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Bovine Helper T Cell Clones Recognize Five Distinct Epitopes on *Babesia bovis* Merozoite Antigens

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Helper T cell clones from two *Babesia bovis*-immune cattle were characterized for use in identification of potentially protective immunogens of *B. bovis* merozoites. Proliferation assays with 11 $CD4^+$ clones revealed a differential pattern of response to soluble cytosolic antigen, membrane-enriched antigen, detergent extracts of the membrane-enriched antigen, soluble culture supernatant exoantigen, and different geographical isolates of *B. bovis* as well as *Babesia bigemina* parasites. When the data were combined, the clones could be grouped according to five different patterns of response. One group recognized only the membrane-enriched fraction of New World and Australian parasites. Four remaining groups recognized antigens found in the cytosolic as well as the membrane-enriched fraction, and clones representative of each group were used to identify cytosolic antigens fractionated by anion-exchange chromatography with the use of fast-performance liquid chromatography. One clone (C97.3C3), which responded to all *B. bovis* isolates and to *B. bigemina*, recognized a single peak of activity that eluted with 0.25 M NaCl and contained protein bands of 70 and 75 kDa. The remaining clones were stimulated by a second antigenic peak that eluted between 0.35 and 0.45 M NaCl and contained protein bands of 42, 47, 56, and 84 kDa. The majority of the clones produced interferon, whereas tumor necrosis factor alpha/tumor necrosis factor beta production was less frequent. These studies provide the basis for using helper T cell clones to identify potentially protective immunogens of *B. bovis* and delineate a minimum of five helper T cell epitopes recognized by two immune cattle.

The tick-borne intraerythrocytic protozoan parasite *Babesia bovis* causes a virulent form of babesiosis, characterized by anemia, fever, anorexia, cachexia, low parasitemia, and a generalized circulatory disturbance, often resulting in high mortality rates among nonimmune cattle. One hallmark of this disease is the sequestration of parasitized erythrocytes in the capillary beds of the brain and lung, leading to cerebral babesiosis and respiratory distress syndrome (48–50). Similarity in the immunopathology caused by this parasite and related apicomplexan parasites in the genus *Plasmodium* has led to the hypothesis that the diseases caused by these hemoparasites share common mechanisms (49, 50). Cytokines, including gamma interferon (IFN-γ) and tumor necrosis factor (TNF) (7), released by parasite antigen-activated T cells and macrophages are implicated in anemia (16, 28), cytoadherence of parasitized erythrocytes to the brain microcapillary endothelia (20, 21), and accumulation of infected erythrocytes and neutrophils in the pulmonary vasculature (17).

Protective immunity in experimental murine malaria and babesial infections is mediated by T cells and macrophages (1, 8, 14, 15, 26, 36, 47), and the same cytokines involved in immunopathology play a role in protective immunity (17, 37–39, 41). For example, IFN-γ inhibited the intrahepatocytic development of *Plasmodium berghei* parasites (37) and was required for protective immunity to sporozoite challenge (38). Heat-stable, soluble exoantigens of *Plasmodium falciparum* and *Plasmodium yoelii* have been shown to induce the production of TNF from activated macrophages in vitro and in vivo (4–6, 33, 40), and in vivo administration of recombinant TNF inhibited parasitemias caused by infection with *P. yoelii* (41) and *Plasmodium chabaudi* (39). Although definitive studies on the role of cytokines in babesiosis have not been performed with *B. bovis*, supernatants from macrophages incubated with culture-derived babesial exoantigens were shown to inhibit parasite growth in vitro (29). Furthermore, the hypothesis that T cell-macrophage interactions are involved in both immunity and pathogenesis is supported by the observations that parasitemias were higher and cerebral sequestration occurred less frequently in splenectomized cattle infected with *B. bovis* (48).

Identification of parasite antigens which evoke immunity and/or immunopathology during the course of hemoparasitic infection is essential for vaccine development. In studies with *B. bovis*, attempts to characterize protective antigens have historically relied upon the use of antibodies to identify immunodominant proteins. Although antibody may play a role in merozoite neutralization (25), it has become increasingly evident that serologically immunodominant antigens are not always protective against a challenge *B. bovis* infection (19, 44, 46, 49); and in some studies protective immunity to *B. bovis* was inversely related to antibody titer (49). These observations, together with the documented relevance of T cells and macrophages in both immunity and immunopathology associated with infection by related hemoparasites, have prompted a detailed investigation of cell-mediated immune responses in *B. bovis*-infected cattle. Because *B. bovis* has no known exoerythrocytic stage which could serve as a target for major histocompatibility complex (MHC)-restricted cytotoxic T cells, our research has focused on the identification of babesial merozoite antigens which induce T helper (Th) cell responses in immune cattle and on characterization of the cytokines produced by *Babesia*-specific Th cells. We recently described the preferential

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stimulation of $CD4^+$ T cells by unfractionated cytosolic and membrane merozoite antigens of *B. bovis* (10). The differential pattern of response by T cell lines derived from three immune cattle suggested the presence of multiple immunodominant epitopes. In the studies reported here, we have further dissected these responses by examining a panel of Th clones derived from several *Babesia*-specific T cell lines. At least five distinct Th epitopes can be defined by the patterns of response to different parasite isolates and antigenic fractions. Preliminary studies on the production of IFN-γ and TNF-α or TNF-β demonstrate a functional heterogeneity among the Th clones. Together, these experiments provide the foundation for using Th clones to identify potentially protective antigens which may be useful in the development of a vaccine for *B. bovis*.

MATERIALS AND METHODS

Babesial parasite strains and cultivation. The Mexican strain of *B. bovis* was originally isolated in 1978 from cattle infected with natural, tick-derived isolates from an area of Mexico in which babesiosis is endemic (11). The Texan strain of *B. bovis* originated from an infected animal in southern Texas in 1978 (32) and was provided by Will Goff (USDA Agricultural Research Service, Pullman, Wash.). The Australian L strain of *B. bovis* originated from an infected animal in New South Wales, Australia, in 1965 (27) and was provided by Stephen Hines (Washington State University, Pullman). The Mexican strain of *Babesia bigemina* was obtained from an infected animal in northeastern Mexico in 1982 (11). In vitro microaerophilous cultures of all babesial strains were maintained in bovine erythrocytes (11).

Experimental cattle. Cattle were infected with the Mexican strain of *B. bovis* as described in detail elsewhere (11). Briefly, 2-year-old crossbred cow C15 was inoculated intravenously three times over the course of 3 years with *B. bovis* merozoites obtained from autologous, infected erythrocyte cultures. Although animal C15 has not been challenged with tick- or blood-derived parasites, challenge inoculation of 10^9 cultured parasites did not evoke a reduction in packed erythrocyte volume or elevation in temperature. Animal C97, a 9-month-old crossbred cow, was infected by infestation with *Boophilus microplus* tick larvae infected with the same isolate of *B. bovis*. The animal was solidly immune to challenge infection 3 months later with an intramuscular inoculation of a virulent, infected 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. Following infection, both animals were serologically positive for *B. bovis* as determined by indirect immunofluorescence staining of cultured parasites and by immunoblotting merozoite antigen (11). Peripheral blood mononuclear cells (PBMC) from cattle C15 and C97 were typed for bovine class I MHC antigens (42) and had the bovine lymphocyte antigen (BoLA) phenotypes w11/w12 and w5/w6, respectively.

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 soluble, cytosolic, high-speed supernatant fraction (HSS) and a fraction enriched in crude membranes (CM) as described previously (11). Uninfected erythrocytes (URBC) were similarly fractionated for use as control antigens. Detergent solubilization of the CM fraction was achieved with 1% 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS) (Pierce, Rockford, Ill.) (10), and the extracts were then dialyzed against phosphate-buffered saline, pH 7.0, at 4°C.

Soluble merozoite HSS was fractionated by anion exchange on a Mono Q (HR 5/5) column using a fast-performance liquid chromatography (FPLC) apparatus (Pharmacia LKB Biotechnology, Piscataway, N.J.). HSS was dialyzed against the starting buffer (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES]) and filtered, and 7 ml containing 1 mg of protein per ml was loaded onto the Mono Q column. The bound proteins were eluted with a 20-ml linear NaCl gradient of 0.0 to 1.0 M NaCl in 20 mM HEPES at a flow rate of 1.0 ml/min, and protein was monitored at 280 nm. Individual 1.0-ml fractions were collected and diluted to isotonicity (150 mM NaCl) for T cell proliferation studies. Supernatants containing parasite exoantigens were collected from cultures of *B. bovis* (Mexico)-infected erythrocytes or URBC and were also assessed for antigenic activity.

The recombinant 42-kDa *B. bovis* protein Bv42 (25) was obtained from Guy Palmer (Washington State University, Pullman). The recombinant 77-kDa fusion protein Bb-1-GST was described previously (43). Tuberculin purified protein derivative (Statens Seruminstitut, Copenhagen, Denmark) was used as a negative control.

The protein content in all antigen preparations was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.) using immunoglobulin G (IgG) as a protein standard. All antigens were aliquoted and stored at −80°C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of FPLC fractions. FPLC fractions (17.5 μl) were diluted with an equal volume of sample buffer, electrophoresed on 10 or 12% acrylamide gels under reducing conditions, and stained with a silver staining kit from Bio-Rad (10).

Generation of *B. bovis*-specific T cell clones. T cell lines established from PBMC from immune cattle C15 and C97 and specific for *B. bovis* merozoites were described in a previous publication (10). Briefly, a cell line from animal C97 was stimulated with *B. bovis* (Mexico) CM for 5 weeks, and a cell line from animal C15 was stimulated for 4 weeks with the same stock of CM and for an additional 2 weeks with HSS prepared from these parasites. An additional cell line from animal C15 was stimulated for 1 week with CM and 2 weeks with HSS prepared from the Mexican isolate of *B. bovis*. All three cell lines were shown to proliferate in a dose-dependent manner to *B. bovis* (Mexico) merozoite antigens and were selected for limited dilution cloning. Cloning was performed in 96-well round-bottomed plates (Costar, Cambridge, Mass.) essentially as described elsewhere (12, 43), with the following modifications. A statistical average of 1 or 0.3 cell per well was stimulated with 25 μg of *B. bovis* (Mexico) CM (for the C97 T cell line) or HSS (for the C15 T cell lines) antigen per ml of complete RPMI 1640 medium (11) containing 10% bovine T cell growth factor (TCGF [9]) and 5×10^4 irradiated autologous PBMC. Six or seven days after the last stimulation, cells were tested for antigen-dependent proliferation.

Cell surface phenotypic analysis. Antigen-specific clones were stained by indirect immunofluorescence as described previously (43) and analyzed by flow cytometry with a Coulter EPICS 741 flow cytometer. Monoclonal antibodies (MAb) specific for bovine leukocyte surface markers were obtained from the International Laboratory for Research on Animal Diseases (Nairobi, Kenya): IL-A51, specific for CD8

(18); IL-A12, specific for CD4 (3); and IL-A26, specific for CD2 (2).

Lymphocyte proliferation assays. Proliferation assays were carried out in duplicate wells of half-area 96-well plates (Costar) at 37°C, in a humidified atmosphere of 5% CO_2 in air for 3 days as described elsewhere (12, 43). Each well (100-μl total volume) contained complete medium, responder cells added at a final concentration of 2×10^5 or 3×10^5 cells per ml (obtained 6 or 7 days following the last stimulation of the cell lines with antigen), and 2×10^6 autologous or allogeneic, irradiated (3,000 rads) PBMC per ml as a source of antigen-presenting cells (APC). Antigens were added to the assay mixes at a final concentration of 1 to 250 μg of protein per ml of complete medium. Bovine TCGF (5%) or recombinant human interleukin 2 (IL-2; Boehringer Mannheim, Indianapolis, Ind.) (5 or 25 U/ml) was included as a positive control for lymphocyte proliferation. Unless indicated otherwise, clone C15.1H6 was cultured in the presence of 2% TCGF, in addition to antigen, in all wells of the proliferation assays. In some experiments, a MAb, designated IL-A21 (International Laboratory for Research on Animal Diseases), specific for a monomorphic determinant on bovine MHC class II, was included to block antigen presentation. The IgG was purified from ascitic fluid (kindly provided by John Ellis, University of Wyoming, Laramie) by binding to protein G (MabTrap G; Pharmacia) according to the manufacturer's directions. Purified IgG was then added to T cells, APC, and antigen or TCGF at a final concentration of 25 μg of IgG per ml. In some experiments an isotype-matched control MAb was included. Proliferation was determined by measuring the incorporation of 0.25 μCi of [^{125}I]iododeoxyuridine (^{125}IUdR; ICN Radiochemicals, Inc., Costa Mesa, Calif.) added during the final 4 h of the assay. The cells were harvested (11), and the radioactivity was counted in a gamma counter. Results are presented as the mean counts per minute and standard deviations of duplicate samples. When mismatched APC were included in the assays, results are also presented as the percentage of the proliferative response of the T cell clone stimulated with antigen or TCGF with autologous APC.

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

Stimulation of T cell clones 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 to 24 h at a concentration of 2×10^6 cells per ml of complete medium containing 5 μg of concanavalin A (Sigma Chemical Co., St. Louis, Mo.) per ml in the absence of APC. Supernatants were harvested by centrifugation and stored at −80°C.

Biological assays for IFN and TNF. IFN activity in supernatants was measured in a microtiter cytopathic effect assay with vesicular stomatitis virus and Madin-Darby bovine kidney (MDBK) cells as described elsewhere (12). MDBK cell survival was evaluated by the 4-h uptake of MTT dye in the cells (22). IFN titers were compared with a human recombinant IFN-α_2 reference reagent (National Institute of Allergy and Infectious Diseases, Bethesda, Md.; catalog no. Gxa01-901-535) with a reported activity of 9,000 or 3.95 $\log_{10}$ IU/ml. In our assay, this standard had a titer of 3.35 ± 0.18 $\log_{10}$ IU/ml.

For determination of TNF-α/TNF-β activities in supernatants of *B. bovis*-specific T cell clones, a TNF-sensitive WEHI-164 subline originating from Edward Lattime (Memorial Sloan-Kettering, New York, N.Y.) was incubated with culture supernatants in a 48-h assay (35). Cytopathicity was determined by the MTT dye reduction assay. TNF titers in the supernatants were compared with the standard human recombinant TNF-α (Upstate Biotechnology Inc., Lake Placid, N.Y.), which had a reported activity of 10^6 U/ml and in our assay had a titer of 5.92 ± 0.15 $\log_{10}$ U/ml.

RESULTS

***B. bovis*-specific T cell clones.** Three *B. bovis*-specific T cell lines were stimulated with either the CM or HSS of merozoites and were cloned by limiting dilution. Cloning frequencies of 16.6 and 25.5%, respectively, were obtained from the C97 and C15 T cell lines when an average of 0.3 T cell was distributed per well. The majority of clones were *B. bovis* specific, totaling 29 C97 clones and 34 C15 clones. For the second C15 T cell line, cultured for 3 weeks and cloned with an average of 1 T cell per well, a cloning frequency of 58% was obtained. Nine clones responded to antigen, and one, designated C15.1H6, was subcloned. All six subclones responded to antigen (data not presented), so the more vigorous parent clone was used in subsequent assays. All of the clones expressed the surface phenotype characteristic of Th cells: $CD2^+$ $CD4^+$ $CD8^-$. The 11 clones described in this study were initially selected on the basis of proliferation to babesial antigen in the absence of exogenous IL-2.

Differential response of Th clones to HSS and CM of homogenized merozoites. Several different patterns of response to crude *B. bovis* antigens were observed, as exemplified by results with four clones shown in Fig. 1. The majority of the clones derived from the C97 cell line responded to the CM but not to the soluble HSS of merozoites (Fig. 1A). However, two clones, C97.3E9 and C97.3E12, responded strongly to both CM and HSS (Fig. 1B), and a third clone, C97.3C3, proliferated weakly in response to HSS as compared with CM (Fig. 1C). All C15 clones responded vigorously to both CM and HSS (Fig. 1D). None of the clones responded to HSS or CM prepared from URBC (data not presented and Tables 1 and 3).

Differential response of Th clones to detergent extracts of merozoite CM. Detergent extraction of merozoite membrane proteins was performed to evaluate the potential to use Th clones to probe for detergent-solubilized proteins during purification procedures. The Th clones exhibited the same pattern of response to CHAPS extracts of *B. bovis* CM as did the parent cell lines (10). With the exception of clone C15.1H6, all C15 clones responded vigorously to CHAPS extract, whereas the C97 clones, including HSS-responsive clone C97.3E9, responded weakly or not at all to this preparation of antigen (Table 1).

Differential response of Th clones to culture supernatant exoantigens. We had previously determined that the parasite-specific cell lines from animal C15, but not C97, were stimulated by antigens in the parasite culture supernatant (10). Th clones derived from these lines reacted in a similar manner, in that the C97 clones responded poorly or not at all to the soluble exoantigen whereas four of six C15 clones were stimulated by the parasite culture supernatant (data from representative clones are presented in Table 2).

Differential response of Th clones to *B. bovis* and *B. bigemina* isolates. It was of interest to determine whether the Th clones recognized antigens shared between different geographical isolates of *B. bovis*, since inclusion of common antigens in a vaccine would be preferential. Selected clones were cultured with the CM of Mexican (immunizing isolate), Texan, or Australian *B. bovis* merozoites all propagated in vitro in erythrocytes from the same uninfected donor animal

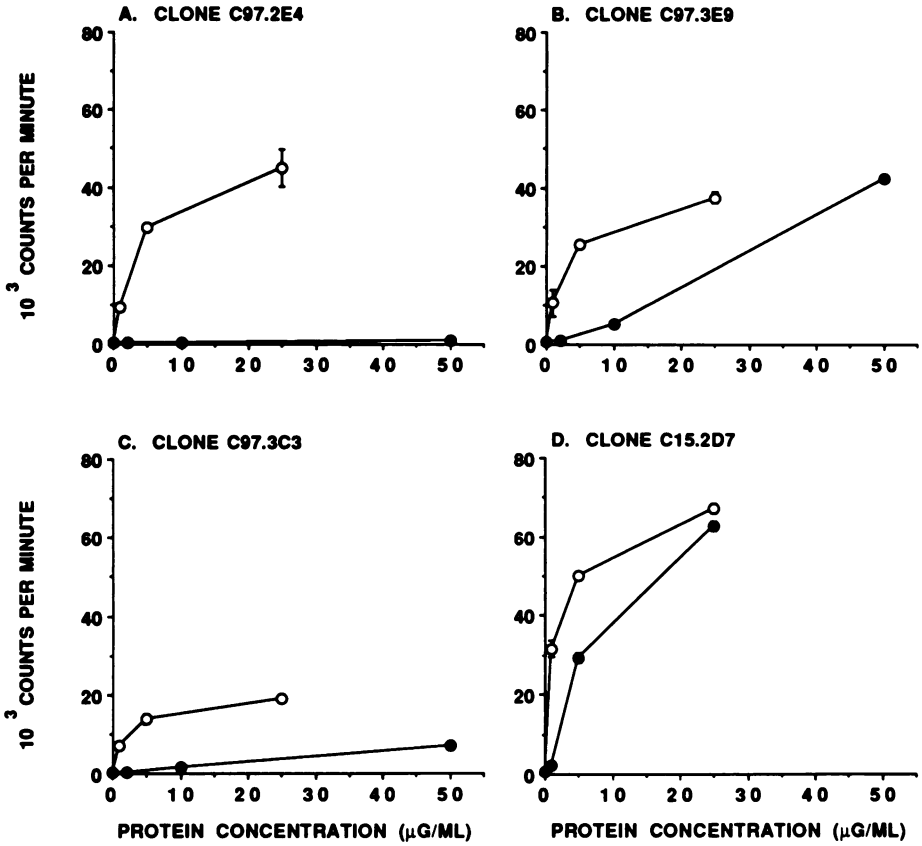

FIG. 1. Dose-dependent proliferation of Th clones against cytosolic and membrane-enriched fractions of *B. bovis* merozoites. Representative proliferative responses of 4 clones, out of a total of over 60, to *B. bovis* (Mexico) HSS (closed circles) or CM (open circles) antigens are presented.

(results with representative clones are shown in Table 3). A differential pattern of response emerged: five of seven C97 clones responded to all *B. bovis* isolates, whereas two clones responded only to the Mexican and Texan isolates. Only one of four C15 Th clones responded to all three *B. bovis* isolates, and the remaining three clones responded to both Mexican and Texan isolates. Previous studies indicated a differential response of T cells from animals C97 and C15 to *B. bigemina* parasites, in that C15 cells responded as well to *B. bigemina* as to *B. bovis* whereas the C97 cells were stimulated weakly or not at all by this parasite (10, 11). However, when the Th clones were examined for proliferation to *B. bigemina*, only one clone (C97.3C3) responded to this parasite, and its proliferation against *B. bigemina* was consistently higher than that against *B. bovis* (Table 3).

Proliferative responses of Th clones to recombinant babesial proteins. None of the Th clones recognized either a recombinant form of the 42-kDa major merozoite surface glycoprotein, Bv42 (25), or the recombinant 77-kDa merozoite fusion protein, Bb-1-GST (43, 45) (data not presented), both of which have been shown to induce proliferation of $CD4^+$ T cells in these two animals (13, 43). As expected, none of the Th clones responded to purified protein derivative of *Mycobacterium bovis* (data not presented).

Th clones are MHC class II restricted. To ensure that the Th clones recognized parasite antigen in an MHC-restricted

TABLE 1. Proliferative responses of *B. bovis*-specific Th cell clones to detergent-solubilized merozoite membranes

Clone	Radioactivity (mean cpm ± SD) incorporated by T cell lines stimulated with[a]:			
	Medium (control)	URBC CM	*B. bovis* CM	CHAPS extract
C97.2E4	235 ± 14	286 ± 9	51,918 ± 507	10,246 ± 43
C97.3C3	2,432 ± 77	2,492 ± 572	23,604 ± 184	896 ± 89
C97.3E9	625 ± 25	652 ± 12	25,314 ± 707	1,649 ± 158
C15.1H6	436 ± 15	596 ± 11	11,370 ± 764	728 ± 120
C15.2D7	587 ± 3	662 ± 2	44,720 ± 934	33,612 ± 1,779

[a] Antigens, prepared from URBC or the Mexican isolate of *B. bovis*, were used at a final protein concentration of 5 μg/ml except that 10 μg of CHAPS per ml was used to stimulate the C97 T cell clones.

TABLE 2. Proliferative responses of *B. bovis*-specific Th cell clones to culture supernatant exoantigen

Clone	Radioactivity (mean cpm ± SD) incorporated by T cell clones stimulated with supernatants[a] collected from:	
	URBC	*B. bovis* (Mexico)-infected erythrocytes
C97.2E4	362 ± 107	1,671 ± 396
C97.3C3	490 ± 10	1,137 ± 58
C97.3E9	826 ± 54	790 ± 22
C15.1H6	678 ± 61	731 ± 19
C15.2D7	697 ± 32	12,961 ± 1,111

[a] A final protein concentration of 200 or 250 μg/ml was used in the assay.

TABLE 4. Proliferative responses of *B. bovis*-specific Th cell clones with autologous or MHC-mismatched APC

Clone	TCGF or antigen[a]	Radioactivity (mean cpm ± SD) incorporated by T cells stimulated with:	
		Autologous APC	MHC-mismatched APC[b] (%[c])
C97.2E4	TCGF	5,476 ± 418	6,645 ± 601 (121)
	CM	45,023 ± 4,720	394 ± 29 (<1)
C97.3C3[d]	CM	18,864 ± 819	164 ± 4 (<1)
C97.3E9	TCGF	38,972 ± 2,336	47,978 ± 1,126 (123)
	CM	37,400 ± 1,509	727 ± 1 (<1)
C15.1H6	TCGF	5,970 ± 723	5,570 ± 283 (93)
	HSS	16,041 ± 581	450 ± 27 (<1)
C15.2D7	TCGF	22,669 ± 1,009	7,603 ± 224 (33)
	HSS	62,693 ± 1,275	368 ± 7 (<1)

[a] TCGF was used in the assay at a final concentration of 5%, with the exception of clone C15.1H6, for which it was used at a final concentration of 12%. *B. bovis* (Mexico) antigen (CM or HSS) was used in the assay at a final concentration of 25 μg of protein per ml.
[b] Irradiated C15 PBMC were used as mismatched APC for C97 Th clones, and irradiated C97 PBMC were used as mismatched APC for C15 Th clones.
[c] Percentage of the response of Th clone with autologous APC.
[d] Results with TCGF were not determined.

manner and were not being stimulated by parasite-derived superantigens (23), selected clones were compared for stimulation with antigen or TCGF as a control, in the presence of either autologous or MHC-mismatched APC which were known to differ in expression of BoLA class I antigens (Table 4). When mismatched APC were used to present *B. bovis* antigen, the proliferative responses of all Th clones were completely abrogated. TCGF-induced proliferation was either not affected or only partially inhibited in the presence of mismatched APC. Inclusion of purified IgG from a MAb (IL-A21) specific for bovine MHC class II resulted in 86 to 99% inhibition of antigen-induced proliferation of all clones tested at a concentration of 25 μg of IgG per ml (data not presented), showing that the Th clones respond to babesial antigen in an MHC class II-restricted manner. Nonspecific toxicity of the IgG was ruled out by the absence of inhibition of the response of the clones to TCGF. An additional control using an isotype-matched (IgG2a) MAb was included in the experiment, and although this antibody nonspecifically inhibited the proliferation to TCGF at a concentration of 25 μg/ml or higher (65% inhibition versus 0% inhibition by MAb IL-A21 of the response of clone C97.3C3), the antigen-specific response was not inhibited by the control MAb to the same extent as by MAb IL-A21 (48% inhibition versus 99% inhibition by MAb IL-A21 of the response of clone C97.3C3).

Fractionation of *B. bovis* HSS antigen by FPLC anion-exchange chromatography. Because many of the *B. bovis*-specific Th clones could be stimulated by soluble antigen present in the HSS of merozoites, several clones were used in an attempt to identify antigenic proteins present in HSS fractionated by anion-exchange chromatography using FPLC. Figure 2A shows that the antigenic activity for clone C97.3C3 eluted in a single peak with 0.25 M NaCl, whereas clones C97.3E9, C15.1H6, C15.2D7, and C15.3A5 were all stimulated by antigen eluting between 0.35 and 0.45 M NaCl (Fig. 2B and C). As a negative control, a Th clone (C97.1C8) reactive with recombinant Bb-1-GST, which did not respond to HSS (43), was similarly tested and did not respond to any of the fractions (data not presented).

Examination of the individual FPLC fractions by SDS-PAGE and silver staining (Fig. 3) revealed the presence of two bands of 70 and 75 kDa, apparently unique to the peak of activity eluting with 0.25 M NaCl (lane B), which stimulated only clone C97.3C3. The peak of antigenic activity in fractions eluting with 0.35 to 0.45 M NaCl (Fig. 3, lane D) contained numerous proteins, including prominent ones of 42, 47, 56, and 84 kDa.

Cytokine production by the Th clones. Biological assays for TNF-α/TNF-β and IFN-γ were used to detect the production of these cytokines following stimulation of the Th clones

TABLE 3. Proliferative responses of *B. bovis*-specific Th cell clones to *B. bigemina* and different isolates of *B. bovis*

Clone	Antigen concn (μg/ml)	Mean cpm of ^{125}IUdR incorporation (SD) with[a]:					
		Medium	URBC	*B. bovis* from:			*B. bigemina*
				Mexico	Texas	Australia (L strain)	
C97.3C3	25	489 (138)	429 (10)	**12,025 (2,129)**	**5,543 (741)**	**18,145 (2,224)**	**30,063 (1,172)**
C97.2E4	25	734 (54)	743 (11)	**43,569 (148)**	**38,798 (1,928)**	**48,052 (1,485)**	731 (10)
C15.1H6[b]	25	11,642 (622)	12,213 (26)	**53,087 (104)**	**48,291 (60)**	**45,722 (1,016)**	10,163 (634)
C97.3E9	25	1,042 (37)	1,152 (26)	**21,462 (292)**	**7,542 (1,250)**	1,342 (44)	1,014 (114)
C15.2D7[c]	25	570 (117)	416 (9)	**31,270 (1,640)**	**28,614 (1,911)**	455 (37)	ND
	5	587 (3)	662 (2)	**44,720 (934)**	ND	ND	622 (21)

[a] Results of duplicate cultures. Results indicated in boldface type were shown to be significantly different from those with control cultures ($P < 0.01$) by the Student one-tailed *t* test. Antigen consisted of the CM of URBC, *B. bovis* merozoites, or *B. bigemina* originating from Mexico. Results are presented for stimulation with 25 μg of antigen per ml, except for clone C15.2D7, for which the *B. bigemina* antigen was tested at 5 μg of protein per ml. Controls are included for this experiment. ND, not determined.
[b] Clone C15.1H6 was assayed in the presence of 5 U of human IL-2 per ml.
[c] Data from two experiments are presented for clone C15.2D7 to include all relevant results.

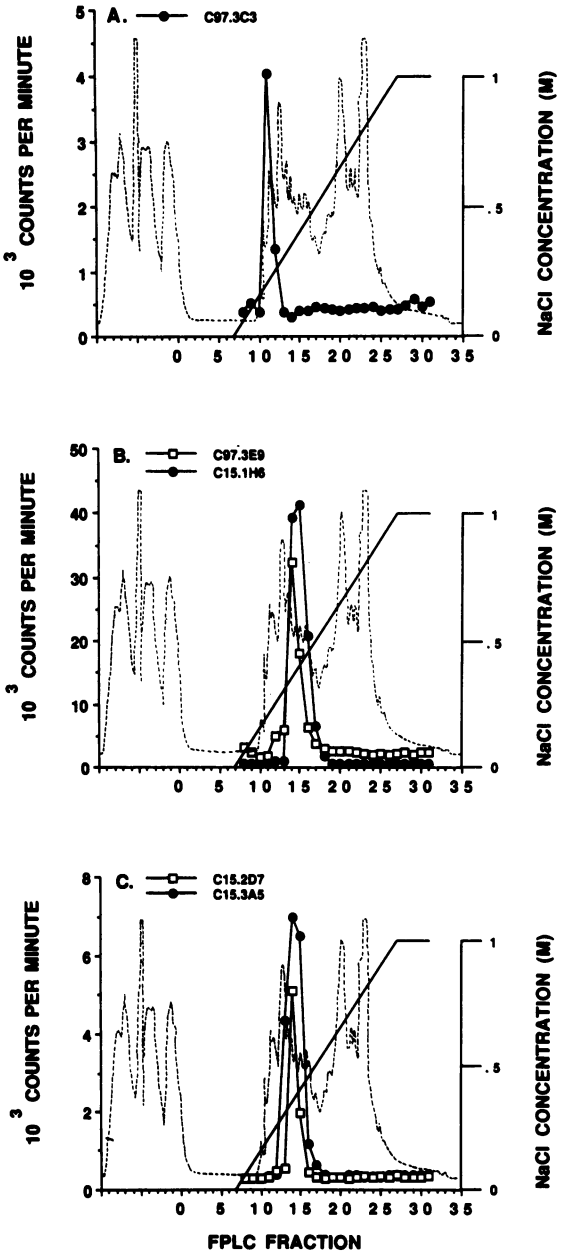

FIG. 2. Proliferative responses of five Th clones to *B. bovis* (Mexico) HSS antigen fractionated by anion-exchange chromatography using FPLC. HSS was applied onto the Mono Q column, eluted with a linear NaCl gradient (——), and monitored for protein at 280 nm (– – –) on a scale of 0 (baseline) to 1.0 (maximum peaks) U of absorbance. FPLC fractions were diluted to isotonicity, and 25-μl samples were assayed in duplicate cultures with the following Th clones and autologous APC: C97.3C3, C97.3E9 (plus 5 U of IL-2 per ml) and C15.1H6 (plus 2.5% TCGF), and C15.2D7 and C15.3A5. The symbols indicate the proliferative responses of the indicated clones to individual fractions.

with concanavalin A (Table 5). The majority of Th clones produced IFN-γ, with activity ranging from barely detectable (2 U/ml) to 226 U/ml. TNF-α/TNF-β production by these clones was also variable, ranging from undetectable to 13 U/ml. Interestingly, three clones produced no detectable cytokines (clones C15.2D7 and C15.3A5 are represented)

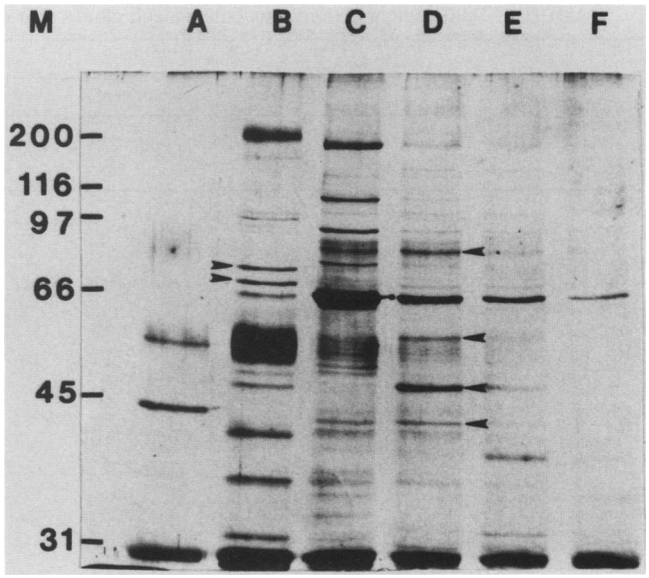

FIG. 3. SDS-PAGE and silver staining of selected FPLC fractions. Fractions eluting with the following concentrations of NaCl are shown: lane A, 0.15 M; lane B, 0.25 M; lane C, 0.35 M; lane D, 0.40 M; lane E, 0.45 M; and lane F, 0.50 M. Molecular size markers are indicated on the left, and individual bands of 84, 75, 70, 56, 47, and 42 kDa are indicated with arrowheads.

following our stimulation protocol. All of the Th clones responded to stimulation with IL-2, and those with undetectable IFN-γ and TNF-α/TNF-β activities had the highest proliferative responses to IL-2.

DISCUSSION

T lymphocytes are known to be important for the development of protective immunity against many intracellular protozoan parasites. However, the nature of protective immunity, including the subsets of T cells induced, in response to the cattle parasite *B. bovis* have not been characterized. We have identified a panel of *Babesia*-specific $CD4^+$ T cell clones for use in identification of T cell immunodominant antigens that could be purified from soluble merozoite extracts and for functional characterization of

TABLE 5. Proliferative responses to IL-2 and production of cytokines by *B. bovis*-specific Th cell clones

Clone	Specificity	Stimulation index[a]	Cytokine produced (U/ml)[b]	
			IFN	TNF
C97.2E4	CM	9	18	<2
C97.3C3	CM, HSS	11	200	13
C97.3E9	CM, HSS	3	226	3
C15.1H6	CM, HSS	18	41	6
C15.2D7	CM, HSS	41	<2	<2
C15.3A5	CM, HSS	93	<2	<2

[a] Calculated as 100 × mean cpm of ^{125}IUdR incorporation of duplicate cultures of a Th cell clone in response to IL-2/mean cpm of ^{125}IUdR incorporation of duplicate cultures of Th cells with medium alone. Human recombinant IL-2 was added at a final concentration of 25 U/ml, except for clones C97.2E4 and C97.3E9, which were stimulated with 5 U of IL-2 per ml.

[b] Cytokine activity was measured in the supernatants of Th cell clones cultured for 17 to 24 h with 5 μg of concanavalin A per ml.

TABLE 6. Summary of *B. bovis*-specific Th cell clones that define at least five antigenic epitopes in crude merozoite homogenate

Group and clones	Response[a] to:							
	Unfractionated *B. bovis* antigen[b]				Parasite isolates			
					B. bovis from:			*B. bigemina* from Mexico
	HSS	CM	CHAPS	EXO	Mexico	Texas	Australia	
I								
C97.2A6	−	+	±	−	+	+	+	−
C97.2D2	−	+	±	±	+	+	+	−
C97.2E4	−	+	±	±	+	+	+	−
C97.3E8	−	+	±	−	+	+	+	−
II								
C97.3C3	+	+	−	±	+	+	+	+
III								
C15.1H6	+	+	−	−	+	+	+	−
IV								
C97.3E9	+	+	±	−	+	+	−	−
C97.3E12	+	+	±	−	+	+	−	−
V								
C15.2B1	+	+	+	+	+	+	−	−
C15.2D7	+	+	+	+	+	+	−	−
C15.3A5	+	+	+	+	+	+	−	−

[a] Proliferative responses of the individual Th clones in each of the five groups are indicated as follows: +, positive response; ±, weakly positive response; and −, negative response.
[b] *B. bovis* HSS, CM, CHAPS extract of the CM, and soluble culture supernatant (EXO) antigens were prepared from merozoites of the Mexican isolate.

the T cells. The results presented here are the first to describe a differential pattern of response of MHC class II-restricted, $CD4^+$ bovine T cell clones stimulated with soluble or membrane-enriched *B. bovis* merozoite antigens.

On the basis of the patterns of proliferative responses against unfractionated merozoite antigens and different geographical isolates of *B. bovis* and *B. bigemina*, 11 Th clones fall into groups which appear to recognize at least five different antigenic epitopes, as summarized in Table 6. The first group of Th clones, all derived from animal C97 (clones 2A6, 2D2, 2E4, and 3E8), responded to the CM of parasites. There was no proliferation to soluble HSS and only little or no response to either CHAPS extract of CM or culture supernatant exoantigen. The epitope(s) recognized by these clones is present in New World and Australian isolates of *B. bovis*, but not *B. bigemina*, parasites.

The remaining Th clones recognized antigens present in both CM and HSS of merozoites and fall into an additional four groups. The second group is represented by clone C97.3C3, which recognizes an antigenic epitope shared by all *B. bovis* and *B. bigemina* parasites that is not extractable from the CM with CHAPS. The third group is represented by clone C15.1H6, which recognizes an antigen in HSS and CM shared by all *B. bovis* isolates which is not found in *B. bigemina*. The fourth group is represented by clones C97.3E9 and C97.3E12, which, unlike clone C15.1H6, recognize an antigen found only in New World isolates of *B. bovis*. Finally, the majority of C15 clones belong to a fifth group which recognize an antigen(s) found only in New World isolates of *B. bovis* and present in HSS, CM, CHAPS extract of the CM, and parasite culture supernatants. Thus, at least five distinct epitopes of *B. bovis* merozoite antigens can be distinguished by the induction of differential Th cell responses.

Four of these different epitopes are present in the cytosolic HSS, and fractionation of this soluble form of antigen by FPLC anion-exchange chromatography has indicated that activity elutes in two major peaks. Clone C97.3C3 is stimulated by fractions eluting with 0.25 M NaCl which contain proteins of 70 and 75 kDa apparently not present in neighboring fractions. The relative molecular masses of two of these proteins are consistent with stress-induced proteins belonging to the hsp 70 group (34). In this respect, it is intriguing that clone C97.3C3 recognizes a determinant shared across divergent babesial species, as parasite stress proteins are often highly conserved across species as well as genera (31). The Bb-1 gene, which is conserved among New World and Australian isolates of the parasite (45) and encodes a 77-kDa merozoite protein that is inducible by oxidative or nutritional stress (34a), does not elicit proliferation of this clone. The identity of the protein that stimulates clone C97.3C3 is not known.

Clones representative of the other three groups (C15.1H6, C97.3E9, and C15.2D7 and C15.3A5) were stimulated by fractions eluting between 0.35 and 0.45 M NaCl that contained prominent bands of 42, 47, 56, and 84 kDa. Since none of the Th clones recognized recombinant Bv42, the 42-kDa protein is not a likely candidate immunogen for these clones, although in its native and recombinant states, this protein is very immunogenic, stimulating both strong humoral and T cell responses in immune cattle (11, 13, 24). Combined protein fractionation protocols, including FPLC anion exchange, gel filtration, and isoelectric focusing, will be used to purify and identify the immunodominant T cell epitopes present in soluble fractions of *B. bovis* merozoites. However, as noted by us (12) and others (49), protozoal antigens which stimulate T cells may not be abundant and therefore may prove difficult to identify by biochemical purification.

Because the nature of the immune response and the induction of immune-mediated pathology elicited by immu-

nization with a defined babesial protein will reflect the cytokines produced by antigen-activated T cells and macrophages, we have measured cytokines produced by the *Babesia*-specific Th clones. Biological assays revealed a differential production of IFN-γ and TNF-α/TNF-β, suggesting functional differences among the Th clones. All clones in groups I to IV produced IFN-γ, and some clones in each group produced TNF, whereas none of the clones in group V produced either cytokine. All of the clones in groups I to IV also produced TCGF activity (data not presented), but bioassays that distinguish bovine IL-2 and IL-4 activities are currently not available. For this reason, analysis of the expression by these cells of mRNA encoding IFN-γ, IL-2, IL-4, TNF-β, and TNF-α is being performed to determine whether these *B. bovis*-specific Th clones can be classified as either Th1 or Th2 cells (30).

As T cell immunogenic antigens of *B. bovis* become identified and available in purified or recombinant form, it will possible to define, at the molecular level, the complex interactions among bovine T cells, macrophages, and parasite antigens important in designing vaccines that will induce protective antiparasite immunity or limit immune-mediated pathology. Our aim is to select, for vaccine trials, antigens that induce an inflammatory or Th1 type of response which in other protozoal infections is associated with protective immunity.

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