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Identification of *Babesia bovis* Merozoite Antigens Separated by Continuous-Flow Electrophoresis That Stimulate Proliferation of Helper T-Cell Clones Derived from *B. bovis*-Immune Cattle

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To characterize *Babesia bovis* merozoite antigens that stimulate anamnestic T helper (Th)-cell responses from *B. bovis*-immune cattle, *B. bovis*-specific Th-cell lines and clones, previously assigned to different antigenic groups (W. C. Brown, S. Zhao, A. C. Rice-Ficht, K. S. Logan, and V. M. Woods, Infect. Immun. 60:4364–4372, 1992), were tested in proliferation assays against fractionated merozoite antigens. The antigenic groups were determined by the patterns of response of Th clones to different parasite isolates and soluble or membrane forms of merozoite antigen. Soluble antigen fractionated by anion-exchange chromatography or gel filtration by using fast-performance liquid chromatography resolved two or three antigenic peaks, respectively. To enable fractionation of membrane-associated proteins and to resolve more precisely the proteins present in homogenized merozoites, a novel technique of continuous-flow electrophoresis was employed. Merozoite membranes or whole merozoites were homogenized and solubilized in sodium dodecyl sulfate-sample buffer, electrophoresed under reducing conditions on 15% or 10% acrylamide gels, eluted, and collected as fractions. Individual fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and tested for the ability to stimulate *Babesia*-specific $CD4^+$ T-cell lines and clones. $CD4^+$ Th-cell lines from two cattle displayed differential patterns of reactivity and detected numerous peaks of antigenic activity, ranging from <14 to 76 kDa. Th-cell clones previously categorized into different antigenic groups detected antigenic peaks unique for clones representative of a given group. Antigens of 29, 51 to 52, and 85 to 95 kDa (group I), 40 kDa (group III), 20 kDa (group IV), 58 to 60 kDa (group VI), and 38, 45, and 83 kDa (group VII) were identified in the stimulatory fractions. Immunization of rabbits with selected fractions produced a panel of antisera that reacted specifically on Western blots (immunoblots) with merozoite antigens of similar sizes, leading to the tentative identification of candidate antigens of *B. bovis* merozoites with molecular masses of 20, 40, 44, 51 to 52 or 95, and 58 to 60 kDa that stimulate proliferation of Th clones representative of five different antigenic groups. These antisera may be useful for isolating recombinant proteins that are immunogenic for Th cells of immune cattle and therefore potentially useful for vaccine development.

Babesia bovis is an economically important hemoprotozoan parasite of cattle that is endemic in many tropical and subtropical regions of the world, including eastern, southern, and central Africa, Australia, and Central and South America. Babesiosis results in high mortality rates among susceptible cattle or the presence of a carrier state in recovered cattle. Control of *B. bovis* has proven difficult since it is widespread, endemic in wild ruminant populations, transmitted by ticks (*Boophilus* spp.), and capable of inducing a chronic carrier state. Despite decades of research, safe and effective vaccines for this and related protozoan parasites, including the human parasites *Babesia microti* (39), a newly recognized pathogenic *Babesia* sp. (44), and *Plasmodium* spp. (28), are still lacking.

Although the nature of acquired resistance to *B. bovis* has not been defined clearly (8), it is well established that $CD4^+$ T cells and activated macrophages are important for protective immunity against related babesial and malaria infections of mice (14, 27, 28, 40, 49). In murine malaria, both subsets of T helper (Th) cells appear to be important for immunity, with Th1 cells (producing gamma interferon [IFN-γ], tumor necrosis factor beta, and interleukin-2 [IL-2]) appearing early and Th2 cells (producing IL-4, IL-5, IL-6, and IL-10) appearing late in infection (27). Cytokines produced by Th2 cells likely function to maintain antiparasitic antibody titers once the initial infection has been controlled by cell-mediated immune mechanisms (27).

The acquired protective immune response to *B. bovis* in cattle depends most likely on both cell-mediated and humoral mechanisms. Cattle that recover from infection with *B. bovis*, either naturally or following chemotherapy with antiparasitic drugs, develop long-lived protective immunity against subsequent exposure to homologous and some heterologous strains (30, 32, 33). Both high antibody titers (50) and cell-mediated immune responses, determined by in vitro proliferation assays of peripheral blood mononuclear cells (PBMC), were observed in cattle immune to challenge (6, 46). Mahoney and coworkers (30) found that passively transferred hyperimmune serum or mixtures of immunoglobulins G1 and G2 effectively lowered parasitemias in splenectomized calves infected 4 days earlier. However, the protective effect was observed only for homologous parasite strains. These results were in contrast to the observation that crude parasite extracts conferred protection against both homologous and heterologous strains (31) and

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suggested that the antigenic determinants that induced cross-protective immunity differed from those that induced antibody-mediated immunity observed in the passive transfer studies. Furthermore, serologically immunodominant antigens present in protective fractions of merozoites were not protective (20, 45, 48, 50, 51). These findings, together with the well-documented role of T cells in other protozoan infections (28, 41), indicated the need to identify protein antigens and their epitopes that induce cell-mediated immune responses against *B. bovis*. Therefore, our laboratory has focused on characterizing T-cell responses and T-cell-dependent antigens of *B. bovis* merozoites as a strategy for developing vaccines that would incorporate T-cell epitopes important in the induction of a protective immune response (12, 13).

B. bovis preferentially induces the proliferation of $CD4^+$ Th cells in short-term cultures of PBMC stimulated with crude membrane (CM) or soluble, cytosolic high-speed supernatant (HSS) antigen prepared from homogenized merozoites (5). Because this parasite does not possess an exoerythrocytic stage that could serve as a target for major histocompatibility complex (MHC)-restricted cytotoxic T cells, we have concentrated our studies on characterizing Th cells. Cytokine analysis of Th-cell clones derived from the $CD4^+$ T-cell lines showed that merozoite antigens preferentially induced Th0- and Th1-like cells that produced the high titers of IFN-γ required for activation of antiparasitic properties of macrophages (11, 12). Proliferation assays with a panel of $CD4^+$ T-cell clones revealed a differential pattern of response to soluble HSS antigen, membrane-enriched CM antigen, detergent extracts of the membrane form of antigen, culture supernatant exoantigen (EXO), and different geographical isolates of *B. bovis* as well as *Babesia bigemina* parasites. The patterns of response by the Th clones indicated the presence of at least five different antigenic epitopes in the merozoite extracts, and the Th clones were assigned correspondingly to one of five antigenic groups (12). Application of gel filtration techniques to separate soluble merozoite antigen revealed the presence of additional antigenic groups, described in the current study, and increased the number of antigenic groups to seven (reviewed in reference 8). Fractionation of the soluble, cytosolic HSS antigen by anion-exchange chromatography or gel filtration revealed only two or three different antigenic peaks and indicated the need for alternative protein separation techniques.

Fractionation of *Plasmodium chabaudi adami* schizont proteins by continuous-flow electrophoresis (CFE) identified protective antigens in the molecular mass range of 25 to 40 kDa (25). Since T cells are required for and have been shown to confer protective immunity in this murine model (1, 21), this finding suggested that protective T-cell antigens could be defined by this technique. Using a similar procedure that is described in the present study, we have identified candidate protein antigens of *B. bovis* merozoites with molecular masses of 20, 40, 44, 51 to 52 or 95, and 58 to 60 kDa that stimulate proliferation of Th-cell clones representative of five of the seven different antigenic groups. Antisera raised against immunostimulatory fractions recognized bands on Western blots (immunoblots) of whole merozoite extracts that corresponded in molecular mass to antigens identified in the fractions. These antisera will be employed to screen a *B. bovis* expression library to isolate recombinant proteins capable of activating potentially protective Th cells.

MATERIALS AND METHODS

Babesial parasite strains and cultivation. The Mexico strain of *B. bovis* was isolated originally in 1978 from cattle infected with natural, tick-derived isolates from a *Babesia*-endemic area of Mexico (6). The Texas strain of *B. bovis* was obtained in 1978 from an infected animal in southern Texas (37) and was provided by Will Goff (USDA Agricultural Research Service, Pullman, Wash.). The Australian L strain of *B. bovis* was isolated in 1965 from an infected animal in New South Wales, Australia (31), and was provided by Stephen Hines (Washington State University, Pullman). The Mexico strain of *B. bigemina* was obtained in 1982 from an infected animal in northeastern Mexico (6). In vitro microaerophilous cultures of all babesial strains were maintained in bovine erythrocytes (6).

Experimental cattle. Animal C15 was inoculated intravenously four times over the course of 6 years with cultured, autologous infected erythrocytes. The final inoculation of 10^9 parasitized erythrocytes did not evoke a fever or a reduction in packed erythrocyte cell volume. Animal C97 was infected by infestation with *Boophilus microplus* tick larvae infected with the same strain of *B. bovis*. The animal recovered from clinical babesiosis following treatment with 3 mg of diaminazine aceturate per kg of body weight and was solidly immune to challenge infection 3 months later with an intramuscular inoculation of a virulent, infected tick stabilate. A *Babesia*-naive control animal used to assess the virulence of the stabilate became clinically ill, experiencing a 57% reduction in packed erythrocyte cell volume on day 14 postchallenge (6).

Parasite antigens. A homogenate (H) was prepared with a French pressure cell (SLM Instruments, Inc., Urbana, Ill.) as described previously (6) from culture-derived *B. bovis* or *B. bigemina* merozoites suspended in phosphate-buffered saline (PBS; pH 7.2) or double-distilled and deionized H_2O containing the protease inhibitors (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) E-64 (25 μg/ml), antipain (25 μg/ml), and phenylmethylsulfonyl fluoride (2 mM). Ultracentrifugation of the homogenized parasites yielded a soluble, cytosolic HSS fraction and a fraction enriched in CM as described previously (6). Uninfected erythrocytes (URBC) were prepared similarly for use as control antigens. Supernatants containing parasite EXO were collected from cultures of *B. bovis* Mexico-infected erythrocytes and assessed for antigenic activity.

Fractionation of HSS antigen by anion-exchange chromatography. Soluble merozoite HSS was fractionated by anion exchange on a Mono Q (HR 5/5) column with a fast-performance liquid chromatography (FPLC) apparatus (Pharmacia LKB Biotechnology, Piscataway, N.J.) as described previously (12). HSS was prepared in H_2O, adjusted to a concentration of 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; starting buffer), and filtered, and 4.5 mg of protein was applied to the Mono Q column. 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, filter sterilized, diluted to isotonicity (150 mM NaCl), and stored at −80°C for T-cell proliferation studies. We determined previously that 25% (vol/vol) of each fraction induced optimal proliferation of T cells (12).

Fractionation of HSS antigen by gel filtration. Soluble merozoite HSS was fractionated by gel filtration on a Superose 12 column with FPLC (Pharmacia). HSS was prepared in PBS and concentrated on a Centricon 10 membrane (Amicon, Danvers, Mass.), and 200 μl of sample containing approximately 1.9 mg of protein was applied to the column. Proteins were eluted with PBS at a flow rate of 0.4 ml/min. Individual 0.4-ml fractions were collected and stored at −80°C for T-cell proliferation assays; they were subsequently used at a final volume of 25% in the assay wells. The column was calibrated with molecular mass standards (Pharmacia) that included aldolase (158 kDa), bovine serum albumin (66 kDa), and chymotrypsin (25 kDa).

Fractionation of CM or merozoite H by CFE. CFE of merozoite antigen was performed with a Prep-Cell Apparatus (Bio-Rad Laboratories, Richmond, Calif.). Approximately 10 to 15 mg of *B. bovis* CM or H was solubilized in sodium dodecyl sulfate (SDS)-sample buffer containing β-2-mercaptoethanol, boiled, electrophoresed under reducing conditions on 28-mm (internal diameter) cylindrical gels consisting of either 15 or 10% acrylamide, eluted, and collected (2.5-ml fractions) over the course of 8.5 h (15% acrylamide gel; 110 fractions) or 24 h (10% acrylamide gels; 220 to 240 fractions). The fractions were stored at −80°C. An aliquot of every 10th fraction was analyzed on silver-stained SDS-polyacrylamide gels. A 1.0- or 1.5-ml aliquot of each fraction was then processed for proliferation assays as described by Kima et al. (25) with the following modifications. For each experiment, a constant volume of fraction was mixed with 8 volumes of ice-cold acetone, placed in a dry ice-ethanol bath for 30 min, and centrifuged at 10,000 × *g* for 30 min. The acetone was aspirated, the precipitates were resuspended in 70% ethanol and centrifuged at 10,000 × *g* for 30 min, and the ethanol was aspirated. The pellets were air dried, suspended in 250 μl of PBS, aliquoted, and stored at −80°C. To examine the potential toxic effects of residual SDS or other gel components on T-cell proliferation, the ability of individual fractions to affect mitogen-induced proliferation of PBMC was determined. PBMC (2×10^5) were cultured in replicate wells with 2.5 μg of concanavalin A (Sigma) per ml and serial dilutions of the reconstituted fractions. It was found that addition of the fractions to a final concentration of 12.5% (vol/vol) had no deleterious effect on the proliferative response to concanavalin A. Subsequent experiments therefore used either 12.5 or 6.2% (vol/vol) of each fraction in the assay wells. A third aliquot of selected fractions was used for the production of rabbit antisera.

TABLE 1. Characterization of *B. bovis*-specific Th-cell clones used in this study

Th-cell clone	Response[a] to the following antigens:								Reference(s)
	Parasite isolates[b]				Unfractionated *B. bovis* antigen[c]				
	B. bovis			*B. bigemina* Mexico	H	CM	EXO	HSS	
	Mexico	Texas	Australia						
Group I									
C97.2D2	+	+	+	−	+	+	±	−	12
C97.2E4	+	+	+	−	+	+	±	−	12
Group III									
C97.3C3	+	+	+	+	+	+	−	+	12
C15.1E3	+	+	+	+	+	+	−	+	11
C15.2G10	+	+	+	+	+	+	−	+	11
Group IV: C15.1H6	+	+	+	−	+	+	−	+	11, 12
Group V: C15.1D10	+	+	+	−	+	+	−	+	11
Group VII: C15.2D7	+	+	−	−	+	+	+	+	11, 12

[a] Proliferative responses of the individual Th clones in each of the antigenic groups listed are indicated as follows: +, positive response; ±, weakly positive response; and −, negative response.
[b] *B. bovis* isolates were obtained from Mexico, Texas, and Australia, and the *B. bigemina* isolate was obtained from Mexico.
[c] *B. bovis* H, CM, EXO, and HSS were prepared from merozoites of the Mexico isolate.

Protein determination. Protein concentrations in subcellular and biochemically separated parasite fractions were determined by the Bradford protein assay (Bio-Rad) with bovine immunoglobulin as a protein standard.

SDS-PAGE analysis of fractionated merozoite antigens. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with a minigel system (Bio-Rad) and 12% acrylamide gels (26). Molecular mass standards and silver stain were obtained from Bio-Rad. Equal volumes (16 μl) of the column flowthrough and selected fractions (1 to 14) of HSS separated by FPLC Mono Q column chromatography and unfractionated HSS (12.5 μg of protein) were diluted with sample buffer and electrophoresed under reducing conditions. Similarly, to analyze CFE fractions eluted from the 15% acrylamide gel, 30 μl of every 10th fraction was analyzed by SDS-PAGE in 12% acrylamide gels. For CFE fractions eluted from 10% acrylamide gels, 37.5 μl of every 10th fraction was analyzed by SDS-PAGE with 4 to 20% acrylamide gradient gels.

***B. bovis*-specific T-cell lines and clones.** *B. bovis*-specific T-cell lines were established approximately 1.5 years (animal C15) and 4 years (animal C97) following the last challenge inoculation with the Mexico isolate of *B. bovis*. To establish cell lines, 4×10^6 PBMC were cultured in 24-well plates (Costar) in a final volume of 1.5 ml of complete RPMI 1640 medium with 25 μg of *B. bovis* Mexico CM antigen per ml (5). At weekly intervals thereafter for 7 weeks, the proliferating T lymphocytes were subcultured to a density of 3.3×10^5 cells per ml with antigen and 2×10^6 irradiated (3,000 rads) autologous PBMC as a source of antigen-presenting cells (APC). When examined after 4 weeks of culture, the C15 T-cell line contained 87% $CD4^+$ T cells, 5% $CD8^+$ T cells, and 7% γ/δ $TcR1^+$ T cells, and the C97 T-cell line contained 98% $CD4^+$ cells.

The derivation of Th-cell clones from *B. bovis*-immune animals C15 and C97 that were used in this study was described in previous publications (11, 12). All of the clones are *B. bovis* specific, $CD4^+$, and MHC class II restricted. A differential pattern of response by the Th clones to different parasite antigens resulted in assigning individual clones to an antigenic group. A total of seven antigenic groups have been defined in this manner (8), and clones representative of five of those groups (I, III, IV, V, and VII) were used in this study (Table 1).

Cell surface phenotypic analysis. *B. bovis*-specific T-cell lines were stained with a panel of monoclonal antibodies by indirect immunofluorescence as described previously (3) and analyzed with a Coulter EPICS 741 flow cytometer. Monoclonal antibodies specific for bovine leukocyte surface markers obtained from the International Laboratory for Research on Animal Diseases (Nairobi, Kenya) included IL-A51, specific for CD8, IL-A12, specific for CD4, and IL-A26, specific for CD2. Monoclonal antibody CACT-61A, specific for the γ/δ TCR1-N12 determinant, was kindly provided by Bill Davis, Washington State University, Pullman.

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 previously (12). Each well (total volume, 100 μl) contained complete RPMI 1640 medium, responder cells added at a final concentration of 3×10^5 cells per ml (obtained 6 or 7 days following the last stimulation of the T-cell lines or clones with antigen), and 2×10^6 autologous or allogeneic, irradiated (3,000 rads) PBMC per ml as a source of APC. *B. bovis* CM and URBC antigens were added to the assay mixes at a final concentration of 25 μg of protein per ml of complete medium. Fractionated antigens were included at a final concentration of 6.25 to 25% (vol/vol). In some experiments, the Th clones were assayed with the addition of either 1 to 5 U of human recombinant IL-2 (Boehringer Mannheim Biochemicals) per ml or 2% (vol/vol) bovine T-cell growth factor (TCGF [4]), in addition to antigen, to all wells of the proliferation assays. Proliferation was determined by measuring the incorporation of 0.25 μCi of [^{125}I]iododeoxyuridine (ICN Radiochemicals, Inc., Costa Mesa, Calif.) added during the final 4 h of the assay. The cells were harvested, and the radioactivity was counted in a gamma counter. Results are presented as the mean counts per minute (cpm) and standard deviation (SD) of duplicate samples.

Production of rabbit antisera against selected CFE fractions and immunoblot analysis. Selected fractions obtained with CFE with 10% acrylamide gels that contained antigenic activity for different Th-cell clones were used to immunize rabbits for the production of antisera. In experiment 1, fractions 1, 11, 30 plus 31, and 60 plus 61 were selected, and in experiment 2, fractions 40, 59, and 100 were selected. Fractions 1, 11, 30 plus 31, and 60 plus 61 were concentrated by Centricon 10 filtration, and a total of 40 to 120 μg of protein was emulsified in RIBI adjuvant (MPL + TDM + CWS Emulsion, R-730; RIBI ImmunoChem Research, Inc., Hamilton, Mont.) and injected three times at 3- to 4-week intervals as suggested by the manufacturer. For fractions 40, 59, and 100, 1.5 ml of each original fraction was emulsified in an equal volume of RIBI adjuvant and divided into three inoculations, 3 to 4 weeks apart. The rabbits were exsanguinated 6 to 7 days following the final immunization, and the sera were absorbed extensively with membranes prepared from URBC. Preimmunization and postimmunization sera were tested for reactivity against homogenized *B. bovis* merozoites or URBC by immunoblotting as described previously (43). Briefly, 15 μg of *B. bovis* or 20 μg of URBC antigen was resolved by SDS-PAGE in 12% acrylamide gels and transferred electrophoretically to nitrocellulose membranes. Pre- or postimmunization rabbit sera were used to develop the immunoblots. Nitrocellulose membranes to which protein was bound were incubated in a PBS blocking solution with 5% nonfat dry milk and 0.02% sodium azide for 1 h at room temperature. The membranes were incubated for 1 to 2 h at 4°C in blocking solution containing rabbit sera diluted 1:400 to 1:1,600. The filters were washed extensively and incubated for 1 h at room temperature with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (heavy plus light chain; Kirkegaard & Perry Laboratories, Gaithersburg, Md.) diluted 1:2,000 in blocking solution. The membranes were again washed, and the chromagenic substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Sigma) were used at a concentration of 50 μg/ml in Tris-buffered saline and added to the membranes to detect bound antibodies.

RESULTS

Characterization of the Th clones used in this study. Previous studies in our laboratory to date have revealed that *B. bovis* merozoites contain at least seven different epitopes immunogenic for T cells (8, 12). These distinctions were based on the differential patterns of proliferative responses of T-cell lines enriched for $CD4^+$ T cells and Th-cell clones to different parasite isolates and to fractions of merozoite antigen (H, CM, HSS, and EXO). The antigen profiles of the clones used in the current study are summarized in Table 1. Th clones in group I reacted with the CM antigen only and recognized all isolates of *B. bovis* tested but not *B. bigemina*. The remaining Th clones

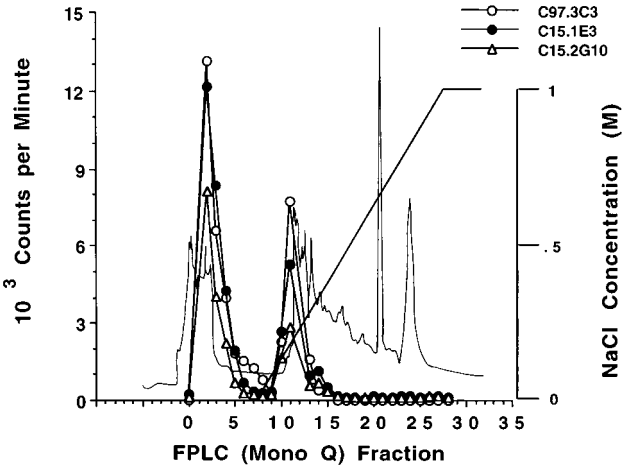

FIG. 1. Proliferative responses of three Th cell clones in antigenic group III to *B. bovis* Mexico HSS antigen fractionated by anion-exchange chromatography with FPLC. HSS was applied to the Mono Q column, the column was washed, and the proteins were eluted with a linear NaCl gradient (diagonal line) and monitored for protein at 280 nm (wavy line) on a scale of 0 (baseline) to 1.0 (maximum peaks) U of absorbance. Fractions were diluted to isotonicity, and 25-μl samples were assayed in duplicate cultures with autologous APC and the following Th-cell clones: C97.3C3 (plus 2 U of human IL-2 per ml), C15.1E3, and C15.2G10. The symbols indicate the proliferative responses to individual fractions. The mean ± SD background proliferative response of clone C97.3C3 cultured with medium plus IL-2 was 2,727 ± 1,593 cpm, which was subtracted from the mean cpm obtained from individual fractions. The mean ± SD background proliferative responses of clones C15.1E3 and C15.2G10 cultured with medium were 201 ± 43 cpm and 136 ± 7 cpm, respectively.

proliferated in response to soluble HSS antigen in addition to CM antigen (groups III to VII). Clones in group VII, unlike the others, responded strongly to the culture supernatant EXO. Other clones were grouped by the soluble antigen fractionation pattern and response to Australia parasites and *B. bigemina*.

Fractionation of soluble HSS antigen by anion-exchange chromatography. Previous fractionation of HSS antigen by anion-exchange chromatography identified two antigenic peaks that induced proliferation of a panel of five clones (12). Clone C97.3C3 (group III) responded to fractions eluted with 0.25 M NaCl, whereas the remaining clones, including clones C15.1H6 (group IV) and C15.2D7 (group VII), responded to fractions eluted with 0.35 to 0.45 M NaCl. In the current study, clone C97.3C3, C15.1E3, and C15.2G10 (group III) recognized antigen in fractions 10 and 11 that eluted with 0.2 to 0.25 M NaCl from the Mono Q column (Fig. 1). Furthermore, cells from all three clones proliferated against HSS antigens present in fractions 2 to 9, which represented material that was not bound by the column during a wash step. Maximum proliferation of all three clones was obtained with fractions 2 and 3. There was no antigenic activity present in the column flowthrough (data not shown). Proteins present in the flowthrough and stimulatory and nonstimulatory fractions (1 to 14) were analyzed by SDS-PAGE to determine if any bands common to the stimulatory fractions could be identified (Fig. 2). A single faint band with an apparent molecular mass of 43 kDa common to fractions 2, 3, 10, and 11 was visualized. These results are consistent with our hypothesis that the three clones previously assigned to antigenic group III recognize the same antigen. The stimulatory protein is poorly reactive with the anion-exchange matrix and appears to have a molecular mass of 43 kDa, although the presence of additional immunostimulatory proteins in these fractions cannot be excluded.

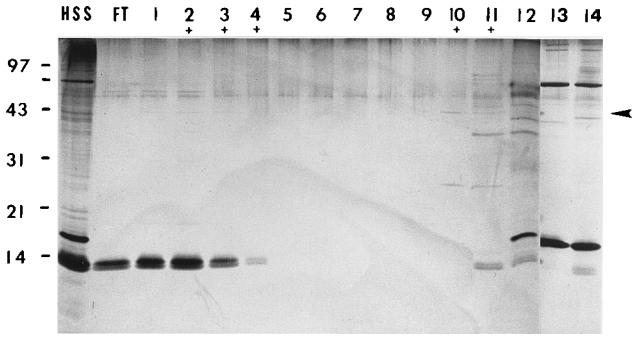

FIG. 2. Selected anion-exchange fractions resolved by SDS-PAGE and silver stained. Unfractionated HSS (12.5 μg of protein) and 16 μl of the column flowthrough (FT) or fractions 1 to 14 were electrophoresed. The relative mobilities of molecular mass standards (in kilodaltons) are indicated on the left. Fractions that contained antigenic activity for clones depicted in Fig. 1 are indicated with a plus at the top of the lane. Fraction 1 was not tested. The arrow on the right indicates a 43-kDa band common to FPLC fractions with stimulatory activity for the Th clones in group III.

Fractionation of soluble HSS antigen by gel filtration. To further characterize the proteins present in soluble HSS, gel filtration was performed by FPLC, and fractions were tested against HSS-reactive clones in antigenic groups III, IV, and V. Three distinct peaks of antigenic activity that stimulated proliferation of clones from the three different antigenic groups were detected (Fig. 3). Activity for clone C15.2G10 (group III) eluted in a broad peak ranging from 30 to 70 kDa as did activity for additional group III clones C97.3C3 and C15.1E3 (data not shown). Clone C15.1H6 (group IV) recognized an antigenic peak that corresponded to the 25-kDa molecular mass marker, and clone C15.1D10 (group V) recognized a major antigenic

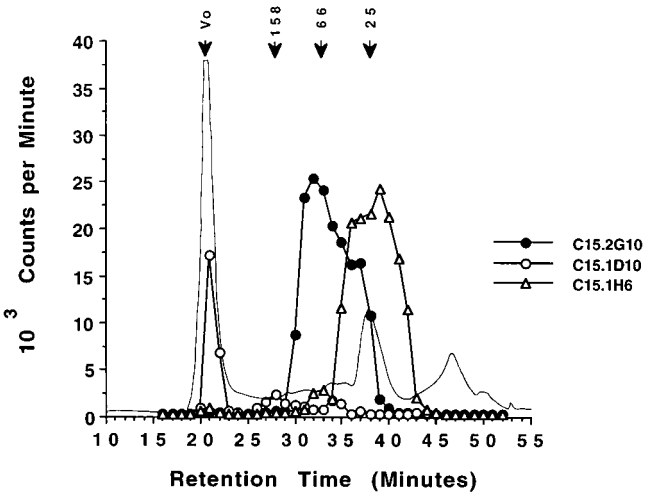

FIG. 3. Proliferative responses of three Th clones representative of three antigenic groups to *B. bovis* Mexico HSS antigen fractionated by gel filtration. HSS was applied to the Superose 12 column, eluted with PBS, and monitored for protein at 280 nm (wavy line) on a scale of 0 (baseline) to 1.0 (maximum peaks) U of absorbance. Fractions (25-μl samples) were assayed in duplicate cultures with the following Th-cell clones and autologous APC: group III clone C15.2G10, group V clone C15.1D10, and group IV clone C15.1H6 (plus 2% [vol/vol] bovine TCGF). The symbols indicate the proliferative responses to individual fractions. The mean ± SD background proliferative responses for clones C15.2G10 and C15.1D10 cultured with medium alone were 350 ± 54 and 367 ± 14 cpm, respectively. The mean ± SD background proliferative response for clone C15.1H6 cultured with medium plus TCGF was 271 ± 23 cpm. The relative retention times of the protein standards (in kilodaltons) and the void volume (Vo) are indicated at the top of the figure.

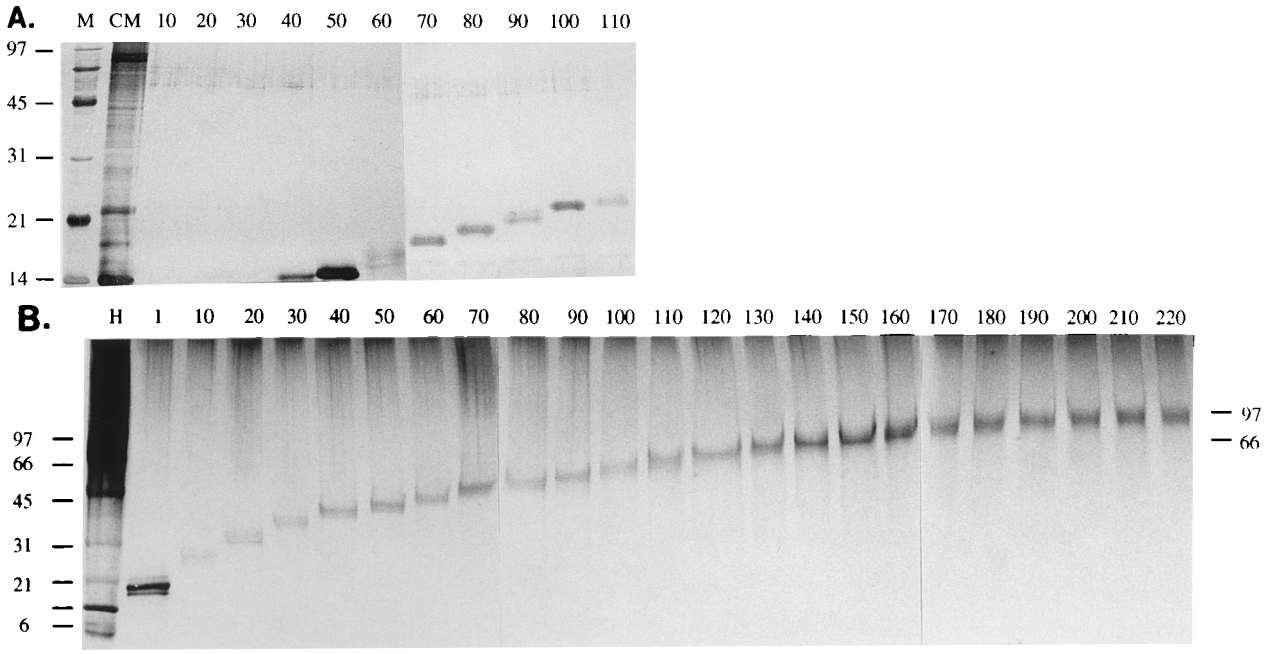

FIG. 4. SDS-PAGE and silver staining analysis of merozoite antigens fractionated by CFE. (A) Merozoite CM antigen was fractionated by CFE with a 15% acrylamide gel, and 30 μl of every 10th fraction or 25 μg of the CM used for fractionation was visualized on the stained analytical gel. (B) Merozoite H was fractionated by CFE with a 10% acrylamide gel, and 37.5 μl of every 10th fraction or 25 μg of the H used for fractionation was visualized on the stained gel. Individual fraction numbers, CM, or H are indicated at the top of each figure, and the relative mobilities of molecular mass standards (lane M) are indicated on the left of each panel (in kilodaltons).

peak that eluted in the column void volume and a minor antigenic peak (2,341 ± 300 cpm as compared with a background proliferation of 367 ± 14 cpm) that eluted with an approximate size of 60 kDa. These data are consistent with the recognition of unique antigens by the Th clones representative of groups III, IV, and V and with the recognition of a common antigen by the three clones in group III.

Fractionation of merozoite antigen by CFE. Both anion-exchange chromatography and gel filtration methods of fractionating parasite antigens are limited to the use of soluble antigen and by the presence of numerous protein bands present in many of the antigenic fractions when analyzed by SDS-PAGE (Fig. 2) (12). These two drawbacks were partially overcome by the use of a preparative CFE apparatus. *B. bovis* merozoite CM antigen was fractionated by electrophoresis under reducing conditions on a 15% acrylamide gel. Analysis of every 10th fraction by SDS-PAGE and silver staining revealed a step-wise progression of bands ranging from 14 to 25 kDa (Fig. 4A). Th-cell lines, which proliferated against babesial antigens but not URBC (data not shown), were tested for proliferation against each fraction. Several peaks of activity were revealed (Fig. 5). The Th-cell line from animal C97 proliferated maximally against fractions containing antigens of <14, 22, and 24 kDa, and the cell line derived from animal C15 proliferated against fractions containing antigens of <14 and 16.5 kDa.

To resolve a broader spectrum of proteins, 10% acrylamide gels were used in two experiments for fractionating merozoite antigens by CFE. Analysis of every 10th fraction by SDS-PAGE again revealed a step-wise progression of bands, ranging from 20 to 95 kDa. The results from experiment 1 are shown in Fig. 4B. When the C97 T-cell line was tested for proliferation against fractions from these latter two experiments, numerous peaks of antigenic activity were detected, and stimulatory fractions contained antigens that ranged in apparent molecular mass from 29 to 70 kDa (experiment 1) and 20 to 76 kDa (experiment 2) (Fig. 6). Many stimulatory fractions that contained antigens of the same apparent sizes were detected in the two CFE experiments.

To verify that the Th cells were stimulated by parasite proteins in an MHC-restricted manner and were not activated to proliferate by potential merozoite superantigens, stimulatory fractions from the 15% acrylamide gel were retested for stimulation of C97 T cells in the presence of either autologous APC or allogeneic APC obtained from animal C15 which do not present antigen to C97 T cells (5). In the presence of allogeneic APC, no proliferation was detected against either CM or CFE fractions, whereas TCGF still induced proliferation. For example, when comparing proliferative responses (mean cpm ± SD) of the T-cell line with autologous versus allogeneic APC, the following results were obtained with the indicated antigens: *B. bovis* CM, 109,000 ± 1,954 versus 28 ± 3; CFE fraction 36, 20,011 ± 608 versus 18 ± 18; CFE fraction 107, 28,610 ± 4,728 versus 17 ± 1 cpm; TCGF, 29,508 ± 1,691 versus 18,827 ± 983. Since the responder C97 T-cell line consisted of 98% $CD4^+$ T cells, the antigen-driven proliferative responses against the CM fraction of *B. bovis* as well as all CFE fractions tested were apparently MHC class II restricted.

We next tested Th clones representative of five different antigenic groups (I, III, IV, V, and VII) for proliferation against homogenized *B. bovis* merozoite antigen fractionated by CFE. Most clones were tested on fractions obtained from two separate CFE experiments. Individual fractions with peak activity were analyzed on silver-stained SDS-polyacrylamide gels to determine the apparent molecular masses of the antigens in the fractions. As shown in Fig. 7, clones representative of the different antigenic groups expressed unique antigenic profiles when tested on the CFE fractions separated by elec-

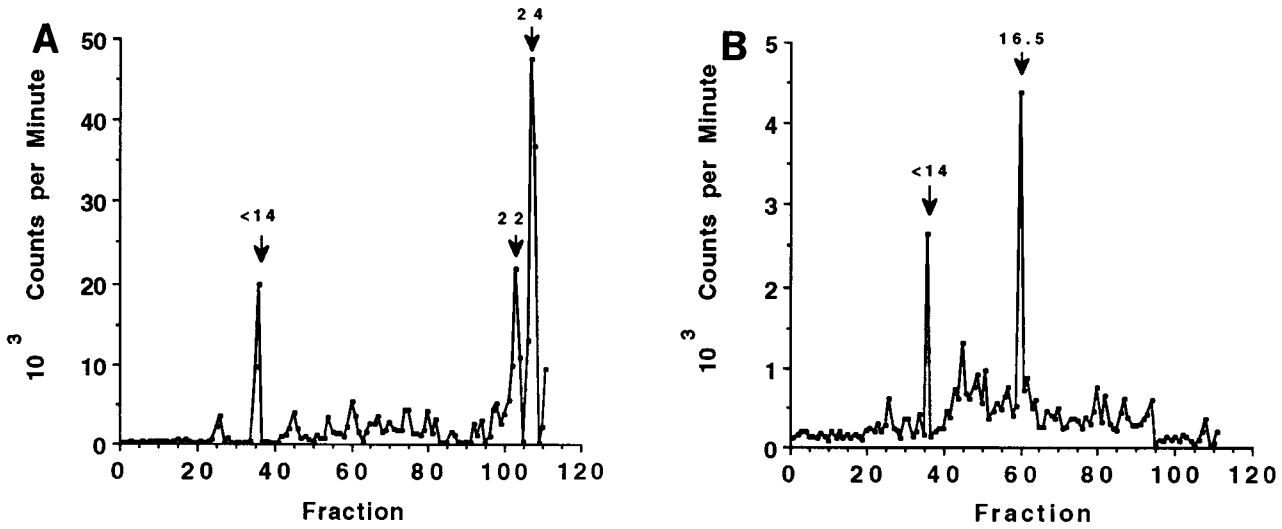

FIG. 5. Proliferative responses of *B. bovis*-specific Th cell lines to *B. bovis* Mexico CM antigen fractionated by CFE. CM was solubilized in SDS-sample buffer and electrophoresed on a 15% acrylamide gel. Proteins were eluted from the gel and collected as 2.5-ml fractions. Proteins in 1.0 ml of each fraction were precipitated and resuspended in PBS, and 12.5-μl samples were assayed in duplicate cultures for stimulation of T-cell lines from animal C97 (A) or C15 (B). Mean ± SD background proliferative responses for C97 and C15 T-cell lines cultured with medium alone were 80 ± 60 and 245 ± 35 cpm, respectively.

trophoresis with 10% acrylamide gels. Interestingly, clones C97.2D2 and C97.2E4 from antigenic group I had superimposable antigenic profiles and recognized two major antigenic activity peaks (Fig. 7A and B) corresponding to fractions that contained antigens of 29 and 51 to 52 kDa. In experiment 2, clone C15.2E4 again responded strongly to fractions containing 51-kDa antigens and to a high-molecular-mass antigen ranging from 85 to 95 kDa, but there was no activity associated with lower-molecular-mass antigens (data not shown). Clones C97.3C3 and C15.2G10, representative of antigenic group III, also displayed nearly identical antigenic profiles in this experiment. Maximum proliferation was obtained with fraction 31 (Fig. 7C and D), which contained an antigen of 40 kDa. In the second CFE experiment, an antigenic peak was observed when clone C15.2G10 was stimulated with fraction 52, which again contained a 40-kDa antigen (data not shown). Clone C15.1H6 (antigenic group IV) recognized antigen with maximal activity in fraction 1 (Fig. 7E), which contained several bands of approximately 20 kDa. To verify the size of the antigen recognized by this clone, C15.1H6 was tested against *B. bovis* CM fractionated with a 15% acrylamide gel, which separated proteins of ≤25 kDa (Fig. 4A). In this experiment, maximum antigenic activity was obtained with fraction 89 (Fig. 7F), which contained antigens of approximately 20 kDa. Clone C15.1D10 recognized a unique antigenic activity peak in fraction 85 that contained a 58-kDa antigen (Fig. 7G). This antigenic profile

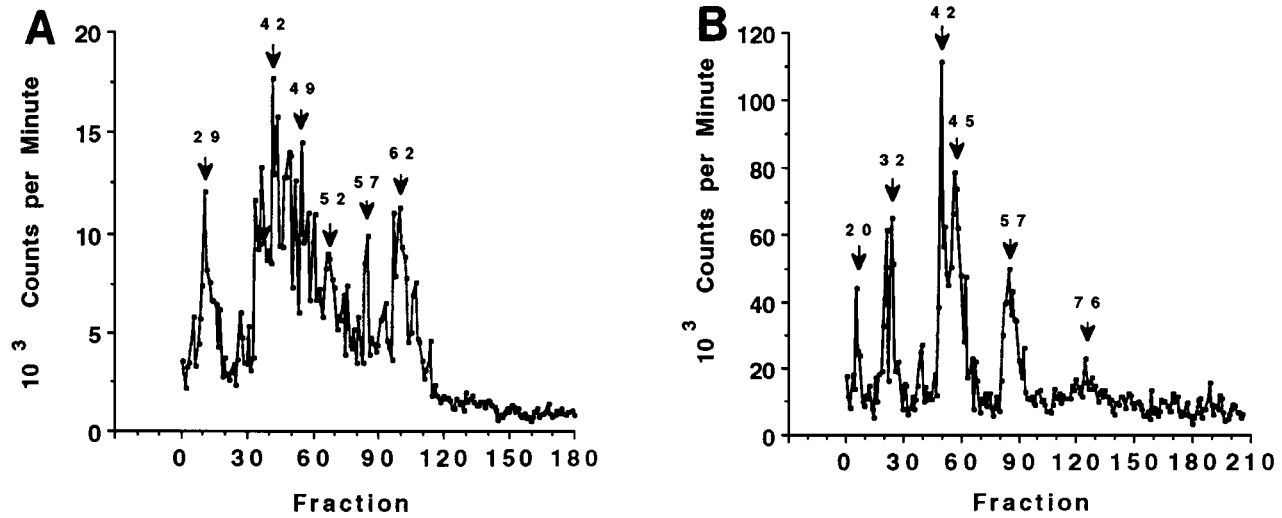

FIG. 6. Proliferative responses of *B. bovis*-specific C97 T-cell line to *B. bovis* Mexico H antigen fractionated by CFE. H was solubilized in SDS-sample buffer and electrophoresed on a 10% acrylamide gel in two experiments. Proteins were eluted from the gel and collected as 2.5-ml fractions. Proteins in 1.0 ml (experiment 1) or 1.5 ml (experiment 2) were precipitated and resuspended in PBS, and 12.5-μl (experiment 1) (A) or 6.2-μl (experiment 2) (B) samples were assayed in duplicate cultures for stimulation of the C97 T-cell line described in the legend to Fig. 5. The apparent molecular masses (in kilodaltons) of molecules present in fractions stimulating peak proliferative responses are indicated by arrows. Mean ± SD background proliferative responses for the T-cell line in experiments 1 and 2 cultured with medium alone were 832 ± 36 and 4,057 ± 923 cpm, respectively.

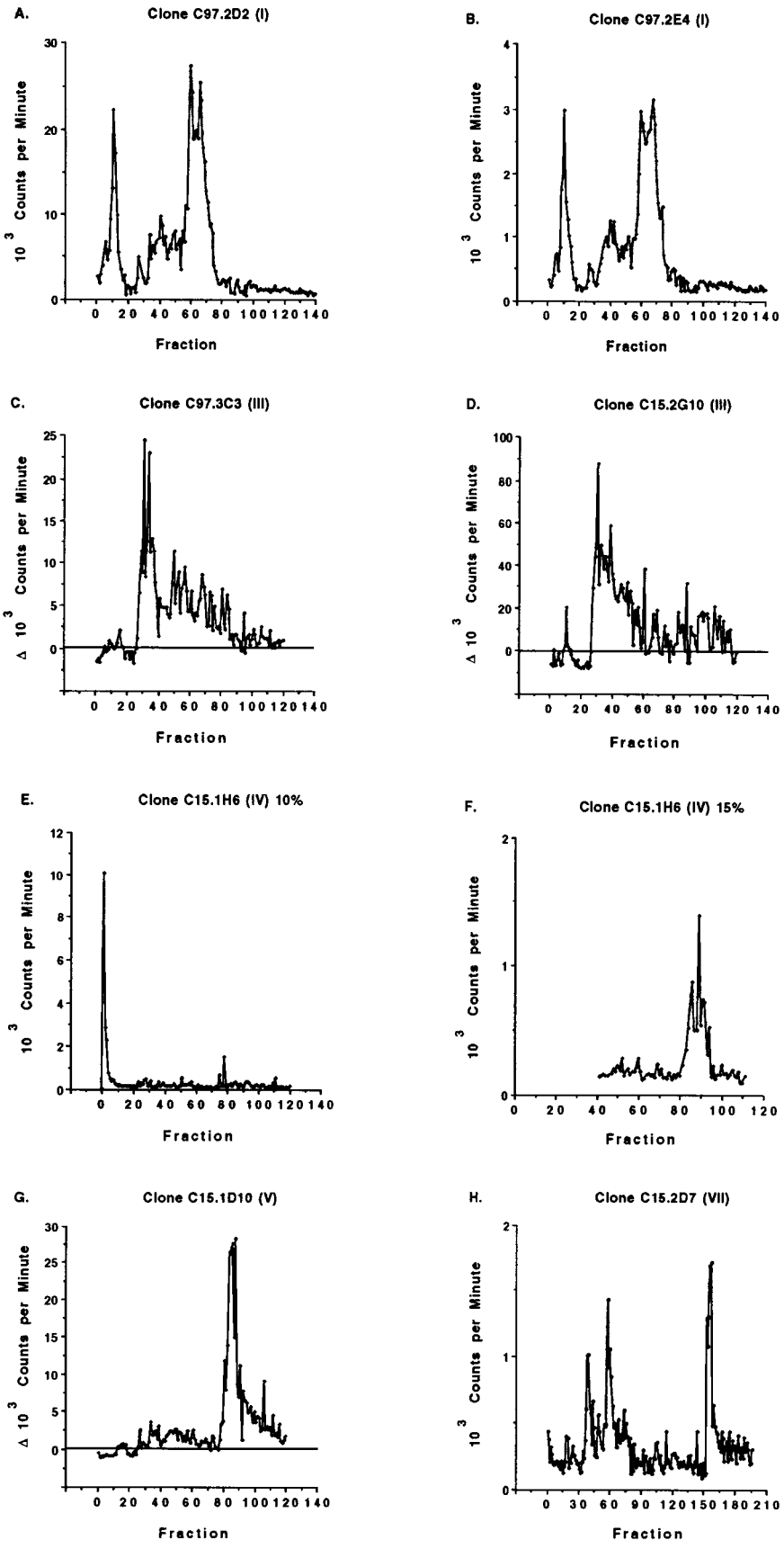

FIG. 7. Proliferative responses of *B. bovis*-specific Th-cell clones to *B. bovis* Mexico H antigen fractionated by CFE. Th-cell clones were assayed in duplicate cultures for proliferation against CFE fractions described in the legends to Fig. 6 and 7. The clones depicted in panels A to E and G were tested with 12.5 μl of CFE fractions from the 10% acrylamide gel in experiment 1. Clone C15.1H6, depicted in panel F, was tested with 12.5 μl of CFE fractions from the 15% acrylamide gel. Clone C15.2D7, depicted in panel H, was tested with 6.2 μl of CFE fractions from the 10% acrylamide gel in experiment 2. Mean ± SD background proliferative responses for the clones were as follows: (A) C97.2D2 with medium, 256 ± 1; (B) C97.2E4 with medium, 164 ± 25; (C) C97.3C3 with 1 U of human IL-2 per ml, 4,732 ± 566; (D) C15.2G10 with 2 U of human IL-2 per ml, 9,247 ± 18; (E) C15.1H6 with 1 U of human IL-2 per ml, 34 ± 14; (F) C15.1H6 with medium, 48 ± 5; (G) C15.1D10 with 1 U of human IL-2 per ml, 2,597 ± 523; (H) C15.2D7 with medium, 185 ± 8. The results are presented as either mean cpm (A, B, E, F, and H) or the difference (Δ) in mean cpm of cultures stimulated with CFE fractions minus the mean cpm of background responses (C, D, and G).

was also verified in the second CFE experiment, wherein fraction 100, which contained antigens of approximately 60 kDa, evoked peak proliferation of this clone (data not shown). Finally, clone C15.2D7, tested against CFE fractions from the second fractionation experiment, detected three activity peaks corresponding to fractions containing antigens of approximately 38, 42, and 83 kDa (Fig. 7H). These results are summarized in Table 2.

Specificity of rabbit antisera produced against CFE fractions with peak activity for Th cells. Rabbits were immunized with fractions containing maximum antigenic activity for Th clones representative of the five different antigenic groups. Antisera were then characterized for reactivity against homogenized *B. bovis* merozoites, and immunoblot analysis was performed with *B. bovis* merozoite antigen obtained from infected erythrocytes or uninfected erythrocytes from the same animal used for merozoite culture (Fig. 8). There was no reactivity of preimmunization sera diluted 1:20 to 1:800 with either antigenic preparation (data not shown). Antisera from immunized rabbits recognized parasite-specific bands, as shown in Fig. 8 and summarized in Table 2. Results with antisera produced against CFE fractions from experiment 1 are shown in Fig. 8A. The antiserum produced against fraction 1, which contained antigens of approximately 20 kDa that stimulated clone 1H6, recognized a unique 20-kDa band. The antiserum raised against fractions 30 plus 31, which contained antigens of approximately 40 kDa, recognized a 40-kDa band, supporting our hypothesis that the antigen recognized by clones representative of group III is approximately 40 kDa. Antiserum produced against the 29-kDa protein(s) present in fraction 11 recognized a 29- to 30-kDa doublet and did not bind to larger molecules on the blots. Immunization with fractions 60 plus 61, which

TABLE 2. Apparent molecular masses of proteins fractionated by CFE that were recognized by *B. bovis*-specific Th-cell clones and by rabbit antisera produced against stimulatory CFE fractions

Th-cell clone	Apparent molecular mass(es) (kDa) of merozoite antigens in CFE fractions recognized by:	
	Th-cell proliferation[a]	Rabbit antisera[b]
Group I		
Expt 1		
C97.2E4	29, 51–52	29, 43, 95
C97.2D2	29, 51–52	
Expt 2: C97.2E4	51, 85–95	
Group III		
C15.1G10	40	40
C97.3C3	40	
Group IV: C15.1H6	20	20
Group V: C15.1D10	58–60	60
Group VII: C15.2D7	38, 45, 83	38, 42–44, 54

[a] Fractions obtained by CFE with 10% acrylamide gels in two experiments were tested for stimulation of the indicated Th-cell clones. The apparent molecular masses (in kilodaltons) of antigens present in fractions with maximal stimulatory activity for a given clone were determined by analysis of silver-stained SDS-polyacrylamide gels and comparison with molecular mass standards.

[b] Antisera were raised in rabbits against individual CFE fractions that induced maximal proliferation of the indicated Th-cell clones. The antisera were tested on immunoblots against a homogenate of *B. bovis* merozoites, and the apparent molecular masses (in kilodaltons) of bands recognized by the antisera were determined by comparison with molecular mass standards.

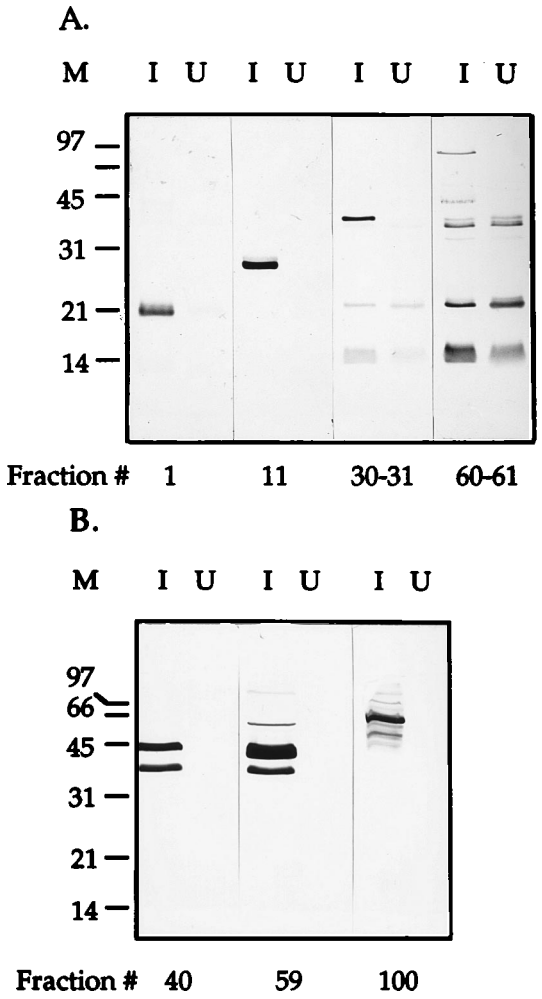

FIG. 8. Immunoblot analysis of rabbit antisera produced against Th-cell-immunostimulatory CFE fractions. Rabbits were immunized with 10% acrylamide CFE fractions from experiment 1 (fractions 1 and 11, a pool of fractions 30 and 31, and a pool of fractions 60 and 61 [A]) or with 10% CFE fractions from experiment 2 (fraction 40, 59, or 100 [B]). The fraction(s) used for immunization is indicated at the bottom of each panel. Following adsorption of the antisera with URBC membranes, sera were diluted 1:800 or 1:1,600 (fraction 100) and used to probe either *B. bovis* Mexico merozoites prepared from infected erythrocyte cultures (I) or control, uninfected erythrocytes used to cultivate the merozoites (U), after electrophoresis of 20 and 25 μg of protein per lane, respectively, on 12% acrylamide gels and transfer to nitrocellulose. The relative mobilities (M) of the molecular mass standards (in kilodaltons) are indicated on the left of each panel.

contained 51- to 52-kDa proteins, produced an antiserum that recognized 43- and 95-kDa antigens not present in URBC. These data suggest that the 29- and 51- to 52-kDa antigens present as activity peaks for clones of group I may be derived from a larger molecule.

Results with antisera produced against CFE fractions from experiment 2 are shown in Fig. 8B. Fractions 40, 59, and 156 stimulated clone C15.2D7. Immunization with fraction 40, which contained a 38-kDa antigen, recognized two bands of 38 and 44 kDa. Immunization with fraction 59, which contained a 45-kDa antigen, reacted intensely with these two bands and a 42-kDa band and less intensely with additional larger bands. Immunization with fraction 156, which contained antigen of approximately 83 kDa, produced an antiserum that recognized numerous high-molecular-mass bands but also recognized an intense band of approximately 44 kDa (data not shown). These results are consistent with the proliferation profile of this clone, which responded to three activity peaks (38, 45, and 83 kDa). Immunization with fraction 100, which stimulated clone C15.1D10 and contained a 58- to 60-kDa antigen, produced an antiserum that was directed predominantly against a 60-kDa band, although additional bands were detected.

DISCUSSION

Immunization with killed *B. bovis* merozoites or biochemically fractionated merozoite antigen has resulted in various degrees of protective immunity against homologous and heterologous *B. bovis* challenge (19, 45, 50). One approach taken to identify vaccine antigens of babesial parasites was to systematically fractionate parasite antigens by biochemical purification strategies, perform immunization trials with parasite fractions, and raise monoclonal antibodies against fractions with protective activity for the production of recombinant proteins (19, 50, 51). It was found that serologically immunodominant antigens isolated from protective parasite fractions were not themselves protective (20, 45, 50, 51), whereas antigens present in small quantities in the parasite that did not induce strong antibody responses did confer some protection (15, 16, 18, 19, 50, 51). These partially protective antigens included a 38-kDa protein (designated 15B1 and subsequently 12D3), a 60- to 70-kDa protein with protease activity (designated T21B4) and shown recently to be identical to rhoptry associated protein-1 (RAP-1) (15, 42), a high-molecular-weight protein designated 11C5 (19), and a 77- to 80-kDa dextran-sulfate-binding protein designated Bv80 (16) and previously designated Bb-1 by our group (13, 22, 43, 47). Secreted parasite EXOs, recovered from either plasma of infected cattle or culture supernatants, have also been shown to protect cattle against homologous and heterologous parasite challenge (36). However, none of these antigens were characterized for the ability to induce Th-cell responses in these studies.

A different approach that we have taken for the selection of potential vaccine antigens of *B. bovis* was to use Th cells as probes to identify antigens that elicit strong anamnestic responses, since Th cells are critical for the induction and maintenance of both humoral and cell-mediated immunity against related protozoan parasites (8, 28, 41). Because the Th-cell clones were derived from T-cell lines cultured for 3 to 6 weeks with CM or soluble merozoite antigens, they are likely to detect antigens that are immunodominant for T cells. The Th clones used in this study were selected on the basis of strong proliferative responses to antigen in the absence of exogenous growth factor, and the majority were characterized previously as being Th0 clones that expressed relatively high levels of IFN-γ (11, 12).

Previous attempts to identify T-cell-dependent *B. bovis* proteins by biochemical fractionation methods revealed distinct patterns of reactivity by Th-cell clones for membrane and soluble forms of merozoite antigen as well as soluble antigen fractionated by anion-exchange chromatography (12). The present study was undertaken to define more precisely the antigens that activated Th-cell clones that recognized distinct epitopes. Gel filtration columns were able to resolve three separate peaks of antigenic activity. However, as with anion-exchange chromatography, this method can be used only with soluble parasite antigens and was generally incapable of purifying a single antigen. Furthermore, we were unable to detect antigenic activity in Th-cell proliferation assays when HSS was subjected to gel filtration and then to anion-exchange chromatography. For these reasons, we applied the technique of CFE to separate homogenized merozoite proteins. Although this single-step procedure has not resulted in complete purification of babesial merozoite antigens, it has enabled the tentative identification of antigens with molecular masses ranging from 20 to 95 kDa that stimulate proliferation of Th-cell lines and clones derived from cattle immune to challenge infection with *B. bovis.*

Many of the same peaks of antigenic activity in merozoite antigen fractionated by CFE and identified by a *B. bovis*-specific T-cell line were identified subsequently by our panel of Th-cell clones. This finding suggests that the clones, selected by in vitro cultivation with unfractionated antigen, are representative of Th cells that proliferate against immunodominant merozoite antigens in the bulk cultures. Clones assigned to antigenic group I responded preferentially to parasite membrane antigens, and CFE enabled the identification of several peaks of antigenic activity which were identical for two clones, C97.2D2 and C97.2E4. Since these clones were derived from the same cell line, it is likely that they recognize the same epitope, which appears to be derived from a high-molecular-weight protein or may be shared by proteins of 51 to 52 and 95 kDa. These clones did not respond to either recombinant 42-kDa merozoite surface antigen-1 (MSA-1) (7) or recombinant 77-kDa spherical body protein Bb-1 of *B. bovis* (9). Clones assigned to antigenic group III appear to recognize an antigen with an apparent molecular mass of 40 kDa, although analysis of antigen fractionated by CFE revealed additional peaks of activity in fractions with larger antigens. The lack of reactivity of antiserum produced against the peak fraction with larger molecules suggests that the promiscuous T-cell response is not due to degradation of a larger molecule. However, the possibility that a 40-kDa fragment derived from a larger protein contains Th-cell epitopes but not B-cell epitopes antigenic for rabbits cannot be ruled out at this time. An alternative explanation for the response of group III clones to more than one antigenic peak is that the T-cell epitope is shared by more than one polypeptide. This possibility is consistent with the broad peak of activity that eluted from the gel filtration column and the broad specificity of these clones when tested against different babesial isolates and species. Clones in group III did not respond to 42-kDa MSA-1, 60-kDa RAP-1, or 77-kDa Bb-1 recombinant proteins of *B. bovis* (9). Clone C15.1H6, which is the only Th-cell clone assigned to group IV, appears to recognize a 20-kDa antigen. Clone C15.1D10, which is the only Th-cell clone assigned to group V, appears to recognize a 58- to 60-kDa protein. Although the majority of activity eluted from the FPLC gel filtration column in the void volume, a small peak of activity which eluted just after the 66-kDa standard was detected. In two experiments using CFE and 10% acrylamide gels, peak immunostimulatory activity for this clone was obtained with fractions containing 58- to 60-kDa proteins.

Antiserum raised against one of these fractions recognized several bands, with a predominant band of 60 kDa. This clone does not respond to the 60-kDa RAP-1 protein of *B. bovis* (9). Finally, clone C15.2D7, representative of group VII, appears to recognize a 44-kDa antigen. This clone responded to three major antigenic activity peaks when *B. bovis* was fractionated by CFE. Rabbit antisera raised against fractions representative of the three peaks all recognized a 44-kDa band, whereas sera produced against the smaller 38- and 45-kDa antigens did not recognize higher-molecular-mass antigens on immunoblots. These data suggest, but do not prove, that the immunogenic T-cell activity detected as three peaks is not due to degraded forms of a larger molecule. Clone C15.2D7 did not proliferate against the 77-kDa Bb-1 protein or the 42-kDa MSA-1 protein (9), ruling out this major antigenic component as the target of recognition by this Th-cell clone. However, a related 44-kDa merozoite surface antigen (MSA-2), which is coexpressed with the 42-kDa MSA-1 antigen on the Mexico and Texas isolates of *B. bovis*, has also been described. The genes encoding MSA-1 and MSA-2 and the Australia BabR genes appear to be members of a multigene family (23, 24). The reactivity of clone C15.2D7 with both the Mexico and Texas but not the Australia isolates of *B. bovis* is consistent with potential recognition of the MSA-2 protein which expresses antibody epitopes and gene sequences that are highly conserved between the Mexico and Texas isolates but either less conserved or absent from the Australia isolates (24, 37).

The identification of antigens immunostimulatory for the Th-cell clones described in this study will be verified by use of the rabbit antisera to isolate DNA clones encoding these proteins from a *B. bovis* expression library (47). Recombinant antigens will be assessed for immunogenicity for the Th-cell clones and T-cell lines from the animals used in this study as well as for Th-cell lines derived from additional *B. bovis*-immune cattle. Furthermore, it will be important to define the cytokines expressed by both Th cells and APC in response to recombinant antigens, both in vitro and in vivo. Studies with *B. bovis*-specific Th-cell clones derived by stimulation with unfractionated merozoite antigens revealed a predominant Th0 cytokine profile among a panel of 20 clones, with coexpression of IL-4 and IFN-γ mRNA and expression of high titers of IFN-γ by the majority of clones. However, the use of crude merozoite antigens to select memory cells in vitro and the use of TCGF, which likely contains IL-4 in addition to IL-2, to maintain the Th clones may have influenced the profile of cytokines expressed in favor of an unrestricted profile (29, 38). In limited studies where the cytokine profiles of Th clones selected with recombinant antigens have been examined, we did obtain evidence for a more restricted profile. For example, clones selected by and reactive with recombinant 77-kDa antigen Bb-1 expressed Th1 cytokines IL-2 and IFN-γ but not IL-4 (13). Recent studies also demonstrated expression of Th1 cytokines by 9 of 12 *Mycobacterium tuberculosis* purified protein derivative-specific bovine Th clones (2). Since cytokines such as IL-10 and IL-12 expressed by APC also influence Th-cell subset differentiation and cytokine expression by differentiated human (17, 34, 35) and bovine (2, 10) T cells, it will be important to examine the immunomodulatory effects of recombinant antigens, selected by their immunostimulatory properties for Th cells, on APC. A comprehensive study of the nature of the T-cell and macrophage responses to defined antigens selected by T-cell immunogenicity both in vitro and in vivo combined with eventual testing of recombinant antigens in vaccine trials will help elucidate the mechanisms of acquired resistance to *B. bovis* and other hemoparasites.

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