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RECOGNITION OF SOLUBLE *Theileria parva* ANTIGEN BY BOVINE HELPER T CELL CLONES: CHARACTERIZATION AND PARTIAL PURIFICATION OF THE ANTIGEN[1]

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Theileria parva-specific bovine BoT4$^+$ Th cell clones were used to characterize Ag associated with _T. parva_ schizont-infected lymphoblastoid cells. All of the clones tested responded to cells infected with the immunizing (Muguga) as well as heterologous stocks of _T. parva_, indicating that the T cells are specific for an Ag shared by several geographically diverse parasites. The response was apparently MHC-restricted, and induced by Ag expressed on the infected cell surface. In the presence of autologous APC, the clones were also stimulated by a soluble high speed supernatant (HSS), but not by a schizont membrane-enriched, subcellular fraction prepared from homogenates of infected cells. The clones produced IFN-γ and T cell growth factor in response to HSS. The soluble Ag was absent in cells from which schizonts had been eliminated by treatment with the anti-theilerial drug, parvaquone. Fractionation of HSS by hydroxylapatite chromatography revealed two antigenic peaks that separated from the majority of the protein. Fractionation of HSS by gel filtration with the use of HPLC revealed several peaks of activity ranging in M_r from 270 kDa to <5 kDa. Further fractionation of HSS by both hydroxylapatite chromatography and gel filtration yielded three major peaks of activity (M_r 43, 12, 4.2 kDa). We conclude that a T cell-dependent schizont-associated soluble Ag is also expressed on the surface of _T. parva_-infected cells.

Bovine theileriosis, or East Coast fever, is a disease of significant economic importance in East Africa, caused by the tick-transmitted protozoan parasite, *Theileria parva*. This intracellular parasite has the exceptional ability to induce unregulated proliferation of host lymphocytes. In susceptible cattle infected with *T. parva*, numerous schizont-infected lymphoblastoid cells invade both lymphoid and nonlymphoid tissues. Widespread lymphocytolysis follows, and most animals die within 4 wk of infection (1).

Cattle can be successfully immunized by the infection and treatment method (2, 3) in areas where the number of field isolates or stocks of *T. parva* is restricted. However, the presence of non-cross-protective stocks of *T. parva* limits the use of this method, and immunization by using viable parasites risks the induction of a carrier state (4).

As with many intracellular organisms, cellular mechanisms of immunity play a key role in aquired immunity to *T. parva*. MHC class I-restricted CTL specific for *T. parva*-infected lymphocytes are involved in both primary and secondary immune responses in cattle undergoing infection or challenge (5, 6). However, the Ag recognized by these effector cells have not yet been identified; in fact, it is not known whether they are schizont-encoded or modified host molecules. An immunodominant schizont surface Ag has been identified with murine mAb (7, 8), but serologically defined Ag on the surface of *T. parva* schizont-infected cells have not yet been described (9). The research in our laboratory has focused on using T cells as reagents to identify *T. parva*-associated Ag that are expressed on the infected cell surface.

We have recently described distinct soluble and schizont membrane-associated Ag, prepared from homogenates of *T. parva*-infected cells, which stimulate Th cells from *T. parva*-immune cattle (10). The soluble Ag contained neither mitogenic nor IL-2 activity. We further showed that Th clones specific for the schizont membrane Ag also recognized Ag associated with the infected lymphocyte cell surface, suggesting a schizont membrane origin of the cell-surface Ag (11). In the present study, we have continued to characterize the soluble Ag by using different Th clones. We have found that, like the schizont membrane-derived Ag, the soluble Ag is expressed on the infected cell surface. Because these Ag stimulate T cells and would be encountered during natural challenge, they may be useful for the development of a recombinant vaccine for theileriosis.

MATERIALS AND METHODS

Animals. The cattle used in this study were castrated Boran (*Bos indicus*) adult males. Animal D768 was immunized against TPM[4] with International Laboratory for Research on Animal Diseases bulk ST 836 by simultaneous inoculation with sporozoites and long acting oxytetracycline (2), and was used for experimental studies 16 mo

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[4] Abbreviations used in this paper: ST, stabilate; BoLA, bovine lymphocyte Ag, TPM, *Theileria parva* (Muguga); H, homogenate; HSS, high speed supernatant; CM-1, crude membrane fraction; TMLC, *Theileria* mixed lymphocyte culture; SI, stimulation index; TCGF, T cell growth factor.

later. Animal B657 was the donor of an IL-2-dependent cell line, designated B657.G6, as described (12). The TPM-infected cell line, C447-TPM, was established from animal C447 (13). The BoLA phenotypes, determined as described previously by using reagents that define class I Ag (14), for animals D768, B657, and C447 were w10/w8, KM10/−, and w10/w7, respectively.

Cell preparation. PBMC and uninfected lymphoblastoid cell lines stimulated with Con A (Sigma, St. Louis, MO) and maintained for several months in medium containing bovine TCGF were prepared as described (12). Uninfected lymphoblastoid cell lines were used as negative controls in experiments utilizing *T. parva*-infected cells and parvaquone-treated infected cells, and in the preparation of subcellular fractions. Cell cultures were carried out at 39°C in 5% CO_2 in air in RPMI 1640 medium (GIBCO, Paisley, Scotland), supplemented as described (10).

Generation of T. parva-infected cell lines. Infected lymphoblastoid cell lines were established as described (15) by in vitro infection of D768 PBM, C447 PBM, and the BoT8$^+$ T cell clone B657.G6 (16) with sporozoites derived from ticks in the same batches as were used to prepare the International Laboratory for Research on Animal Diseases bulk ST of the Muguga (ST 836), Marikebuni (ST 3014), Mariakani (ST 3029), Boleni (ST 3039), and Uganda (ST 3066) stocks of *T. parva*. Cell lines are identified by the animal number, followed by the designation of the cloned T cell line, if used, followed by the parasite stock (TPM).

Parvaquone treatment of infected cells. The anti-theilerial hydroxynapthoquinone derivative, parvaquone (17), was used to eliminate schizonts from C447-TPM cells as described (11, 18).

Subcellular fractionation. Cultures of 1.0 to 1.7 × 10^9 uninfected D768 or C447 lymphoblastoid cells, 1.1 to 3.8 × 10^9 TPM-infected D768-TPM or C447-TPM cells, and 1.7 × 10^8 parvaquone-treated infected C447-TPM cells were used to prepare a cellular H and subcellular fractions as described (10, 11). Briefly, cells were washed and resuspended in 10 to 15 ml PBS, pH 7.4, containing 25 μg/ml of the protease inhibitors leupeptin and E-64 (Cambridge Research Biochemicals, Cambridge, Cambridgeshire, England) and were disrupted by passage through a French® Pressure Cell (SLM Instruments, Urbana, IL) to obtain the H. The soluble HSS and membrane-containing CM-1 fractions were prepared by differential centrifugation of the H. The HSS was collected after centrifugation at 15,000 × g_{av} for 10 min and 125,000 × g_{av} for 60 min. The pellet obtained after the second centrifugation was resuspended in 37% (w/w) SHKE buffer (37% sucrose, 20 mM HEPES, 25 mM KCl, and 1 mM EDTA, pH 7.4), overlayered with 5 ml PBS, and centrifuged at 125,000 × g_{av} for 90 min. The CM-1 fraction was collected at the PBS/37% SHKE interface, washed, and resuspended in PBS. Fractions were stored at −80°C.

Protein determination. The protein concentrations in the subcellular fractions and fractions obtained during the purification of HSS were determined by using the Bradford protein assay (BioRad Laboratories, Richmond, CA) as described (19).

Generation of T. parva-specific cloned T cell lines. T cell lines specific for *T. parva*-infected cells were established from PBMC of animal D768 as described (5, 10). Briefly, 2 × 10^6 PBMC were cultured with 2 × 10^5 γ-irradiated (10,000 rad from a ^{137}Cs source) D768-TPM cells in 2-ml cultures. From 6 to 8 days later, viable cells were subcultured to a density of 1 × 10^6 cells/ml and were restimulated with 2 × 10^5 irradiated (10,000 rad), infected cells and irradiated (5000 rad), autologous PBMC. The *Theileria* MLC were thus restimulated weekly, and were used as a source of responder cells in proliferation assays 6 to 8 days after the last stimulation. One culture, designated D768 TMLC-4, proliferated vigorously in response to HSS prepared from autologous infected, but not uninfected, lymphoblastoid cells (data not shown). This TMLC was stimulated 5 times with irradiated D768-TPM cells, and then once with 100 μg/ml D768-TPM HSS to select HSS-specific T cells for limiting dilution cloning. One week after the last stimulation, clones were established from cultures seeded at 0.5 or 1 cell/well in 96-well round bottomed plates (Costar, Cambridge, MA) with 2 × 10^4 irradiated autologous PBMC, 10% bovine TCGF, and either 1 × 10^3 irradiated D768-TPM cells or 50 μg/ml HSS prepared from D768-TPM cells. Proliferating cells were transferred to 48-well Costar plates and were screened for the capacity to proliferate in response to HSS in the absence of exogenous TCGF. Selected clones were maintained in 24-well Costar plates with irradiated autologous PBMC as a source of APC, 10% bovine TCGF, and either irradiated D768-TPM cells or HSS as Ag.

Staining with mAb. Selected T cell clones were stained by indirect immunofluorescence and analyzed by flow cytometry by using a FACS II (Becton Dickinson, Sunnyvale, CA) (20). The following mAb were used: IL-A17, which recognizes BoT8, the bovine analogue of CD8 (21); IL-A12, which recognizes BoT4, the bovine analogue of CD4 (22); and IL-A26, which recognizes BoT2, the bovine analogue of CD2 (23).

Proliferation assays. Proliferation assays were carried out as described (11) by using 3 × 10^4 cloned T cells, the adherent fraction of 1 × 10^5 irradiated autologous or MHC-mismatched PBMC as a source of APC, and Ag in a volume of 200 μl in 96-well flat-bottomed Costar microtiter plates. Ag, tested in doubling dilutions, included irradiated (10,000 rad) uninfected or *T. parva*-infected stimulator cells, glutaraldehyde-fixed (24) stimulator cells, H, HSS, and CM-1 fractions prepared from uninfected or *T. parva*-infected cells, and HSS fractionated by hydroxylapatite chromatography or gel filtration. In the latter experiments, 0.5% Mycostatin (GIBCO, Grand Island, NY) was added to the assay wells. Unless indicated otherwise, TCGF was not added to the assay wells. Proliferation was determined by adding 0.5 μCi [^{125}I]UdR (Amersham International, Aylesbury, Buckinghamshire, England) to each well and measuring the incorporation of radioactivity added during the last 4 h of the assay. Results are presented as the mean radioactivity in cpm of duplicate samples ± 1 SD, or as the SI, which was derived by dividing the mean cpm of cultures with Ag by the mean cpm of cultures without Ag.

Production of TCGF and IFN-γ by the T cell clones. Supernatants were collected from T cell clones cultured for 18 h at a density of 1 × 10^6 cells/ml with 50 μg/ml HSS prepared from D768-TPM cells and 2.5 × 10^5 cells/ml irradiated autologous PBMC as a source of APC. Control supernatants were obtained from cultures of HSS and HSS plus APC. TCGF activity was measured in supernatants at a concentration of 50% v/v in a proliferation assay with IL-2-dependent B657.G6 cells (12). This cell line responds to human rIL-2 (W. C. Brown and K. S. Logan, unpublished observations), but its sensitivity to IL-4 is not known. To measure IFN-γ activity, fourfold dilutions of each supernatant were tested for the ability to protect a primary bovine fibroblast line (BT6) from the cytopathic effect of Semliki Forest virus, essentially as described (25). Sensitivity to treatment, pH 2, was also determined. Titers are expressed as the inverse of the dilution giving a 50% reduction in viral cytopathic effect and were determined by comparison with a National Institutes of Health reference standard human IFN-α Ga23-902-530, Research Resources, National Institute of Allergy and Infectious Diseases, National Institute of Health, Bethesda, MD.

Hydroxylapatite chromatography of HSS and protease sensitivity. The HSS fraction (106.4 to 136.5 mg protein) prepared from D768-TPM cells was diluted 1/10 in H_2O and immediately applied onto a 16 × 2.7-cm hydroxylapatite (BioGel HPT, BioRad) column equilibrated in 10% v/v PBS in H_2O. Elution was performed with a 0 to 400 mM $NaPO_4$ (pH 7.4) gradient at room temperature, and protein was monitored at 280 nm in an LKB 8300 Uvicord II UV monitor. From 1- to 2.3-ml fractions were collected, sterilized by filtration (0.45-μm Nalgene filter), and diluted to isotonicity (118 mM $NaPO_4$). The samples were tested for antigenic activity in a proliferation assay with HSS-specific T cell clones or uncloned TMLC. Fractions were stored at 4°C or at −80°C.

Active fractions recovered from hydroxylapatite column chromatography were tested for susceptibility to proteolysis with papain (mercuripapain, United States Biochemical Corp., Cleveland, OH) at a 5% w/w papain/substrate ratio. After 2-h incubation at 22°C the reactions were terminated by the addition of a fivefold excess of the protease inhibitors leupeptin and E-64 (0.1 mg/ml in H_2O) over the equivalent amount needed to effect the complete inhibition of the activity of papain against Z-Phe-Arg-NH-Mec as described (26).The treated samples were stored at −80°C until use in a proliferation assay with HSS-specific clone 2C3.

Gel filtration of HSS with the use of HPLC. HSS prepared from D768-TPM cells was fractionated the same day by gel filtration by using HPLC. A 200-μl aliquot of HSS containing 16.5 to 19.9 mg protein/ml was applied directly onto two 200- × 7.5-mm Bio-Rad BIO-SIL TSK-250 and TSK-125 columns that were coupled in tandem. The columns had been previously equilibrated in the elution buffer (PBS, pH 7.2) by using the following protein standards (Bio-Rad): thyroglobulin (M_r 670,000), IgM (M_r 158,000), egg albumin (M_r 44,000), myoglobin (M_r 17,000), and vitamin B_{12} (M_r 1,350). Elution was performed at room temperature with a flow rate of 0.75 ml/min, and protein was monitored at 235 nm. Fractions (400 μl) were collected every 0.53 min and were tested the same day for antigenic activity.

RESULTS

Selection of HSS-specific T cell clones. In an earlier study (10), we determined that *T. parva*-specific T lymphocytes from animal D768 were preferentially stimulated by a soluble, rather than a membrane-containing

fraction prepared by differential centrifugation of a homogenate of autologous schizont-infected cells. To further characterize the Ag present in the soluble HSS fraction, cloned T cell lines specific for HSS were established from a *Theileria*-specific TMLC. The six clones described here that were stimulated either with irradiated infected cells (2B2, 2C3, and 2G12) or with HSS (3E2, 3F10, and 3H12) were selected on the basis of proliferation in response to HSS without exogenous IL-2, of noncytolytic activity, and of expression of the phenotype $BoT2^+$, $BoT4^+$, $BoT8^-$. In some experiments, only several of the clones were assayed, depending on the availability of cells.

Proliferative responses to membrane-associated and soluble fractions of T. parva-infected cells. The subcellular fractionation procedure yielded a soluble (HSS) and a membrane (CM-1) fraction that could be distinguished antigenically by immunoblot analysis with the use of a schizont surface Ag-specific mAb (8). This antibody (mAb 4) detected an 89- to 92-kDa band in the homogenate and CM-1 fraction that was absent or present occasionally as a weak band in the HSS fraction prepared from TPM-infected cells (10, 11). An additional 170-kDa band was also detected in the CM-1 fraction. We compared the proliferative responses of the T cell clones to crude H, HSS, and CM-1 fractions prepared from autologous D768-TPM cells and from uninfected lymphoblastoid cells. None of the clones responded to the H, HSS, or CM-1 fractions prepared from uninfected cells (data not presented). All six clones proliferated in a dose-dependent manner to the crude H and HSS fraction, but not to the CM-1 fraction, prepared from infected cells (Fig. 1). The responses to the HSS fraction were generally higher than to the H, which is expected because this fraction was derived from the H.

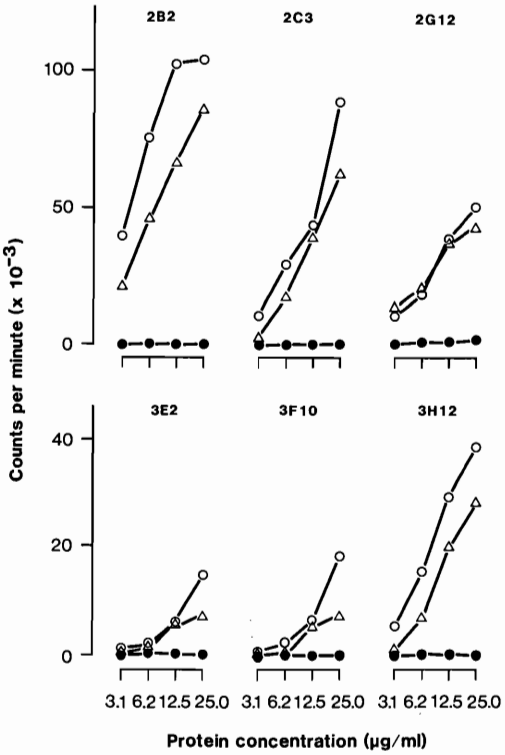

Figure 1. Proliferative responses of the T cell clones to the crude H (△), soluble HSS (○) and CM-1 (●) fractions obtained from autologous D768-TPM cells. Results are expressed as the mean cpm of duplicate cultures of cells stimulated with four concentrations of Ag. Background responses were: 2B2, 1628 ± 213; 2C3, 1023 ± 174; 2G12, 1370 ± 102; 3E2, 458 ± 297; 3F10, 119 ± 34; and 3H12, 249 ± 52 cpm.

We had previously observed that infected cells treated with the anti-theilerial drug, parvaquone, lost the ability to induce proliferation of and to be lysed by *T. parva*-specific T cell lines (10). Therefore, we compared the responses of the T cell clones to HSS prepared from uninfected lymphoblastoid cells, from TPM-infected cells, and from parvaquone-treated infected cells, which did not contain any schizonts as determined by examining Giemsa-stained smears. Table I shows that HSS prepared from both uninfected lymphoblastoid cells and parvaquone-treated *T. parva*-infected cells did not contain detectable antigenic activity, whereas HSS prepared from untreated *T. parva*-infected cells was antigenic.

Responses to cells infected with different T. parva stocks. Proliferative responses of the HSS-specific T cell clones to autologous uninfected lymphoblastoid cells and cell lines infected with the immunizing stock (Muguga) and four different stocks of *T. parva* were examined (Table II). In the presence of autologous APC, none of the clones responded to uninfected cells, and all of the clones responded to all stocks of *T. parva*-infected cells, showing that the response was parasite-specific, but not restricted to the Muguga stock. In fact, all of the clones responded better to heterologous stocks (especially Mariakani) than to the immunizing one with the exception of clone 2B2, which responded better to the Muguga stock than to the Boleni stock.

MHC-restricted response to T. parva Ag. Because the clones responded to intact, infected cells when APC were present, the clones could be recognizing exogenous parasite-derived Ag processed and presented by APC in the culture, or endogenous Ag processed by the infected cell and presented in association with MHC molecules on the infected cell surface. The following experiments were designed to address this question. The requirement for MHC-restricted Ag processing and presentation was determined for the following Ag: soluble HSS (Fig. 2*A*), autologous *T. parva*-infected cells (Fig. 2*B*), autologous, glutaraldehyde-fixed infected cells (Fig. 2*C*), and MHC-mismatched, infected cells (Fig. 2*D*). Because reagents for defining MHC class II Ag in cattle were not available, we could not determine whether the responses were MHC class II restricted. Glutaraldehyde fixation was used because cross-linking agents preserve Ag-MHC complexes on the cell surface (24, 27), but render the cells metabol-

TABLE I

Proliferative responses of T cell clones to HSS prepared from parvaquone-treated T. parva-infected cells

Clone	Radioactivity Incorporated[a] by Th Cell Clones Stimulated with HSS Prepared from the Following Cells[b]			
	None (medium)	Uninfected	Infected	Parvaquone-treated
2B2	608 ± 114	376 ± 78	19,713 ± 640	1,081 ± 50
2C3	1,023 ± 174	1,831 ± 458	49,100 ± 2741	1,140 ± 47
3E2	458 ± 257	538 ± 449	6,887 ± 237	179 ± 39
3F10	119 ± 34	364 ± 212	2,666 ± 303	129 ± 9
3H12	249 ± 52	336 ± 192	20,225 ± 894	492 ± 9

[a] Incorporation of radioactivity is expressed as the mean cpm of [^{125}I] UdR in duplicate samples ± SD.

[b] HSS was prepared from uninfected C447 lymphoblastoid cells, from C447-TPM cells, and from C447-TPM cells 6 days after treatment with parvaquone. In the results shown here, HSS was used at a concentration of 25 µg protein/ml.

TABLE II

Proliferative responses of Th cell clones against cell lines infected with different stocks of T. parva

Stimulator Cells[a]	Radioactivity Incorporated[b] by T Cell Clone				
	2B2	2C3	3E2	3F10	3H12
None	368 ± 230 *1,628[c] ± 213	1,496 ± 88	367 ± 38	466 ± 153	344 ± 33
Uninfected	*1,835 ± 186	609 ± 51	421 ± 87	256 ± 22	220 ± 38
Infected					
Muguga	35,054 ± 1,334	7,794 ± 524	2,435 ± 251	2,299 ± 1	3,072 ± 163
Marikebuni	95,782 ± 1,959	95,857 ± 9,553	35,287 ± 2,317	25,654 ± 5,564	22,018 ± 2,397
Mariakani	133,929 ± 3,469	123,139 ± 4,891	134,058 ± 5,558	52,248 ± 2,230	41,146 ± 3,093
Boleni	3,365 ± 914	29,874 ± 1,621	41,215 ± 8,151	16,833 ± 1,449	25,002 ± 2,506
Uganda	64,958 ± 704	92,216 ± 6,535	99,809 ± 7,839	42,849 ± 1,636	45,731 ± 4,594

[a] A total of 3×10^4 irradiated stimulator cells per well was used.
[b] Incorporation of radioactivity is expressed as the mean cpm of [^{125}I]UdR in duplicate samples ± SD.
[c] Mean cpm preceded by (*) are from a separate experiment.

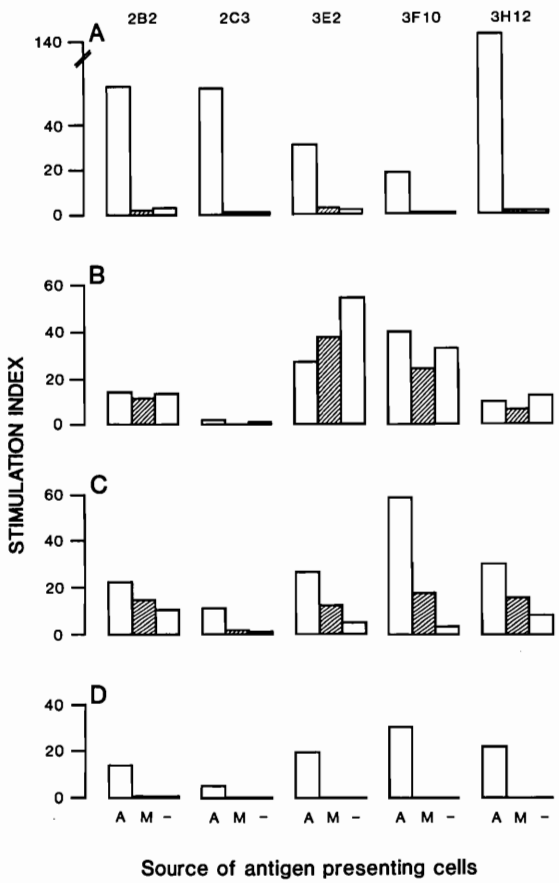

Figure 2. Proliferative responses of the T cell clones to Ag in the presence or absence of APC as indicated on the abscissa: autologous D768 APC (A), MHC class I-mismatched B657 APC (M) or no APC (–). Ag included: *A*, 50 μg/ml soluble HSS Ag; *B*, 3×10^4 irradiated autologous D768-TPM cells; *C*, 2.5×10^5 glutaraldehyde-fixed D768-TPM cells; and *D*, 3×10^4 irradiated MHC class I-mismatched B657.G6-TPM cells. Cultures in *B*, *C*, and *D* contained 0.5% TCGF. Results are expressed as SI, derived by dividing the mean cpm of cultures containing HSS or stimulator cells by the mean cpm of cultures containing no Ag.

ically inert and unable to synthesize or secrete proteins (24, 28, 29).

The responses of all five clones tested to the HSS Ag required autologous APC (Fig. 2*A*), showing that the T cell clones were incapable of presenting soluble, exogenous Ag to themselves and that the response was self-MHC restricted. Four of the clones proliferated in response to autologous *T. parva*-infected cells (Fig. 2*B*) and glutaraldehyde-fixed infected cells (Fig. 2*C*) in the presence and absence of APC, showing that *Theileria*-infected cells express and can present Ag in association with self-MHC. In contrast, the response of all clones to MHC-mismatched *T. parva*-infected cells required autologous APC (Fig. 2*D*), showing that the response is self MHC-restricted. The fifth clone, 2C3, had low SI to stimulation by all infected cells, probably as a result of high background proliferation induced with TCGF alone (9906 ± 1101 cpm).

Production of lymphokines. Clones 2B2, 2C3, 3E2, 3F10, and 3H12 were tested for TCGF and IFN-γ production after stimulation with HSS and APC. All supernatants contained measurable TCGF activity as compared with a bovine TCGF standard, resulting in a 3.3- to 19.0-fold increase in proliferation of the IL-2-dependent cloned T cell line, B657.G6, above background levels. In addition, all of the supernatants contained detectable levels of IFN-γ as compared with a human IFN-α standard, resulting in 50% endpoint titers in the range of 128 to 2048. The activity was sensitive to treatment at pH 2.0. Supernatants from cultures containing HSS alone or HSS and APC contained neither TCGF nor IFN-γ activity.

Characterization and purification of the antigenic activity in the HSS fraction. In order to determine whether liquid chromatographic separation of HSS would yield fractions enriched in antigenic activity, freshly prepared HSS was fractionated by hydroxylapatite column chromatography and/or by HPLC gel filtration. Antigenic activity routinely eluted from the hydroxylapatite column in three peaks separated from the majority of the protein. In the experiment depicted in Figure 3, antigenic activity eluted with approximately 250, 280, and 310 mM PO_4, whereas the bulk of the protein eluted in two peaks with approximately 40 and 180 mM PO_4. Similar antigenic profiles were obtained when the fractions were assayed on clones 2B2, 2C3 (shown in Fig. 3), and 3F10. Antigenic activity in the first two peaks was sensitive to treatment with papain, resulting in 76% and 85% inhibition of activity, respectively (data not shown). Protease sensitivity of Ag in the third peak was not determined.

When freshly prepared HSS was fractionated by gel filtration by using HPLC, and fractions were tested for antigenic activity, several peaks of activity eluted from the column, as shown in Figure 4. The M_r of the Ag peaks shown here (tested with clone 3F10) were 270, 38, 9, 5.4, 3.1, and 1.2 kDa; however, the majority of the activity was associated with protein eluting between 26 and 33 min (M_r 2 kDa to 20 kDa). Similar antigenic profiles were obtained when these fractions were tested on clones 2B2, 2C3, and 3H12, with peak activities eluting with M_r 270

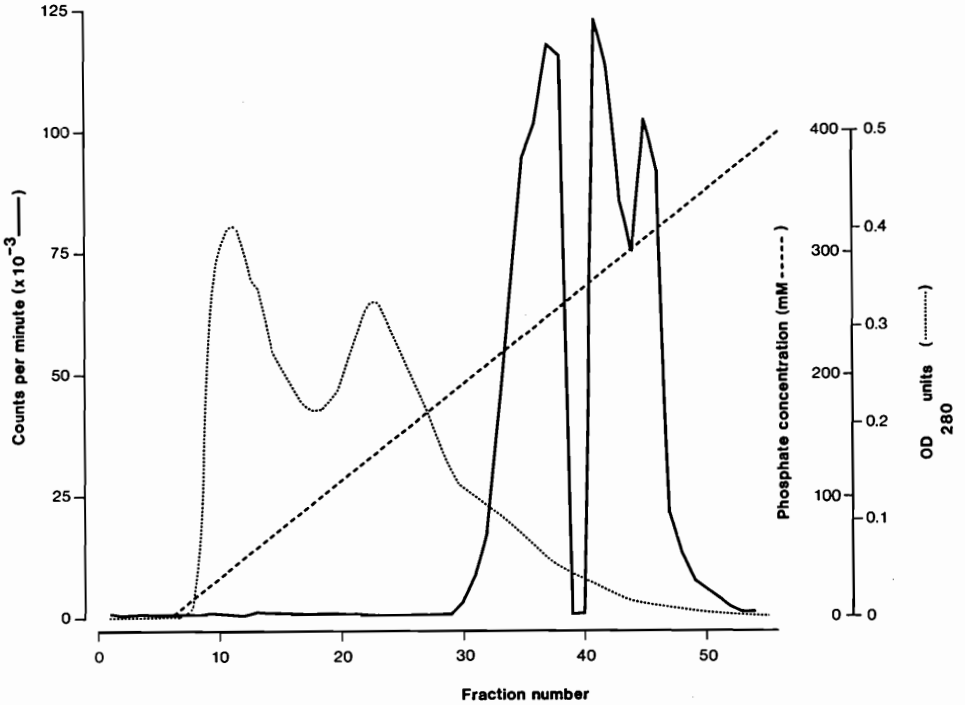

Figure 3. Fractionation of HSS by hydroxylapatite column chromatography. HSS (106.4 mg) was applied to the column, the material was eluted with a 0 to 400 mM PO_4 gradient (*dashed line*), and protein was monitored at 280 nm (*dotted line*). Fractions (20 μl) were tested for the ability to stimulate clone 2C3 in a proliferation assay (*solid line*). Results are expressed as the mean cpm of cultures containing the antigenic fractions. Background cpm of cells cultured in medium were 857 ± 82.

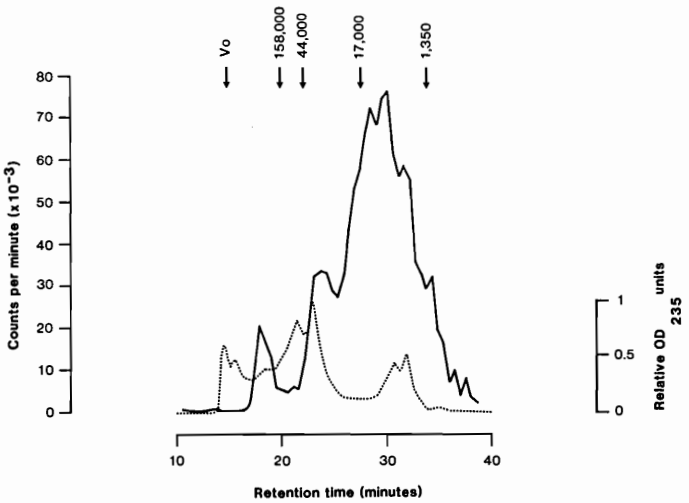

Figure 4. Gel filtration of D768-TPM HSS by using HPLC. HSS (3.3 mg protein) applied onto the column was eluted with PBS and monitored for protein at 235 nm (*dashed line*). Fractions (100 μl) were tested for the ability to stimulate clone 3F10 in a proliferation assay (*solid line*). Background cpm of cells cultured with medium and PBS were 453 ± 84. Protein standards are indicated at the top of the figure.

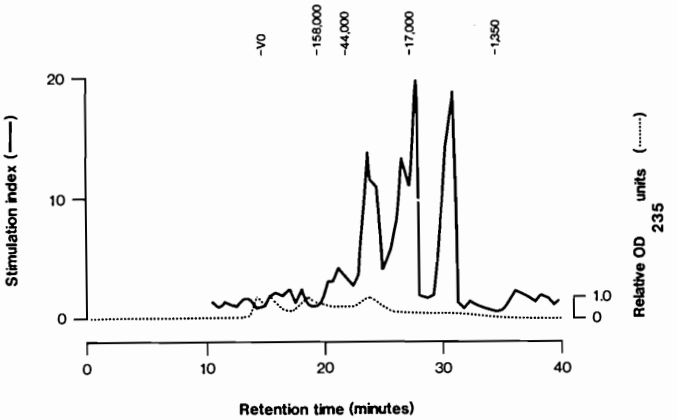

Figure 5. Gel filtration by hydroxylapatite-fractionated HSS with the use of HPLC. HSS was partially purified by hydroxylapatite column chromatography and a sample containing 60 μg protein was applied onto the HPLC column and was eluted with PBS. Protein was monitored at 235 nm (*dashed line*). Fractions were tested for the ability to stimulate clone 3F10 in a proliferation assay (*solid line*). Results are expressed as the SI. Background cpm of cells cultured with medium and PBS were 51 ± 19. Protein standards are indicated at the top of the figure.

kDa, 41 to 49 kDa, 5.4 to 9 kDa, 1.7 to 2.5 kDa, and less than 1 kDa.

In a different experiment, HSS was fractionated by hydroxylapatite column chromatography and further purified by HPLC. The material eluted from hydroxylapatite with 200 mM PO_4, contained 300 μg protein/ml, and stimulated peak levels of proliferation of uncloned D768 TMLC-4 cells (6.2 μg/ml protein induced 23,143 ± 2,998 cpm as compared with a background of 221 ± 88 cpm). After storage at −80°C, a sample of this fraction containing 60 μg protein was applied to HPLC, and eluted fractions were tested for the ability to stimulate clone 3F10. Antigenic activity was detected in three major peaks with M_r of 43 kDa, 12 kDa, and 4.2 kDa (Fig. 5). When samples from these peaks were subjected to SDS-PAGE and silver staining, no bands were visible (data not shown).

DISCUSSION

Although the importance of T cell-mediated immune responses in controlling *T. parva* infection in cattle has been well documented (for a review, see Reference 30) little is known about the nature of the target Ag recognized by either parasite-specific CTL or Th cells. In an attempt to better understand the role of Th cells in immunity to this organism, and to define parasite-associated Ag that stimulate T cell responses, we have characterized Th clones specific for Ag derived from schizont-infected cells. In a related study (11), we identified a

schizont membrane-associated Ag that appears to be expressed as well on the infected cell surface. In the present study, we have characterized a different Ag found in the soluble extract prepared from a H of *T. parva*-infected lymphocytes.

The T cell clones described here were derived by stimulation with either intact, *T. parva*-infected cells, or with the soluble HSS fraction from these cells. Nevertheless, all of the clones exhibited MHC-restricted specificity for Ag presented by the infected cells themselves and for soluble HSS, and none responded to the CM-1 fraction enriched for schizont membranes. Studies with parvaquone-treated cells have shown that the presence of viable parasites is required for expression of parasite Ag in soluble HSS as well as on the infected cell surface (10). Together, these results are consistent with the possibility that the parasite Ag, released upon homogenization into the soluble cytosol fraction, is endogenously processed by the infected cell and presented as a peptide in association with MHC at the cell surface. However, these data do not preclude the possibility that peptides released by the infected cells bind to MHC determinants. Because the clones express BoT4 (CD4), we assume that the Ag is associated with MHC class II molecules, known to be expressed on the surface of *T. parva*-infected lymphocytes (31).

In other systems, MHC class II-restricted $CD4^+$ T cells exhibit specificity for both virus-infected cells and exogenously processed viral Ag (32, 33). In addition, class-II restricted $CD4^+$ T cells specific for intracellular pathogens are often cytotoxic for infected target cells (32, 34). However, all of the *Theileria*-specific CTL clones described expressed the $BoT8^+$ ($CD8^+$) phenotype (13, 35), and none of the $BoT4^+$ ($CD4^+$) clones described, including those in this study, were cytolytic (11, 36). The production of both IFN-γ and TCGF by the T cell clones in response to stimulation by HSS Ag suggests that they may represent inflammatory Th cells. IFN-γ-producing $CD4^+$ T cell clones specific for other intracellular protozoa, including *Plasmodium* (37, 38), and *Leishmania* (39, 40), have been described. This lymphokine has been implicated in protective immunity by inhibiting the growth of exoerythrocytic forms of malarial parasites (41, 42) and by activating macrophages to kill intracellular *Leishmania* (43, 44). The role of immune IFN in response to infection with *Theileria* is unclear; it appears that IFG-γ does not impair the viability of intracellular schizonts in vitro (J. C. DeMartini and C. L. Baldwin, unpublished observations); however, the effects of IFN-γ on schizonts in vivo and on other stages of the parasite life cycle, and the role of activated macrophages in protective immunity, have not been examined.

The Ag recognized by the Th clones is shared by several different geographic isolates of *T. parva*, because parasitized cells of all stocks tested were stimulatory. Although different patterns of response to infected cells were observed with the clones (i.e., clone 2C3 responded very poorly to D768-TPM cells as compared with clones 2B2, 3F10, and 3H12), no striking differences were detected in the responses of these clones to HSS fractionated by either gel filtration or hydroxylapatite, suggesting that they all recognize the same or cross-reacting antigenic determinants. The protein Ag is heterogeneous with respect to affinity for hydroxylapatite and size, as estimated by gel filtration and, after sequential purification by using both methods, still contained three activity peaks. These may represent degradative products or peptide fragments of a large molecule, aggregates of a small molecule, or different molecules expressing a shared epitope.

Experiments are underway to purify to homogeneity the *Theileria parva*-associated soluble Ag so that we can continue to investigate both Th and CTL responses to the purified molecules in vitro. This Ag is a good vaccine candidate because it is expressed by geographically diverse parasites, expressed on the infected cell surface where it would be recognized by memory T cells during challenge, and present in a soluble subcellular fraction from which it can be purified. Our eventual aim is to examine the potential protective properties of both the soluble and schizont membrane-associated Ag in cattle in order to evaluate their suitability for use in developing a recombinant vaccine for theileriosis.

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