INFECTION AND IMMUNITY, Mar. 1994, p. 818–827
0019-9567/94/$04.00+0

Vol. 62, No. 3

CD4$^+$ T-Cell Clones Obtained from Cattle Chronically Infected with *Fasciola hepatica* and Specific for Adult Worm Antigen Express Both Unrestricted and Th2 Cytokine Profiles

WENDY C. BROWN,[1]* WILLIAM C. DAVIS,[2] DIRK A. E. DOBBELAERE,[3] AND ALLISON C. RICE-FICHT[4]

Department of Veterinary Pathobiology[1] and Department of Medical Biochemistry and Genetics,[4] Texas A&M University, College Station, Texas 77843[1]; Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington 99164[2]; and Institute of Parasitology, University of Bern, Bern, Switzerland[3]

Received 9 September 1993/Returned for modification 5 November 1993/Accepted 16 December 1993

The well-established importance of helper T (Th)-cell subsets in immunity and immunoregulation of many experimental helminth infections prompted a detailed study of the cellular immune response against *Fasciola hepatica* in the natural bovine host. T-cell lines established from two cattle infected with *F. hepatica* were characterized for the expression of T-cell surface markers and proliferative responses against *F. hepatica* adult worm antigen. Parasite-specific T-cell lines contained a mixture of CD4$^+$, CD8$^+$, and γ/δ T-cell-receptor-bearing T cells. However, cell lines containing either fewer than 10% CD8$^+$ T cells or depleted of γ/δ T cells proliferated vigorously against *F. hepatica* antigen, indicating that these T-cell subsets are not required for proliferative responses in vitro. Seventeen *F. hepatica*-specific CD4$^+$ Th-cell clones were examined for cytokine expression following concanavalin A stimulation. Biological assays to measure interleukin-2 (IL-2) or IL-4, gamma interferon (IFN-γ), and tumor necrosis factor and Northern (RNA) blot analysis to verify the expression of IL-2, IL-4, and IFN-γ revealed that the Th-cell clones expressed a spectrum of cytokine profiles. Several Th-cell clones were identified as Th2 cells by the strong expression of IL-4 but little or no IL-2 or IFN-γ mRNA. The majority of Th-cell clones were classified as Th0 cells by the expression of either all three cytokines or combinations of IL-2 and IL-4 or IL-4 and IFN-γ. No Th1-cell clones were obtained. All of the Th-cell clones expressed a typical memory cell surface phenotype, characterized as CD45R^{low}, and all expressed the lymph node homing receptor (L selectin). These results are the first to describe cytokine responses of *F. hepatica*-specific T cells obtained from infected cattle and extend our previous analysis of Th0 and Th1 cells from cattle immune to *Babesia bovis* (W. C. Brown, V. M. Woods, D. A. E. Dobbelaere, and K. S. Logan, Infect. Immun. 61:3273–3281, 1993) to include *F. hepatica*-specific Th2 cells.

The digenetic trematode parasite *Fasciola hepatica* is a commonly occurring liver fluke that infects a wide variety of mammals, including cattle, sheep, and humans. Fascioliasis can be a chronic disease, with adult worms surviving for up to 12 years in the host. The major pathology caused by *F. hepatica* includes fibrosis due to severe trauma induced by juvenile worms migrating through the liver and inflammation, edema, and fibrosis of the bile ducts due to the presence of the adult flukes. Acquired immunity to this parasite differs in different species (reviewed in reference 22). Whereas sheep and rabbits do not appear to acquire resistance to infection by *F. hepatica*, cattle, goats, and rats exhibit substantial immunity to challenge infection with *F. hepatica* when they have had a previous infection. In cattle, it was shown that worm rejection occurred 24 weeks after a primary infection (18), leading to resistance to a secondary infection characterized by a decrease in the size and number of recovered flukes.

Protective immunity can be elicited in cattle, goats, and rats by infection with irradiated metacercariae, somatic fluke extracts, and excretory/secretory fluke antigens (22). More recent studies have indicated the feasibility of achieving partial protective immunity in cattle and sheep vaccinated with purified or recombinant proteins of *F. hepatica*, including a 12-kDa antigen (24) and a 26-kDa protein, glutathione *S*-transferase (46). However, in spite of these accomplishments, the nature of the protective immune response to *F. hepatica* has not been clearly defined. Both humoral and cell-mediated mechanisms of immunity appear to be important for resistance to *F. hepatica* infection in different species (22). However, the following observations suggest that antibody alone is not sufficient for protective immunity. First, passive transfer of resistance in rats by immune serum was achieved only with large volumes (1), and an insignificant level of immunity was achieved in a study involving the transfer of immune serum from one calf to its naive twin (13). Second, serum antibody titers of infected rats did not correlate with the number of flukes recovered at necropsy (30). Finally, resistance in sheep vaccinated with *F. hepatica* glutathione *S*-transferase did not correlate with antibody titers (46).

In mice and humans, helminth infections are generally characterized by high levels of immunoglobulin E (IgE) and eosinophils, induced by interleukin-4 (IL-4) and IL-5. These cytokines are produced by the helper T2 (Th2) subset of CD4$^+$ T cells (20). However, protective immunity in experimental murine schistosomiasis caused by *Schistosoma mansoni* appears to be dependent on the Th1 subset of T cells that produce IL-2 and gamma interferon (IFN-γ) (10, 48). Protective immunity is apparently effected by IFN-γ-activated macrophages that, in vitro, are able to kill schistosomula (28). In addition, downregulation of Th1-cell responses by Th2-cell-derived IL-10 in mice undergoing egg-laying infections appears

* Corresponding author. Mailing address: Department of Veterinary Pathobiology, Texas A&M University, College Station, TX 77843-4467. Phone: 409-845-4207. Fax: 409-845-9972. Electronic mail address: wbrown@vthvax.tamu.edu.

to play an important role in the chronicity of infection (47). In contrast to the mouse model, studies performed with the rat model of *S. mansoni* have strongly suggested a major role for Th2 cells in protective immunity through the stimulation of IgE, eosinophils, and mast cells (11). In this model, cellular immunity is mediated by eosinophils and macrophages, which in the presence of specific antibody including IgE, are activated to kill schistosomula in vitro. It thus appears that the cytokine-dependent effector mechanisms against *S. mansoni* and *F. hepatica* may differ in different host species, underscoring the need to define the mechanisms of immunity against these parasites in the natural host.

The importance of T cells in protective immunity against *F. hepatica* was indicated by studies performed with rats and cattle, which demonstrated successful transfer of resistance with spleen and lymph node cells from infected donors to syngeneic recipients (1, 13, 40). However, few studies have attempted to characterize T-lymphocyte responses in cattle against *F. hepatica*. In vitro proliferation assays revealed early and transient responses of peripheral blood mononuclear cells (PBMC) against *F. hepatica* adult worm antigen, which disappeared by 5 weeks postinfection (37). Furthermore, skin tests performed after 4 weeks postinfection failed to demonstrate delayed-type hypersensitivity in fluke-infected cattle (19). The reasons for the discrepancy between the ability to transfer resistance with lymphoid cells and the inability to detect cell-mediated immune responses by 5 weeks following experimental infection are unclear. One possibility is that by 4 to 5 weeks postinfection, cell-mediated immune responses, effected by Th1-like cells, are downregulated by IL-10 produced in response to antigens released by migrating worms. Clearly, a detailed investigation of the molecular mechanisms of immunity to *F. hepatica* is needed before rational vaccine strategies can be devised.

The importance of cytokines in response to different helminth infections, including the related trematode parasite *S. mansoni*, and the need to understand the molecular basis of immunity in fascioliasis in the natural host prompted a detailed investigation of the bovine T-cell response against *F. hepatica*. Since polyclonal lymphoid-cell responses are often unreliable because of complex cellular interactions, parasite-specific T-cell lines and T-cell clones were derived from PBMC of infected cattle for phenotypic and functional characterization of the *F. hepatica*-specific T-cell response. Adult worm extract was selected as an antigen for these studies, since this preparation was capable of stimulating protective immunity in cattle (22). We found that bovine Th-cell clones specific for helminth antigens expressed both unrestricted and Th2 cytokine profiles, as reported for Th-cell clones derived from humans and mice (12, 17, 20, 50). The experiments described in the present study are the first to characterize the bovine T-cell response against *F. hepatica* as well as Th2 cells in cattle and provide a basis for further studies of the immune response in cattle resistant to challenge infection with *F. hepatica*. T-cell clones may be useful for subsequent identification of potentially protective antigens of this parasite.

MATERIALS AND METHODS

Experimental infection and serological responses. Two 15-month-old Charolais cattle (one heifer, designated animal G1, and one steer, designated animal G8) were purchased from Granada Biosciences, Inc., Houston, Tex. These animals were produced by embryo cloning and were genetically identical to heifer G3 and steer G6, respectively, which were infected and challenged with *Babesia bovis* as described in an earlier publication (7). Cattle G1 and G8 were infected per os with 1,000 metacercariae of the Oregon strain of *F. hepatica* (Baldwin Aquatics, Monmouth, Oreg.). Serum samples obtained prior to and at 3- to 10-month intervals following infection were obtained for use in immunoblot assays. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting were performed essentially as described elsewhere (6) and employed the minigel and transblot systems (Bio-Rad Laboratories, Richmond, Calif.), with 10% acrylamide gels and 30 μg of *F. hepatica* worm antigen per lane. Molecular mass standards obtained from Bio-Rad included β-galactosidase (116 kDa), rabbit muscle phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), hen egg white ovalbumin (45 kDa), and bovine carbonic anhydrase (31 kDa). Fractionated proteins were electrophoretically transferred from acrylamide to nitrocellulose filters for 1 h at 100 V. Preinfection and postinfection bovine sera were used to develop the immunoblots. Sera were diluted 1:100 in Tris-buffered saline (10 mM Tris, 150 mM NaCl [pH 7.5]) containing 1% gelatin, and serologically reactive proteins were detected with a 1:5,000 dilution of alkaline phosphatase-conjugated goat anti-bovine IgG (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) and the chromogenic substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Sigma Chemical Co., St. Louis, Mo.) as instructed by the manufacturers.

Parasite antigens. Adult *F. hepatica* flukes were obtained from infected bovine livers collected at a local abbatoire (Braunfels Meat, Inc., Sealy, Tex.). Flukes were teased from the bile ducts after transverse slices were made across the entire ventral surface of the liver. Flukes were washed three times in 0.15 M phosphate-buffered saline (PBS), pH 7.4, frozen, and stored at −70°C until use. To prepare the adult worm antigen extract, 15 frozen adult flukes were ground to a fine powder and suspended in 10 ml of PBS in the presence of 2 mM phenylmethylsulfonyl fluoride and 5 mM iodoacetamide. The suspension was homogenized with a tissue homogenizer and centrifuged at 15,000 × *g* at 4°C for 1 h. The supernatant was diluted to a concentration of 1 mg of protein per ml and sterilized by filtration through a 0.22-μm-pore-size filter. For lymphocyte proliferation assays, the antigen was prepared as described, except that iodoacetamide was not included. A crude membrane preparation of *B. bovis* merozoites was prepared from the Mexico isolate as described elsewhere (6) and used as a control antigen for lymphocyte proliferation studies. The protein contents in both parasite antigenic preparations were determined by the Bradford protein assay (Bio-Rad) with bovine IgG as a protein standard. Aliquots of the different antigens were stored at −70°C.

Generation of *F. hepatica*-specific T-cell lines and clones. T-cell lines specific for *F. hepatica* adult worm antigen were established from PBMC of infected cattle G1 and G8 essentially as described for T-cell lines specific for *B. bovis* (2, 5). In brief, PBMC were obtained from infected animals at 27 weeks (cell lines G1.5 and G8.5), 34 weeks (cell lines G1.6 and G8.6), and 40 weeks (G8.7) following infection. PBMC were stimulated in 1.5-ml cultures in 24-well plates (Costar, Cambridge, Mass.) at a density of 4×10^6 PBMC per well with 25 or 50 μg of adult worm antigen per ml of complete RPMI 1640 medium (6). After 7 days, viable cells were subcultured to a density of 5×10^5 cells per well and were restimulated with antigen and 2×10^6 irradiated (3,000 rads) autologous PBMC as a source of antigen-presenting cells (APC).

Cell lines G1.6 and G8.6, which had been cryopreserved after 1 week of culture and thawed, were treated with a monoclonal antibody (MAb) specific for the bovine γ/δ T-cell receptor complex, designated TcR1-N12 (14), and rabbit com-

plement to remove γ/δ T cells. Briefly, 10^7 T cells were incubated for 30 min at 4°C with MAb CACT-61A diluted in PBS to a final concentration of 15 μg of protein per ml. The cells were washed once in PBS and were then incubated for 30 min at 37°C with an equal volume of rabbit complement (Sigma) diluted 1:8 in PBS. The cells were then washed three times in complete medium and stimulated with antigen and APC as described. These cell lines were designated G1.6Rx and G8.6Rx. Cell line G8.7Rx was derived from freshly isolated PBMC that were subjected to two consecutive treatments with MAb CACT.61A and complement and was cultured with *F. hepatica* antigen as described.

Cell lines were maintained in culture for up to 7 weeks by weekly stimulation with antigen and APC and were assessed for antigen-specific proliferation or the presence of surface markers 7 or 8 days following the last stimulation with antigen and APC. Lines G1.6Rx, G8.6Rx, and G8.7Rx were cloned by limiting the dilution after 2.5 weeks of culture. Cloning was performed in 96-well round-bottom plates (Costar) essentially as described elsewhere (8), with the following modifications. A statistical average of 1 or 0.3 cells per well was stimulated with 25 μg of *F. hepatica* adult worm antigen per ml of complete medium containing 10% bovine T-cell growth factor (TCGF) (3) and 5×10^4 irradiated (3,000 rads) autologous PBMC. Proliferating cells were transferred successively to 48-well (Costar) and 24-well plates, stimulated with antigen, TCGF, and APC, and tested for antigen-dependent proliferation. Cloning frequencies of 60 to 75% and 15 to 25%, respectively, were obtained from the cell lines when an average of 1.0 or 0.3 T cells was distributed per well.

Cell surface phenotypic analysis. PBMC, *F. hepatica*-specific cell lines, and T-cell clones were stained with a panel of MAbs by indirect immunofluorescence as described elsewhere (5) and were analyzed with a Coulter EPICS 741 flow cytometer. MAbs 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), IL-A26 (specific for CD2), and IL-A150 (considered specific for a polymorphic determinant on bovine CD45RO) (32, 33). MAbs obtained from Chris Howard at the Agriculture and Food Research Council Institute for Animal Health in Compton, United Kingdom, included CC76 (specific for bovine CD45R; 26) and CC32 (specific for bovine L selectin; 25). MAb CACT-61A (specific for the γ/δ TcR1-N12 determinant; 14) was used to enumerate γ/δ T cells in PBMC and T-cell lines. Goat anti-mouse immunoglobulin [affinity-purified $F(ab')_2$ fragments; Cappel/Organon-Teknika, Inc., Malvern, Pa.] was used as a second reagent. The percentages of positive cells were determined by subtracting the percentages of cells stained with the second reagent only from the percentages of cells stained with a given MAb and the second reagent, as described by Overton (38). The proportion of $CD4^+$, $CD8^+$, and γ/δ T cells was determined by the following equation:

$$100 \times \frac{\%\text{ cells stained with a given MAb}}{\%\text{ IL-A12}^+\text{ cells} + \%\text{ IL-A51}^+\text{ cells} + \%\text{ CACT.61A}^+\text{ cells}}$$

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 (5). Each well (total volume, 100 μl) contained complete medium, with responder cells added at a final concentration of 3×10^5 cells per ml (obtained 7 days following the last stimulation of the T-cell lines or clones with antigen) and 2×10^6 autologous or allogeneic (animal C97), irradiated PBMC per ml as a source of APC. Antigens were added to the assay mixes at a final concentration of 1 to 50 μg of protein per ml of complete medium. Bovine TCGF (4 or 5%) was included as a positive control for lymphocyte proliferation. In some experiments, clones G8.3B7 and G1.2H4 were assayed in the presence of 5% TCGF, in addition to antigen, in all wells of the proliferation assays. 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, and the radioactivity was counted in a gamma counter. The results are presented as the mean counts per minute and standard deviations of duplicate samples.

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

Stimulation of cells and biological assays for cytokine production. T cells obtained 6 or 7 days after the last stimulation with antigen and APC were washed in complete medium and cultured for 17 to 18 h at a concentration of 1.3×10^6 cells per ml of complete medium containing 5 μg of concanavalin A (ConA; Sigma) per ml in the absence of APC. In one experiment, *F. hepatica*-specific T-cell clone G1.3E11 was cultured with either ConA or with 25 μg of antigen per ml and APC for 18 h, and supernatants were compared. Controls included ConA diluted to 5 μg per ml of complete medium and supernatants collected from APC cultured with antigen in the absence of T cells. Supernatants were harvested by centrifugation and stored at −80°C.

Biological assays for cytokines have been described in detail elsewhere (7–9). In brief, IFN-γ activity was measured in a microtiter cytopathic-effect assay with vesicular stomatitis virus and Madin-Darby bovine kidney (MDBK) cells. MDBK cell survival was evaluated by the 4-h uptake of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye in the cells. IFN-γ titers were compared with a human recombinant IFN-α_2 reference reagent (National Institute of Allergy and Infectious Diseases, Bethesda, Md.; catalog number, Gxa01-901-535). For determination of tumor necrosis factor (TNF) activity in the supernatants of T-cell clones, a TNF-sensitive WEHI-164 subline was incubated with culture supernatants in a 48-h assay, and cytopathicity was determined by the MTT dye reduction assay. TNF titers were compared with a standard human recombinant TNF-α (Upstate Biotechnology, Inc., Lake Placid, N.Y.). A cloned $CD8^+$, IL-2-dependent bovine T-cell line, designated 99.G1.G3, was used to measure IL-2 or IL-4 activity. The cell line did not discriminate IL-4 from IL-2 activity, since it expressed both IL-2 and IL-4 mRNA and proliferated upon ConA activation (7). Delectinated culture supernatants of *F. hepatica*-specific T-cell clones stimulated with ConA and supernatant from antigen-stimulated clone G1.3E11 were diluted 1:2 to 1:256 in complete medium and added to duplicate wells of 99.G1.G3 cells which had been distributed at a density of 3×10^4 cells per well in 96-well half-area microtiter plates. In all assays, recombinant human IL-2 (Boehringer Mannheim, Indianapolis, Ind.) was serially diluted from 100 to <1 U/ml for use as a reference standard. Cells were cultured for 48 to 72 h, radiolabeled, harvested, and counted as described above. A semiquantitative estimate of IL-2 and IL-4 activity in the culture supernatants was obtained by comparison with a standard curve of human IL-2.

Northern (RNA) blot analysis. Analysis of mRNA expression of IL-2, IL-4, and IFN-γ was performed by Northern blotting as described elsewhere (7, 9). Total RNA was obtained from unstimulated bovine turbinate (BT) cells as a negative

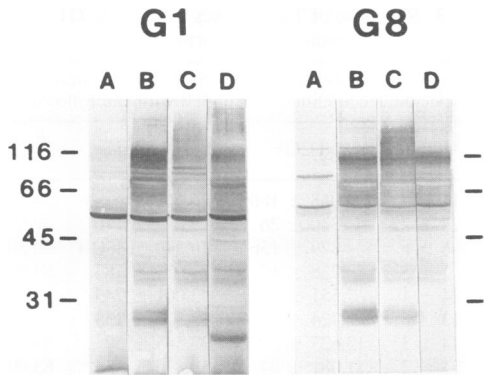

FIG. 1. Serologic reactivity of serum samples from *F. hepatica*-infected cattle with adult worm antigen. Western blots were performed by using 30 μg of *F. hepatica* adult worm antigen per lane and preimmune sera from animals G1 and G8 (lane A) and sera obtained 3 months (lane B), 5 months (lane C), or 10 months (lane D) following oral infection of cattle G1 and G8 with 1,000 metacercariae. Molecular size standards are indicated on the left and right of the figure.

control, from PBMC stimulated for 18 h with ConA as a positive control, and from T-cell clones that had been cultured for 8 h with ConA, by using 2 ml of RNazol B (Biotecx Laboratories, Inc., Houston, Tex.) per 10^7 cells, as specified by the manufacturer. Total RNA was also obtained from T-cell clones stimulated for 13 h with 50 μg of adult worm antigen per ml and APC or from control APC cultured in the absence of T cells. RNA (15 to 20 μg) was subjected to electrophoresis in a formaldehyde–morpholinepropanesulfonic acid (MOPS)–1.6% agarose gel, transferred to GeneScreen nylon filters (Dupont NEN, Boston, Mass.), and hybridized with the following cDNA probes. The IL-2 cDNA probe consisted of a 0.79-kb *Eco*RI fragment of the bovine IL-2 cDNA (41) and was a generous gift from Raymond Reeves, Washington State University. The IL-4 cDNA probe consisted of a 0.4-kb *Eco*RI-*Sal*I fragment of bovine IL-4 cDNA (23). The IFN-γ probe consisted of a 0.5-kb *Xba*I-*Dra*I fragment of bovine IFN-γ cDNA and was kindly provided by Arjun Singh, Genentech, San Francisco, Calif. The actin cDNA probe consisted of a 1.0-kb *Eco*RI fragment of bovine actin cDNA (15) and was kindly provided by Angelika Ehrfeld, Max Planck Institute for Immunology, Freiburg, Germany. The cDNAs were labeled with ^{32}P by the random primer method with a kit from Boehringer Mannheim, yielding probes with specific activities of 1×10^9 to 2×10^9 cpm/μg. Filters were prehybridized and hybridized with radiolabeled probes (7, 9) and were then exposed to Hyperfilm^{MP} (Amersham Corporation, Arlington Heights, Ill.) at −80°C. The approximate sizes of the transcripts in kilobases were determined from nucleic acid molecular size standards (0.24- to 9.5-kb RNA ladder; GIBCO-BRL, Gaithersburg, Md.) that were electrophoresed, transferred to nylon, and stained with methylene blue.

RESULTS

Serologic responses of *F. hepatica*-infected cattle. Serum samples obtained from animals G1 and G8 before infection and at 3, 5, and 10 months following oral infection with 1,000 metacercariae of *F. hepatica* were compared for reactivity with adult worm antigenic extract. Figure 1 shows that, compared with preinfection serum samples (lane A), postinfection serum samples (lanes B to D) reacted on immunoblots, with numerous bands ranging in apparent molecular mass from approximately 26 to >116 kDa. However, during the course of infection, the pattern of reactivity with *F. hepatica* varied. For example, 5 months postinfection serum from animal G1 (lane C) displayed decreased reactivity with bands ranging from 60 to 120 kDa, compared with 3-month (lane B) and 10-month, (lane D) postinfection sera, whereas 5-month postinfection serum from animal G8 (lane C) recognized additional high-molecular-mass bands of ≥116 kDa that were less visible in 3-month (lane B) and 10-month (lane D) postinfection serum samples. In addition, 10 months postinfection, serum from animal G1 recognized a novel band of <26 kDa. These results show that at the time at which the T-cell lines were established from these animals (approximately 7 to 10 months postinfection), serologic reactivity, indicative of an active or recent infection, was still present.

TABLE 1. Response of *F. hepatica*-specific T-cell lines to *F. hepatica* adult worm antigen

Cell line and no. of wks in culture	Radioactivity (mean cpm ± SD) incorporated by T-cell lines stimulated with the following antigens[a]:		
	None (medium)	*B. bovis*	*F. hepatica*
G1.5			
2	434 ± 36	414 ± 76	34,894 ± 87
7	2,936 ± 66	706 ± 48	14,418 ± 99
G8.5			
2	328 ± 64	325 ± 26	29,391 ± 254
7	2,332 ± 26	1,796 ± 41	5,341 ± 271
G1.6			
1	203 ± 65	315 ± 20	18,074 ± 5,915
3	2,902 ± 341	2,197 ± 116	24,851 ± 1,780
G1.6Rx (γ/δ T-cell depleted)[b]			
5	396 ± 78	519 ± 74	88,783 ± 217
G8.6			
1	243 ± 37	263 ± 45	5,245 ± 931
3	503 ± 155	238 ± 3	31,121 ± 1,875
G8.6Rx (γ/δ T-cell depleted)[b]			
5	1,382 ± 266	588 ± 106	83,349 ± 1,962

[a] Results are presented for 25 μg of *B. bovis* merozoite membrane antigen and 50 μg of *F. hepatica* adult worm antigen per ml. The results of proliferative responses of cell lines cultured with *F. hepatica* antigen were shown to be significantly different from responses of cell lines cultured with either complete medium alone or *B. bovis* ($P < 0.01$) by the Student one-tailed *t* test.

[b] Cell lines G1.6 and G8.6 were cryopreserved after 1 week of culture, thawed, treated with MAb CACT.61A and complement, and cultured for an additional 4 weeks. The γ/δ T-cell-depleted lines are designated G1.6Rx and G8.6Rx.

F. hepatica-specific T-cell lines. To characterize the nature of the T-cell response to *F. hepatica* adult worm antigens, *F. hepatica*-specific T-cell lines were established from PBMC obtained from cattle infected 7 to 10 months earlier. All cell lines responded to either 10 or 50 μg of *F. hepatica* adult worm antigen per ml of medium (Table 1 and data not presented) but did not respond to any concentration of control antigens, including membrane antigen prepared from either *B. bovis* merozoites (Table 1) or uninfected erythrocytes (data not presented). Cell lines G1.5 and G8.5 were cloned after 3 weeks of culture, and 10 T-cell clones were obtained. None of the 10 clones proliferated in response to *F. hepatica* adult worm antigen, and all of the clones expressed the γ/δ TcR (data not presented). This finding prompted a careful evaluation of the relative proportions of $CD4^+$, $CD8^+$, and γ/δ TcR^+ T cells in the cell lines stimulated with *F. hepatica*. In the majority of *F.*

TABLE 2. Cell surface phenotypic analysis of *F. hepatica*-specific T-cell lines

Cell line and no. of wks in culture	Percentage of the total T-cell population stained by a MAb[a]		
	IL-A12 (CD4)	IL-A51 (CD8)	CACT.61A (γ/δ TcR1)
G1.5			
0 (PBMC)	51.1	25.9	23.0
6	22.4	27.7	49.9
G8.5			
0 (PBMC)	41.3	28.5	30.2
6	22.9	26.2	50.9
G1.6			
1	55.6	14.1	30.4
3	35.6	15.4	48.9
G1.6Rx (γ/δ T-cell depleted)[b]			
5	85.5	6.3	8.4
G8.6			
1	35.5	27.6	36.9
3	34.8	7.8	57.4
G8.6Rx (γ/δ T-cell depleted)[b]			
5	88.9	2.2	8.8

[a] Freshly isolated PBMC or T cells obtained from cell lines cultured for 1 to 6 weeks with *F. hepatica* worm antigen were stained with the indicated MAb. The proportion of $CD4^+$, $CD8^+$, and γ/δ T cells was determined as described in the Materials and Methods section.

[b] Cell lines G1.6Rx and G8.6Rx are described in footnote *a* to Table 1.

TABLE 3. Response of T-cell clones from cattle G1 and G8 to *F. hepatica* adult worm antigen

T-cell clone	Radioactivity (mean cpm ± SD) incorporated by *F. hepatica*-specific T-cell clones stimulated with the following antigens[a]:			
	Medium	TCGF	*F. hepatica*	*F. hepatica* + TCGF
G8.2G10	16 ± 4	6,068 ± 160	46,668 ± 6,626	56,751 ± 3,244
G8.3B7	23 ± 22	340 ± 26	9,627 ± 110	49,811 ± 1,795
G1.3G10	6 ± 6	879 ± 136	10,340 ± 1,410	70,500 ± 1,348
G1.3G4	7 ± 5	238 ± 17	22,923 ± 183	84,912 ± 1,630
G1.3E11	24 ± 1	7,551 ± 513	43,001 ± 745	82,990 ± 4,626
G1.3B11	13 ± 6	2,724 ± 11	13,877 ± 133	38,216 ± 189
G1.1H5	44 ± 3	15,627 ± 1,133	32,545 ± 59	59,011 ± 4,079
G1.1H12	51 ± 2	1,081 ± 94	59,873 ± 256	83,906 ± 4,687
G1.2H4	8 ± 6	2,240 ± 30	42 ± 2	23,278 ± 471
G8.2C12	27 ± 9	1,218 ± 191	19,991 ± 1,955	44,865 ± 94
G8.2C1	24 ± 8	8,802 ± 633	56,144 ± 2,118	81,887 ± 618
G8.2C3	25 ± 2	428 ± 11	44,638 ± 1,238	41,060 ± 434
G8.2B1A	28 ± 3	4,102 ± 312	90,940 ± 3,210	102,610 ± 1,345
G8.2D9	13 ± 1	6,808 ± 192	18,054 ± 1,397	27,767 ± 756
G1.3G5	16 ± 4	687 ± 37	1,597 ± 371	43,269 ± 1,887
G1.3F7	25 ± 9	300 ± 24	475 ± 47	7,117 ± 887
G1.2B6	67 ± 6	3,658 ± 234	5,676 ± 144	20,211 ± 1,111

[a] Antigen consisted of 50 μg of *F. hepatica* adult worm antigen per ml. TCGF was used at a final concentration of 4%. Proliferative responses of T-cell clones cultured with *F. hepatica* antigen were shown to be significantly different from responses of T cells cultured with medium alone ($P < 0.01$), and proliferative responses of T-cell clones cultured with antigen and TCGF were shown to be significantly different from proliferative responses of T-cell clones cultured with TCGF alone ($P < 0.01$) by the Student one-tailed *t* test.

hepatica-specific T-cell lines examined, the proportion of $CD4^+$ T cells increased during a 4-week culture period, whereas the proportion of $CD8^+$ T cells decreased during this time (data not presented), and cell lines containing fewer than 10% $CD8^+$ T cells proliferated well to antigen (Table 2). However, γ/δ T cells constituted between 15 and 40% of the total T-cell population in T-cell lines stimulated with antigen for 2 to 4 weeks and reached levels of 50 and 51% of the T cells in lines G1.5 and G8.5, respectively, by 6 weeks of culture (Table 2). The high percentage of γ/δ T cells observed in several of these cell lines could be partially attributed to the relatively high numbers of γ/δ T cells in the PBMC of these young cattle. When sampled four times over a 2-month period, the percentages of γ/δ T cells, expressed as percentages of the total T-cell population, averaged 18.3% ± 2.9% for animal G1 and 30.0% ± 3.6% for animal G8. Because the γ/δ T cells were possibly interfering with the ability to clone antigen-responsive $CD4^+$ T cells from the lines, the γ/δ T cells were lysed with specific antibody and complement. This treatment was generally effective at removing the majority of γ/δ T cells from T-cell lines and PBMC. The γ/δ T cell-depleted cell lines exhibited strong proliferative responses against antigen, as shown for cell lines G1.6Rx and G8.6Rx (Tables 1 and 2). The panel of T-cell clones described here was derived from T-cell lines depleted of γ/δ T cells.

Antigen-specific responses of *F. hepatica*-specific T-cell clones. To obtain T-cell clones useful for future identification of stimulatory parasite antigens and to characterize cytokines produced by *F. hepatica*-specific T cells, several *F. hepatica*-specific cell lines were cloned by limiting dilution and screened for antigen-specific proliferative responses. Although the clonal nature of the cells cannot be conclusively confirmed, the majority of Th-cell clones were obtained from wells with cloning frequencies of ≤25%, which is consistent with the distribution of a statistical average of ≤1 cell per well. Furthermore, the Th-cell clones have retained their antigen responsiveness over a period of several months in culture. Twenty-two T-cell clones that responded specifically and in a dose-dependent manner to *F. hepatica* adult worm antigen were obtained. All clones proliferated in response to 10 and 50 μg of antigen per ml of medium, with optimal responses achieved with the highest antigen concentration (17 clones are represented in Table 3). One clone, G1.2H4, responded to antigen only in the presence of TCGF, although the majority of clones were capable of proliferating in response to antigen in the absence of exogenous growth factor. None of the clones responded to *B. bovis* merozoite antigen, and none responded to a recombinant 66-kDa fibrous tegument protein of *F. hepatica* produced as a fusion polypeptide with *Schistosoma japonicum* glutathione *S*-transferase (data not presented). All of the clones expressed the surface phenotype characteristic of Th cells, i.e., $CD2^+$ $CD4^+$ $CD8^-$ γ/δ $TcR1^-$ (data not presented; see Fig. 3).

To rule out the possibility that the proliferative responses of the Th-cell clones were due to nonspecific mitogens or superantigens present in the crude worm extract, proliferation assays were performed with APC obtained from either autologous or allogeneic donor cattle. APC from animal C97 were previously shown to be unable to present antigen to T-cell lines from animals G1 and G8. The proliferative response to *F. hepatica* antigen was nearly or completely abrogated in the presence of allogeneic APC, whereas the proliferative response to TCGF was either enhanced or only marginally inhibited when allogeneic APC were present in the cultures (data not presented). Thus, the $CD4^+$ T-cell clones appear to be major histocompatibility complex restricted.

Analysis of cytokines produced by *F. hepatica*-specific Th-cell clones. Previous studies have indicated that maximal IL-2, IL-4, IFN-γ and TNF activities were detected in supernatants of T cells stimulated with ConA for 18 to 22 h compared with

TABLE 4. Cytokine production by *F. hepatica*-specific CD4+ T cell clones

T-cell clone	Cytokine activity (U/ml)[a]		
	IL-2/IL-4	IFN-γ	TNF
G8.2G10	1	128	<6
G8.3B7	6	128	12
G1.3G10	1	8	<6
G1.3G4	1	64	6
G1.3E11	4	128	2
G1.3B11	2	128	<6
G1.1H5	1	64	6
G1.1H12	6	128	12
G1.2H4	1	<4	6
G8.2C12	6	<2	<2
G8.2C1	26	32	12
G8.2C3	26	128	6
G8.2B1A	26	8	12
G8.2D9	3	192	<2
G1.3G5	0	128	2
G1.3F7	0	24	<2
G1.2B6	1	12	<2

[a] Cytokine activity was measured in the supernatants of Th-cell clones cultured for 17 to 18 h with 5 μg of ConA per ml.

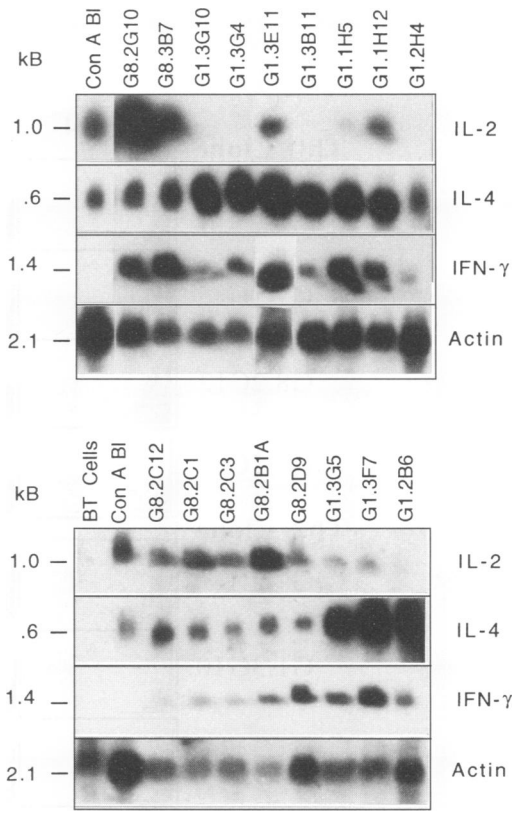

FIG. 2. Northern blot analysis of RNA from CD4+ *F. hepatica*-specific T-cell clones from infected animals G1 and G8. As a positive control, RNA was prepared from ConA-stimulated PBMC (ConA B1), and as a negative control, RNA was prepared from BT cells. A 20-μg amount (or 15 μg for clones G8.2C12 and G8.2C1) of total cellular RNA was electrophoresed on agarose gels, transferred to nylon membranes, and probed with the indicated cytokine probes, including actin as a control for semiquantification of RNA. Filters were exposed for 4 days (IL-2 and IL-4) or 2 h (IFN-γ and actin). A positive IFN-γ signal was observed with ConA B1 RNA when filters were exposed for 9 h (data not presented). The approximate sizes (in kilobases) of the indicated cytokine transcripts are indicated on the left of each panel.

8 h (7). An additional comparison revealed higher levels of all three cytokines in supernatants of T cells stimulated with mitogen compared with specific antigen (*B. bovis*) and APC (7). Analysis of cytokines secreted by *F. hepatica*-specific clone G1.3E11 similarly revealed that higher supernatant levels of IFN-γ and IL-2 or IL-4 activities were detected when ConA was used as a stimulus. T cells stimulated for 18 h with 50 μg of *F. hepatica* adult worm antigen per ml of medium and APC secreted 32 U of IFN-γ, 2 U of IL-2 or IL-4, and 2 U of TNF per ml, whereas cells stimulated with ConA secreted 128 U of IFN-γ, 4 U of IL-2 or IL-4, and 2 U of TNF per ml. Control supernatants of APC cultured with antigen and medium supplemented with ConA did not contain detectable levels of any cytokine. Because several studies have reported that the same cytokines are induced in T cells stimulated with either ConA or antigen and APC (21, 29, 35, 44), and since ConA induced quantitatively higher levels of cytokines compared with antigen and APC in bovine T-cell clones, this mitogen was used to induce cytokines in the panel of *F. hepatica*-specific T-cell clones described in the present study (Table 4). In general, the levels of IL-2 or IL-4 activity in the supernatants were low (≤6 U/ml), with the exception of clones G8.2C1, G8.2C3, and G8.2B1A, which produced 26 U of IL-2 or IL-4 per ml. IFN-γ titers ranged from <2 to 192 U/ml. TNF titers in the supernatants ranged from undetectable to 12 U/ml.

Because neutralizing antibodies directed against bovine IL-2 and IL-4 are not available for use in distinguishing IL-2 and IL-4 activities detected by our bioassay, Northern blotting was performed to identify cytokine mRNAs expressed by the panel of *F. hepatica*-specific T-cell clones. In addition to hybridizing mRNA with cDNA probes for bovine IL-2, IL-4, and IFN-γ, a bovine actin cDNA probe was used to verify the relative quantities of RNAs on the filters. RNA extracted from PBMC stimulated for 18 h with ConA was used as a positive control, and RNA extracted from unstimulated BT cells was used as a negative control for cytokine mRNA expression in the hybridization studies. We had previously determined that expression of IL-2, IL-4, and IFN-γ mRNA in activated Th-cell clones was much stronger at 8 h than at 18 to 24 h poststimulation (7, 9), and similar kinetics of cytokine mRNA expression were observed with *F. hepatica*-specific Th-cell clones G1.1H5 and G1.1H12 (data not presented). For this reason, total cellular RNA was harvested from T-cell clones 8 h following ConA stimulation. A heterogeneous pattern of cytokine expression by the panel of 17 *F. hepatica*-specific Th-cell clones was observed, with the majority of clones expressing an unrestricted (Th0) or intermediate cytokine profile (Fig. 2). Six clones expressed all three cytokine mRNAs (G8.2G10, G8.3B7, G1.3E11, G1.1H12, G8.2B1A, and G8.2D9), three clones expressed IL-2 and IL-4 mRNAs with barely detectable IFN-γ mRNA (G8.2C12, G8.2C1, and G8.2C3), and two clones expressed IL-4 and IFN-γ mRNAs with barely detectable IL-2 mRNA (G1.1H5 and G1.3F7). The remaining six clones exhibited relatively strong expression of IL-4 mRNA with little or no IL-2 or IFN-γ mRNA (G1.3G10, G1.3G4, G1.3B11, G1.2H4, G1.3G5, and G1.2B6), a pattern characteristic of the Th2 cytokine profile described for murine (35) and human (43) T cells. mRNAs obtained from clones G1.1H5 and G1.3G10 following stimulation with *F. hepatica* antigen and irradiated PBMC as a source of APC exhibited cytokine profiles identical to those observed following ConA stimulation (data not presented).

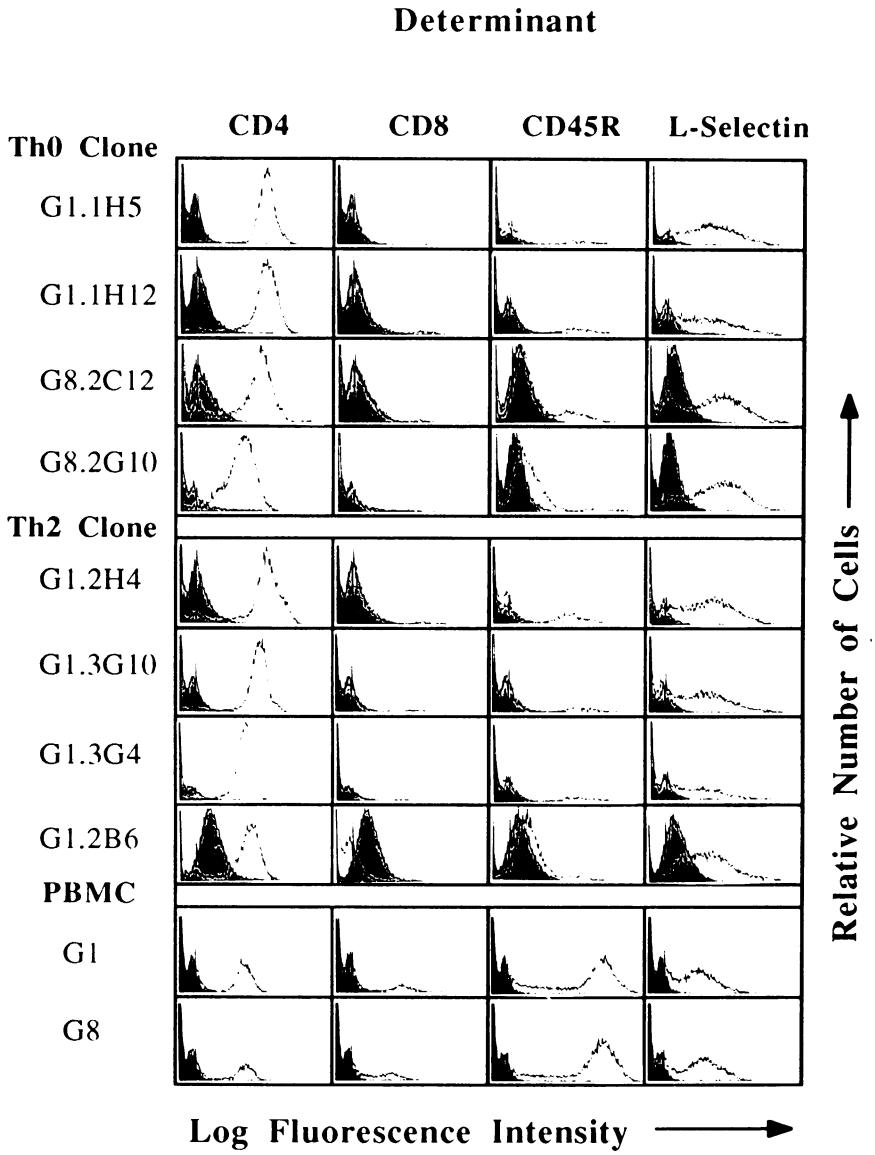

FIG. 3. Comparison of the expression of CD4, CD8, CD45R, and L selectin on *F. hepatica*-specific Th-cell clones and autologous PBMC. T-cell clones or PBMC, indicated on the left, were stained with MAb directed at the cell surface determinants indicated at the top of the figure. Data are presented for 10,000 cells stained with MAb and fluorescein isothiocyanate goat anti-mouse immunoglobulin. Histograms show the log fluorescence intensity (abscissa) and the relative number of positive cells (ordinate). Negative profiles, for which only the secondary antibody was used, are indicated by the shaded areas of the curve.

Surface expression of CD45 isoforms and L selectin. To determine whether functional differences in the Th-cell clones, characterized by the differential expression of cytokines, correlated with differences in surface phenotype, the Th-cell clones were examined for expression of high- and low-molecular-weight isoforms of the common leukocyte surface antigen CD45 and the peripheral lymph node homing receptor L selectin. Figure 3 compares the surface expression of CD4, CD8, CD45R, and L selectin on PBMC and on Th-cell clones selected for differences in cytokine mRNA expression. All Th-cell clones derived from both cattle expressed low levels of the high-molecular-weight isoform of CD45 (CD45R), which is expressed on naive T cells in cattle (26). In contrast, 71 and 80% of PBMC from animals G1 and G8, respectively, expressed the CD45R isoform. In addition, all Th-cell clones of animal G8 expressed high levels of the low-molecular-weight isoform of CD45, designated CD45RO, which is a polymorphic determinant present on freshly isolated memory T cells (32, 33). Thirty-six percent of PBMC from animal G8 expressed this determinant, whereas leukocytes from animal G1 did not express the CD45RO determinant identified by MAb ILA-150. L selectin was expressed on PBMC and on all T-cell clones examined.

DISCUSSION

The basis for acquired resistance to the cattle liver fluke *F. hepatica* has not been defined, but it is likely that both humoral and cell-mediated responses are important for acquired immunity. Through the elaboration of cytokines, T cells play a

central role in immunity to the related parasite *S. mansoni*, acting as either helper cells for antibody production or as inflammatory cells that participate in the events leading to activation of larvicidal macrophages (11, 28, 48). Furthermore, through the production of important immunoregulatory cytokines, including IL-10, T cells may modulate a protective host response. Therefore, as a first step in unraveling the complex cellular interactions leading to resistance in bovine fascioliasis, we have performed studies designed to characterize the T-cell response against *F. hepatica*. Cattle G1 and G8 used for experimental infections were genetically identical to animals G3 and G6, respectively, which were infected with *B. bovis* and used as a source of *B. bovis*-specific Th-cell clones described in a related study (7).

Between 5 and 7 months following oral infection with *F. hepatica* metacercariae, PBMC from cattle G1 and G8 were tested for parasite-specific proliferation. As reported by others (37), *F. hepatica*-specific responses were not detected during this time (data not presented). However, chronic or recent infection was indicated by the presence of *F. hepatica*-specific antibodies in serum sampled from the cattle from 3 to 10 months following infection, which, in the case of animal G1, detected a previously unrecognized band on a Western blot at 10 months postinfection.

To overcome the problems associated with in vitro microproliferation assays performed with polyclonal PBMC (49), short-term T-cell lines were established and cloned by limiting the dilution. Unlike PBMC, T-cell lines did proliferate specifically to antigen as early as 1 week after their initiation and exhibited strong antigen-specific responses for an additional 2 to 3 weeks of culture. However, many of the cell lines could not be maintained in culture for more than 3 or 4 weeks, and two cell lines maintained for 7 weeks displayed reduced levels of proliferation. Similar observations were made with *B. bovis*-specific T-cell lines (2, 5), in which antigen-specific proliferation ceased by 11 to 15 weeks of culture. In the studies performed with *B. bovis*, decreased antigen responsiveness correlated with the disappearance of $CD4^+$ T cells and the predominance of γ/δ T cells in the cell lines (2). γ/δ T cells were also identified in the cell lines described in the present study that were stimulated with *F. hepatica* adult worm antigen, reaching levels of approximately 50% by 6 weeks of culture, coincident with a decline in *F. hepatica*-specific proliferation. Depletion of the majority of γ/δ T cells by antibody- and complement-mediated cell lysis did not prevent the ability of the surviving cells to respond to antigen and in fact often resulted in enhanced proliferative responses. Furthermore, when two cell lines that were not depleted of γ/δ T cells were cloned after 3 weeks, only γ/δ T-cell clones were obtained, and these clones did not proliferate in response to *F. hepatica*. In contrast, when T-cell lines depleted of the majority of γ/δ TcR^+ T cells were cloned, 22 *F. hepatica*-specific $CD4^+$ T-cell clones were obtained, whereas no $CD8^+$ T-cell clones and only one γ/δ T-cell clone were obtained (data not presented). T-cell lines containing fewer than 10% $CD8^+$ T cells were also capable of vigorous antigen-specific proliferation. Collectively, these observations indicate that adult worm extract of *F. hepatica* preferentially stimulates the proliferation of $CD4^+$ T cells in vitro and, although not formally proven, suggest that the presence of γ/δ T cells may impede the ability of $CD4^+$ T cells to proliferate to *F. hepatica*, as shown for *B. bovis* (2).

A spectrum of cytokine mRNA profiles was expressed by the *F. hepatica*-specific Th-cell clones, ranging from a Th2 profile, consisting of strong IL-4 with weak or undetectable IL-2 and IFN-γ mRNA expression, to an unrestricted pattern of all three cytokine mRNAs. Intermediate patterns of cytokines were also observed, including combinations of IL-2 and IL-4 but no IFN-γ, and IL-4 and IFN-γ but no IL-2. The $CD8^+$ IL-2-dependent cell line C99.G1.G3 expresses IL-4 as well as IL-2 mRNA upon activation; however, we cannot presently determine whether the cell line actually responds to IL-4. For this reason, it is difficult to correlate IL-2 or IL-4 secretion with mRNA expression. However, *F. hepatica*-specific Th-cell clones that expressed high levels of IL-4 mRNA and reciprocally low levels of IL-2 mRNA (i.e., clones G1.3G10, G1.3G4, G1.3B11, G1.1H5, G1.2H4, G1.3G5, G1.3F7, and G1.2B6) produced only 0 to 2 U of IL-2 or IL-4 activity per ml, whereas clones with moderate IL-2 and low IL-4 mRNA expression (i.e., clones G8.2C1, G8.2C3, and G8.2D9) produced 26 U of IL-2 or IL-4 activity per ml upon ConA stimulation. These results indicate that cell line C99.G1.G3 responds poorly, if at all, to IL-4. IFN-γ titers in the culture supernatants correlated, in most cases, with the level of IFN-γ mRNA expressed by these clones. However, higher titers of IFN-γ were detected in the supernatants of clones G1.3B11 and G1.3G5 than would be anticipated on the basis of mRNA expression. The reason for this discrepancy is unclear; however, in studies performed with murine Th-cell clones, a lack of positive correlation between IL-4 and IFN-γ activities and mRNA expression was often observed as well (31).

Previous studies in our laboratory also revealed a heterogeneity in cytokine mRNA expression by a panel of Th-cell clones specific for *B. bovis*, whereby the majority of Th-cell clones expressed various combinations of cytokines that did not fit into classical Th1 or Th2 profiles (7). In contrast to the results presented here, however, no Th2-cell clones specific for *B. bovis* were detected, whereas several Th1-cell clones were identified (7, 9). The use of ConA as a stimulus for cytokine induction does not explain the relative abundance of clones with unrestricted cytokine profiles, since this mitogen activates T cells through triggering of the TcR-CD4 complex in the same way that antigen does and results in qualitatively similar cytokine expression (21, 29, 35, 44). *F. hepatica*-specific Th0- and Th2-like cell clones also had similar cytokine profiles following stimulation with either ConA or antigen and APC. The predominance of T-cell clones expressing neither Th1 nor Th2 cytokine profiles may reflect the fact that the cells were analyzed after being in culture for relatively short periods of time (i.e., several weeks). Similar unrestricted cytokine patterns were observed with short-term Th-cell clones from humans and mice (21, 31, 34, 39, 45, 50, 52), whereas the original description of restricted Th1 and Th2 cytokine profiles was based on analysis of a panel of murine T-cell clones in culture for months or years (35, 36). In vitro selection of either Th1- or Th2-like cells from cells expressing unrestricted cytokines was reported to occur after several months of culture (36).

An alternative explanation for the relative abundance of clones with unrestricted cytokine profiles may be the nature of antigen used for in vitro stimulation. In experiments performed with both *B. bovis* and *F. hepatica*, we employed unfractionated parasite extracts that could potentially stimulate numerous clones of T cells with specificity for distinct antigens, leading to simultaneous production of IL-4 and IFN-γ in the cultures. The presence of immunoregulatory cytokines IL-4 and IFN-γ in bulk cultures has been shown to influence the profile of cytokines subsequently expressed by T cells cloned from the population (34, 51), resulting in Th-cell clones that do not fit into reciprocal Th1- and Th2-cell subsets. In support of this possibility, very high levels of IL-2, IL-4, and IFN-γ mRNA were detected in polyclonal T cells, obtained from PBMC of infected cattle G1 and G8, that were stimulated

for 6 days with *F. hepatica* and then 8 h with ConA (data not presented).

A comparison of IFN-γ titers in the supernatants of Th cell clones specific for *B. bovis* (7, 8) with those of Th-cell clones specific for *F. hepatica* revealed that the latter set of clones secreted relatively low levels of IFN-γ. Only 1 (6%) of the 17 *F. hepatica*-specific clones produced more than 128 U of IFN-γ per ml, whereas 12 (57%) of the 21 *B. bovis*-specific Th-cell clones, which were generated and tested under the same culture conditions, secreted between 200 and 800 U of IFN-γ per ml. In fact, 6 of 8 Th-cell clones that secreted >200 U of IFN-γ per ml were derived from cattle G3 and G6 (7), the genetically identical siblings of cattle G1 and G8. These findings indicate that differences in the levels of IFN-γ produced by the Th-cell clones generated from cattle infected with the different parasites cannot be solely explained by host genetic differences and may be related to the parasite infection itself.

As anticipated, all of the Th-cell clones expressed a memory T-cell surface phenotype ($CD45R^{low}$ and, on G8 clones, $CD45RO^{high}$), and there were no apparent differences in expression of CD45R on T-cell clones with unrestricted or Th2-like cytokine profiles. The Th-cell clones also expressed L selectin. We similarly showed no differences in expression of either CD45R or L selectin on Th0- or Th1-cell clones specific for *B. bovis* (7). Thus, Th cells displaying different cytokine profiles could not be distinguished by the differential expression of either CD45 isoforms detected by available MAb or the lymph node homing receptor L selectin. The relationship between a Th-cell subtype, defined by cytokine production in vitro, and the state of cell differentiation leading from naive T cells to memory T cells in vivo is not clearly defined (36, 50, 51). Furthermore, the events that drive a T cell down a Th1 or Th2 cell pathway are not completely understood. However, recent evidence supports the theory that naive $CD4^+$ T cells specific for a given antigenic epitope are not precommitted to differentiate into either Th1 or Th2 cells, but that differentiation into one subtype or the other is influenced by the environment (APC), where antigen is encountered in vivo (27, 42).

In summary, T-cell clones derived from cattle infected with *F. hepatica* expressed Th2 as well as less-restricted Th cytokine profiles. These data show that helminth antigens can induce Th2-like T cells in cattle, as described previously for mice (20, 50) and humans (12, 17), but also extend our earlier observation that the majority of parasite-specific Th-cell clones do not fit into reciprocal Th1 and Th2 cell subtypes (7). Nevertheless, the differential expression of IFN-γ by Th-cell clones specific for *F. hepatica* or *B. bovis* is consistent with a differential induction of parasite-specific Th cells in response to infection with helminth or protozoan parasites. It may be that the relative amounts of the different cytokines produced is more important in determining the function of a given Th cell than the absolute presence or absence of a given cytokine (16, 53).

The role of the functionally different subsets of Th cells in resistance to *F. hepatica* remains to be discovered. Preliminary results suggest that IL-10 is expressed by several *F. hepatica*-specific Th clones (4), and experiments are planned to determine the effects of IL-10 on antigen-driven T-cell proliferation. Studies are also planned to compare Th-cell clones that express different patterns of cytokines for the capacity to act as helper cells for *F. hepatica*-specific B cells. Specifically, induction of different immunoglobulin subclasses (IgG1, IgG2, IgA, and IgE) will be examined. These and additional T-cell clones can also be used to identify adult worm proteins that elicit T-cell responses in cattle resistant to challenge infection, as a means of targeting potential vaccine antigens for this parasite.

ACKNOWLEDGMENTS

We would like to thank Barbara Doughty for infecting the two cattle used in this study with *F. hepatica* metacercariae; Chris Howard and Niall MacHugh (ILRAD) for providing MAbs; Roger Smith and Betty Rosenbaum for performing flow cytometry; and Kathleen Logan, Vivienne Woods, and Emily Francis for expert technical assistance.

This research was supported by U.S. Department of Agriculture CRGO grant 90-37266-5623 (A.C.R.-F.) and NRICGP grant 91-37206-6821 (W.C.B.).

REFERENCES

1. **Armour, J., and J. D. Dargie.** 1974. Immunity to *Fasciola hepatica* in the rat. Successful transfer of immunity by lymphoid cells and by serum. Exp. Parasitol. **35:**381–389.
2. **Brown, W. C., W. C. Davis, S. H. Choi, D. A. E. Dobbelaere, and G. A. Splitter.** Functional and phenotypic characterization of $WC1^+$ γ/δ T cells isolated from *Babesia bovis*-stimulated T cell lines. Cell. Immunol., in press.
3. **Brown, W. C., and D. J. Grab.** 1985. Biological and biochemical characterization of bovine interleukin-2. Studies with cloned bovine T cells. J. Immunol. **133:**3184–3190.
4. **Brown, W. C., S. M. Hash, and A. C. Rice-Ficht.** 1993. Expression of bovine interleukin-10 by Th0, Th1 and Th2 T cell clones, abstr. 104. Conference of Research Workers in Animal Diseases, Chicago, Ill.
5. **Brown, W. C., and K. S. Logan.** 1992. *Babesia bovis*: bovine helper T cell lines reactive with soluble and membrane antigens of merozoites. Exp. Parasitol. **74:**188–199.
6. **Brown, W. C., K. S. Logan, G. G. Wagner, and C. L. Tetzlaff.** 1991. Cell-mediated immune responses to *Babesia bovis* merozoite antigens in cattle following infection with tick-derived or cultured parasites. Infect. Immun. **59:**2418–2426.
7. **Brown, W. C., V. M. Woods, D. A. E. Dobbelaere, and K. S. Logan.** 1993. Heterogeneity in cytokine profiles of *Babesia bovis*-specific bovine $CD4^+$ T cell clones activated in vitro. Infect. Immun. **61:**3273–3281.
8. **Brown, W. C., S. Zhao, A. C. Rice-Ficht, K. S. Logan, and V. M. Woods.** 1992. Bovine helper T cell clones recognize five distinct epitopes on *Babesia bovis* merozoite antigens. Infect. Immun. **60:**4364–4372.
9. **Brown, W. C., S. Zhao, V. M. Woods, C. A. Tripp, C. L. Tetzlaff, V. T. Heussler, D. A. E. Dobbelaere, and A. C. Rice-Ficht.** 1993. Identification of two Th1 cell epitopes on the 77 kDa *Babesia bovis* merozoite protein (Bb-1) by use of truncated recombinant fusion proteins. Infect. Immun. **61:**236–244.
10. **Callada-Benedetti, Z., F. Al-Zamel, A. Sher, and S. James.** 1991. Comparison of Th1- and Th2-associated immune reactivities stimulated by single versus multiple vaccination of mice with irradiated *Schistosoma mansoni* cercariae. J. Immunol. **146:**1655–1660.
11. **Capron, A., J. P. Dessaint, M. Capron, J. H. Ouma, and A. E. Butterworth.** 1987. Immunity to schistosomes: progress toward vaccine. Science **238:**1065–1072.
12. **Colina, K. F., F. B. Perler, I. Matsumura, M. Meda, and T. B. Nutman.** 1990. The identification of an *Onchocerca*-specific recombinant antigen containing a T cell epitope. J. Immunol. **145:**1551–1556.
13. **Corba, J., J. Armour, R. J. Roberts, and G. M. Urquhart.** 1971. Transfer of immunity to *Fasciola hepatica* infection by lymphoid cells. Res. Vet. Sci. **12:**292–295.
14. **Davis, W. C., M. J. Hamilton, Y. H. Park, R. A. Larsen, and C. R. Wyatt.** 1990. Ruminant leukocyte differentiation molecules, p. 47–70. *In* O. Barta (ed.), MHC, differentiation antigens, and cytokines in animals and birds. Monographs in animal immunology. BAR-LAB, Inc., Blacksburg, Va.
15. **Degen, J. L., M. G. Neubauer, S. J. Friezner Degen, C. E. Seyfried, and D. R. Morris.** 1983. Regulation of protein synthesis in mitogen-activated bovine lymphocytes. Analysis of actin-specific and total mRNA accumulation and utilization. J. Biol. Chem. **258:**12153–12162.

16. **Del Prete, G., E. Maggi, P. Parronchi, I. Chretien, A. Tiri, D. Macchia, M. Ricci, J. Banchereau, J. de Vries, and S. Romagnani.** 1988. IL-4 is an essential factor for the IgE synthesis induced in vitro by human T cell clones and their supernatants. J. Immunol. **140:**4193–4198.
17. **Del Prete, G. F., M. de Carli, C. Mastromauro, R. Biagiotti, D. Macchia, P. Falagiani, M. Ricci, and S. Romagagni.** 1991. Purified protein derivative of *Mycobacterium tuberculosis* and excretory-secretory antigen(s) of *Toxocara canis* expand in vitro human T cells with stable and opposite (type 1 helper or type 2 helper) profile of cytokine production. J. Clin. Invest. **88:**346–350.
18. **Doyle, J. J.** 1972. Evidence of an acquired resistance in calves to a single experimental infection with *Fasciola hepatica*. Res. Vet. Sci. **12:**527–534.
19. **Doyle, J. J.** 1973. Skin hypersensitivity reaction induced in calves by experimental infections with *Fasciola hepatica*. Int. Arch. Allergy Appl. Immunol. **45:**752–758.
20. **Finkleman, F. D., E. J. Pearce, J. F. Urban, Jr., and A. Sher.** 1991. Regulation and biological function of helminth-induced cytokine responses, p. A62–A66. *In* C. Ash and R. B. Gallagher (ed.), Immunoparasitology today. Elsevier Trends Journals, Cambridge.
21. **Firestein, G. S., W. D. Roeder, J. A. Laxer, K. S. Townsend, C. T. Weaver, J. T. Hom, J. Linton, B. E. Torbett, and A. L. Glasebrook.** 1989. A new murine $CD4^+$ T cell subset with an unrestricted cytokine profile. J. Immunol. **143:**518–525.
22. **Haroun, E. T. M., and G. V. Hillyer.** 1986. Resistance to fascioliasis—a review. Vet. Parasitol. **20:**63–93.
23. **Heussler, V. T., M. Eichorn, and D. A. E. Dobbelaere.** 1992. Cloning of a full-length cDNA encoding bovine interleukin 4 by the polymerase chain reaction. Gene **114:**273–278.
24. **Hillyer, G. V., E. M. Haroun, and M. Soler de Galanes.** 1987. Acquired resistance to *Fasciola hepatica* in cattle using a purified adult worm antigen. Am. J. Trop. Med. Hyg. **37:**363–369.
25. **Howard, C. J., P. Sopp, and K. R. Parsons.** 1992. L-selectin expression differentiates T cells isolated from different lymphoid tissues in cattle but does not correlate with memory. Immunology **77:**228–234.
26. **Howard, C. J., P. Sopp, K. R. Parsons, D. J. McKeever, E. L. N. Taracha, B. V. Jones, N. D. MacHugh, and W. I. Morrison.** 1991. Distinction of naive and memory BoCD4 lymphocytes in calves with a monoclonal antibody, CC76, to a restricted determinant of the bovine leukocyte-common antigen, CD45. Eur. J. Immunol. **21:**2219–2226.
27. **Hsieh, C.-S., S. E. Macatonia, C. S. Tripp, S. F. Wolf, A. O'Garra, and K. M. Murphy.** 1993. Development of T_H1 $CD4^+$ T cells through IL-12 produced by *Listeria*-induced macrophages. Science **260:**547–549.
28. **James, S. L., and A. Sher.** 1990. Cell-mediated immune response to schistosomiasis. Curr. Top. Microbiol. Immunol. **155:**21–31.
29. **Ju, S.-T., N. H. Ruddle, P. Strack, M. E. Dorf, and R. H. DeKruyff.** 1990. Expression of two distinct cytolytic mechanisms among murine CD4 subsets. J. Immunol. **144:**23–31.
30. **Keegan, P. S., and A. Trudgett.** 1992. *Fasciola hepatica* in the rat: immune responses associated with the development of resistance to infection. Parasite Immunol. **14:**657–669.
31. **Kelso, A., A. B. Troutt, E. Maraskovsky, N. M. Gough, L. Morris, M. H. Pech, and J. A. Thomson.** 1991. Heterogeneity in lymphokine profiles of $CD4^+$ and $CD8^+$ T cells and clones activated in vivo and in vitro. Immunol. Rev. **123:**85–114.
32. **MacHugh, N. D. (International Laboratory for Research on Animal Diseases).** 1993. Personal communication.
33. **MacHugh, N. D., E. O. Awino, and D. J. McKeever.** 1992. A monoclonal antibody specific for CD45RO differentiates memory from naive CD4 positive bovine T-cells, abstr. PS 4.8. 3rd Int. Vet. Immunol. Symp., August 1992. Budapest.
34. **Maggi, E., P. Parronchi, R. Manetti, C. Simonelli, M.-P. Piccinni, F. S. Rugiu, M. de Carli, M. Ricci, and S. Romagnani.** 1992. Reciprocal regulatory effects of IFN-γ and IL-4 on the in vitro development of human Th1 and Th2 clones. J. Immunol. **148:** 2142–2147.
35. **Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Gieldin, and R. L. Coffman.** 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J. Immunol. **136:**2348–2357.
36. **Mosmann, T. R., and R. L. Coffman.** 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu. Rev. Immunol. **7:**145–173.
37. **Oldham, G., and L. Williams.** 1985. Cell mediated immunity to liver fluke antigens during experimental *Fasciola hepatica* infection of cattle. Parasite Immunol. **7:**503–516.
38. **Overton, W. R.** 1988. Modified histogram subtraction technique for analysis of flow cytometry data. Cytometry **9:**619–626.
39. **Paliard, X., R. de Waal Malefijt, H. Yssel, D. Blanchard, I. Chretien, J. Abrams, J. de Vries, and H. Spits.** 1988. Simultaneous production of IL-2, IL-4, and IFN-γ by activated human $CD4^+$ and $CD8^+$ T cell clones. J. Immunol. **141:**849–855.
40. **Rajasekariah, G. R., and M. J. Howell.** 1979. *Fasciola hepatica* in rats: transfer of immunity by serum and cells from infected to *F. hepatica* naive animals. J. Parasitol. **65:**481–487.
41. **Reeves, R., A. G. Spies, M. S. Nissen, C. D. Buck, A. D. Weinberg, P. J. Barr, N. S. Magnuson, and J. A. Magnuson.** 1986. Molecular cloning of a functional bovine interleukin 2 cDNA. Proc. Natl. Acad. Sci. USA **83:**3228–3232.
42. **Reiner, S. L., Z.-E. Wang, F. Hatam, P. Scott, and R. M. Locksley.** 1993. T_H1 and T_H2 cell antigen receptors in experimental leishmaniasis. Science **259:**1457–1460.
43. **Romagnani, S.** 1991. Human Th1 and Th2 subsets: doubt no more. Immunol. Today **12:**256–257.
44. **Ruddle, N. H., C. M. Bergman, K. M. McGrath, E. G. Lingenheld, M. L. Grunnet, S. J. Padula, and R. B. Clark.** 1990. An antibody to lymphotoxin and tumor necrosis factor prevents transfer of experimental allergic encephalomyelitis. J. Exp. Med. **172:**1193–1200.
45. **Salmon, M., G. D. Kitas, and P. A. Bacon.** 1989. Production of lymphokine mRNA by $CD45R^+$ and $CD45R^-$ helper T cells from human peripheral blood and by human $CD4^+$ T cell clones. J. Immunol. **143:**907–912.
46. **Sexton, J. L., A. R. Milner, M. Panaccio, J. Waddington, G. Wijffels, D. Chandler, C. Thompson, L. Wilson, T. W. Spithill, G. F. Mitchell, and N. J. Campbell.** 1990. Glutathione S-transferase. Novel vaccine against *Fasciola hepatica* infection in sheep. J. Immunol. **145:**3905–3910.
47. **Sher, A., D. Fiorentino, P. Caspar, E. Pearce, and T. Mosmann.** 1991. Production of IL-10 by $CD4^+$ T lymphocytes correlates with down-regulation of Th1 cytokine synthesis in helminth infections. J. Immunol. **147:**2713–2716.
48. **Sher, A., R. T. Gazzinelli, I. P. Oswale, M. Clerici, M. Kullberg, E. J. Pearce, J. A. Berzofsky, T. R. Mosmann, S. L. James, H. C. Morse III, and G. M. Shearer.** 1992. Role of T-cell derived cytokines in the downregulation of immune responses in parasitic and retroviral infection. Immunol. Rev. **127:**183–204.
49. **Stadecker, M. J.** 1992. The role of T-cell anergy in the immunomodulation of schistosomiasis. Parasitol. Today **8:**199–204.
50. **Street, N. E., J. H. Schumacher, A. T. Fong, H. Bass, D. F. Fiorentino, J. A. Leverah, and T. R. Mosmann.** 1990. Heterogeneity of mouse helper T cells. Evidence from bulk cultures and limiting dilution cloning for precursors of Th1 and Th2 cells. J. Immunol. **144:**1629–1639.
51. **Swain, S. L., L. M. Bradley, M. Croft, S. Tonkonogy, G. Atkins, A. D. Weinberg, D. D. Duncan, S. M. Hedrick, R. W. Dutton, and G. Huston.** 1991. Helper T-cell subsets: phenotype, function and the role of lymphokines in regulating their development. Immunol. Rev. **123:**115–144.
52. **Umetsu, D. T., H. H. Jabara, R. H. DeKruyff, A. K. Abbas, J. S. Abrams, and R. S. Geha.** 1988. Functional heterogeneity among human inducer T cell clones. J. Immunol. **140:**4211–4216.
53. **Yssel, H., K. E. Johnson, P. V. Schneider, J. Wideman, A. Terr, R. Kastelein, and J. de Vries.** 1992. T cell activation-inducing epitopes of the house dust mite allergen *Der p* I. Proliferation and lymphokine production patterns by *Der p* I-Specific $CD4^+$ T cell clones. J. Immunol. **148:**738–745.