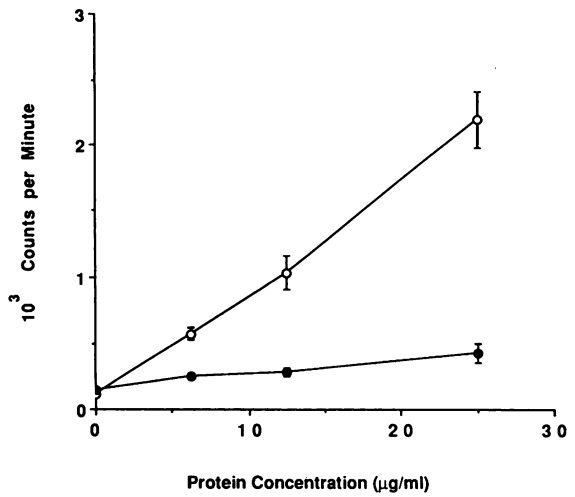

FIG. 7. Requirement for MHC-matched APC in the response of C15 Bb-1-specific T cells. T cells (2×10^4) from the C15 Bb-1-specific cell line cultured with Bb-1-GST for 7.5 weeks were stimulated in the proliferation assay for 3 days with 6.2, 12.5, or 25 μg of Bb-1-GST per ml with 2×10^5 autologous APC (○) or MHC-mismatched APC obtained from animal C97 (●).

antigen is not behaving as either a mitogen or a foreign superantigen. Mitogens stimulate T cells in an MHC-unrestricted manner, and superantigens can be presented by allogeneic or xenogeneic MHC class II molecules (17).

Bb-1 is not an immunodominant antigen for T cells of the animals used in this study. $CD4^+$ T-cell lines and clones derived from animals C97 and C15 after stimulation with crude CM or HSS antigen did not respond to Bb-1-GST, indicating that other antigens present in crude parasite extracts preferentially stimulate $CD4^+$ T cells (4). Furthermore, T-cell lines reactive with Bb-1-GST failed to proliferate against crude merozoite fractions. This could be explained by the relatively low concentration of the native

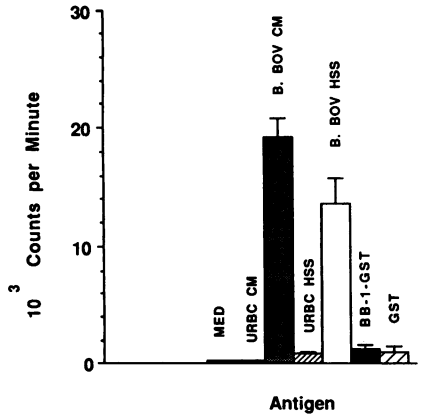

FIG. 8. Proliferative responses of a *B. bovis*-specific C15 T-cell line against merozoite fractions and recombinant Bb-1-GST. PBMC from animal C15 were cultured for 4 weeks with the CM fraction of *B. bovis* and for 3 weeks with the HSS fraction of *B. bovis* merozoites. To assay proliferation, 2×10^4 T cells were stimulated for 3 days with 1 to 100 μg of antigen per ml. Results are presented for the following concentrations of antigen: 1 μg of URBC or *B. bovis* CM per ml; 2 μg of URBC or *B. bovis* HSS per ml; and 25 μg of GST or Bb-1-GST per ml. MED, medium (control).

77-kDa protein in these homogenized parasite fractions. In a parallel study, we found that T-cell clones specific for the 42-kDa immunodominant merozoite surface glycoprotein of *B. bovis* responded weakly or not at all to crude parasite antigen (7). After analysis by SDS-PAGE or immunoblotting, the 42-kDa protein is much more prominent than the 77-kDa protein (5).

It is intriguing that the cell types comprising the cell lines induced by Bb-1-GST are quite different from those stimulated by crude merozoite antigens, which were predominantly $CD4^+$ in these and additional immune cattle. Bb-1-GST stimulated a mixture of $CD8^+$ and $CD4^+$ T cells (C97) or cells which predominantly expressed the γδ T-cell receptor (C15). T-cell clones established by limiting dilution of Bb-1-GST-reactive cell lines from these cattle reflected the mixed composition of the cultures; we obtained three $CD4^+$ clones (C97.1C8, 1B10, and 1D1) and three $CD8^+$ clones (C15.3E6, 2C11, and 1H5) which reacted specifically to Bb-1. Additional γδ T-cell clones ($WC1^+$ $CD2^-$ $CD4^-$ $CD8^-$) obtained at the same time reacted preferentially but not exclusively to Bb-1-GST (data not presented). The function of γδ T cells in the bovine immune response is not understood, although evidence suggests that these $CD2^-$ $CD4^-$ $CD8^-$ T cells participate in autologous mixed-lymphocyte reactions in vitro (9). We are exploring the possibility that Bb-1, which is a stress-induced protein, may stimulate γδ T cells. In mice, heat shock proteins appear to be selective targets for this type of response (29).

The GST moiety of the recombinant Bb-1 fusion protein does not appear to influence the induction of antibody or T-cell responses to Bb-1. We were unable to establish T-cell lines reactive with recombinant GST from immune animals C15 and C97, which is not surprising, since PBMC from these animals reacted only weakly to this protein. As an immunogen, GST does not appear to dominate or downregulate immune responses to the foreign protein moiety of the fusion polypeptide, since rabbits immunized with recombinant Bb-1-GST produced antibody which reacted with the native babesial protein as well as with recombinant Bb-1-GST or GST. GST fusion proteins might be successfully used as bivalent vaccines for immunizing ruminants with recombinant parasite or bacterial proteins. GST purified from *Fasciola hepatica* induced protective immunity to this parasite in vaccinated sheep (39), and GST-*Taenia ovis* fusion proteins stimulated partial protective immunity in sheep against challenge infection with *T. ovis* eggs (22). In addition, a GST-*Borrelia burgdorferi* fusion protein induced high-titered anti-*Borrelia* antibody and protective immunity against spirochetes in vaccinated mice (12).

Studies are in progress with the Bb-1-specific T-cell clones to identify the T-cell epitopes on the protein. Of equal interest will be identification of the cytokines produced by these T cells in response to antigen and examination of their effects on the growth and development of the parasite. Since T cells and macrophages are implicated as mediators of both immunity and pathology in *B. bovis* infections (49), studies of the host T cell-macrophage-parasite interaction at the molecular level are critical for vaccine development.

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