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Interleukin-10 Is Expressed by Bovine Type 1 Helper, Type 2 Helper, and Unrestricted Parasite-Specific T-Cell Clones and Inhibits Proliferation of All Three Subsets in an Accessory-Cell-Dependent Manner

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Murine interleukin-10 (IL-10) is produced by type 2 helper (Th2) cells and selectively inhibits cytokine synthesis by type 1 helper (Th1) cells, whereas human IL-10 is produced by and inhibits proliferation and cytokine synthesis by both Th1 and Th2 subsets. This study reports that bovine IL-10 mRNA is expressed by Th0, Th1, and Th2 clones of bovine T cells specific for either *Babesia bovis* or *Fasciola hepatica* but not by two CD8$^+$ T-cell clones. The antigen-induced proliferative responses of all three subsets of CD4$^+$ cells were inhibited by human IL-10, and low levels (10 U/ml) of exogenous human IL-2 restored the suppressed response. However, proliferation of one Th1 clone was never inhibited but was enhanced by IL-10. Human IL-10 also inhibited the expression of gamma interferon and IL-4 mRNA in Th0 clones. In the absence of accessory cells (AC), the responses of Th clones to concanavalin A or IL-2 were not inhibited by IL-10, whereas antigen-specific responses of Th1 and Th2 cells were reduced when IL-10-pretreated macrophages were used as AC. Together, our results with bovine T cells support the concept that IL-10 primarily affects AC function and does not directly inhibit CD4$^+$ T cells and demonstrate that the immunoregulatory effects of IL-10 are not selectively directed at Th1 populations, as they are in mice.

Interleukin-10 (IL-10) is a multifunctional cytokine, first described as cytokine synthesis inhibitory factor (17), that is produced primarily by CD4$^+$ T cells, monocytes/macrophages (Mϕ), and B cells (reviewed in references 21, 26, 30, and 36). In addition, IL-10 has a number of immunostimulatory properties in vitro, including the enhancement of growth and differentiation of B cells (9, 29), thymocytes and peripheral T cells (23), mast cells (32), and cytotoxic T cells (8). In the original studies by Fiorentino and colleagues, IL-10 was shown to be produced in the mouse by the type 2 helper (Th2) subset of CD4$^+$ T cells and to selectively inhibit cytokine production by type 1 helper (Th1) cells (17). IL-10 also inhibited proliferation of antigen-driven Th1 clones in the presence of irradiated spleen cells or purified Mϕ (22). Studies with murine Th1 clones showed that synthesis of gamma interferon (IFN-γ) was consistently suppressed, whereas synthesis of additional cytokines, including IL-2, was inhibited in some clones but not in others (17). These results paralleled those demonstrating inconsistent or variable inhibition by IL-10 of antigen-driven Th1 cell proliferation (17, 22). IL-10 did not inhibit cytokine expression by Th2 clones, including IL-4 and IL-5, leading to the concept that IL-10 is an important immunoregulatory cytokine for delayed-type hypersensitivity reactions.

In contrast to what was found in the mouse, studies with human Th clones revealed that IL-10 was produced by Th0, Th1, and Th2 cells (11, 33, 35) and inhibited cytokine production and proliferation by all three Th subsets (11–13). In both species, reduced proliferation of resting T cells against mitogens in the presence of IL-10 correlated with reduced production of IL-2 (15, 31). In the mouse, IL-2 partially restored the response (15), whereas both IL-2 and IL-4 were effective at reversing the suppressive effects of IL-10 on human T-cell responses (31).

Additional differences in the mode of action of IL-10 in the human and mouse systems were observed in studies demonstrating the effects of IL-10 on antigen-presenting or accessory cells (AC). In both species, IL-10 downregulated cytokine synthesis by Th cells only in the presence of AC, inhibiting Mϕ, but not B cells as AC for presentation of antigen or superantigen (12, 17, 18) or as costimulatory cells for mitogen- and IL-2-driven responses (15). However, inhibition of human Mϕ function correlated with diminished major histocompatibility complex (MHC) class II expression (12), whereas murine MHC class II expression on Mϕ was not affected by IL-10 (14, 15, 18). It was subsequently found that the diminished AC function of IL-10-treated mouse Mϕ was caused by suppressed induction of the activation antigen B7 (14).

Because IL-10 is an important immunoregulatory cytokine with distinctly different effects on T cells from mice and humans, characterization of IL-10 in other species is important to fully understand the biology of this cytokine and its potential for clinical application. We recently described a cDNA encoding bovine IL-10 (19). The nucleotide sequence has 84 and 79% homology with the human and mouse cDNAs, respectively, and the predicted amino acid sequence is also highly conserved, with 77 and 71% identity, respectively. Whereas Yssel et al. reported that maximal expression of human IL-10 occurred relatively late (after 24 h) following T-cell activation (35), we observed that maximal expression of the bovine transcript occurred early (8 h) after stimulation of peripheral

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TABLE 1. Characteristics of bovine T-cell clones used in this study

Donor cattle	Clone	Phenotype	Antigen specificity	T-cell subtype[a] (cytokine mRNA[s] expressed)	Reference
Normal	99.2G7	$CD8^+$	Unknown	T0 (IL-2, IL-4, IFN-γ)	This study
	99.2H5	$CD8^+$	Unknown	T0 (IL-2, IL-4, IFN-γ)	This study
	G2.1G8	$CD4^+$	Unknown	T0 (IL-2, IL-4, IFN-γ)	This study
B. bovis immune	C97.1C8	$CD4^+$	*B. bovis* Bb-1	Th1 (IL-2, IFN-γ)	7
	G3.2F7	$CD4^+$	*B. bovis*	Th1 (IL-2, IFN-γ)	5
	C15.2G10	$CD4^+$	*B. bovis*	Th0 (IL-2, IL-4, IFN-γ)	5
	C15.1D10	$CD4^+$	*B. bovis*	Th0 (IL-2, IL-4, IFN-γ)	5
F. hepatica immune	G1.1H5	$CD4^+$	*F. hepatica*	Th0 (IL-4, IFN-γ)	2
	G1.1H12	$CD4^+$	*F. hepatica*	Th0 (IL-2, IL-4, IFN-γ)	2
	G1.3E11	$CD4^+$	*F. hepatica*	Th0 (IL-2, IL-4, IFN-γ)	2
	G1.3G10	$CD4^+$	*F. hepatica*	Th2 (IL-4)	2
	G1.3B11	$CD4^+$	*F. hepatica*	Th2 (IL-4)	2
	G1.2H4	$CD4^+$	*F. hepatica*	Th2 (IL-4)	2
	G1.3G4	$CD4^+$	*F. hepatica*	Th2 (IL-4)	2

[a] The T-cell subtype is designated as unrestricted (T0 or Th0), Th1, or Th2. The subtype was determined by the profile of cytokines expressed following activation by either ConA or antigen, as indicated in parentheses.

blood mononuclear cells (PBMC), remained elevated until 24 h, and then declined (19).

The present study was designed to further characterize IL-10 expression by and regulation of different bovine T-cell subsets and employed clones of bovine Th cells specific for either the hemoprotozoan parasite *Babesia bovis* (5, 7) or the trematode parasite *Fasciola hepatica* (2). The $CD4^+$ clones were recently characterized as either Th0, Th1, or Th2 cells by the expression patterns of IL-2, IL-4, and IFN-γ mRNA. Unlike murine Th clones, the majority of more than 25 bovine Th clones expressed unrestricted cytokine profiles, reminiscent of what has been reported for some antigen-specific human Th clones (24, 25, 27). Selected bovine T-cell clones, including $CD8^+$ T cells and $CD4^+$ T-cell clones representing different Th subsets, were characterized for the expression of IL-10 mRNA and the regulation of proliferation and cytokine expression by human IL-10. We report that, like human T cells, IL-10 was expressed by Th0, Th1, and Th2 subsets of bovine Th cells irrespective of their cytokine repertoires. Furthermore, antigen-specific proliferation of Th clones belonging to all three subsets was strongly suppressed by exogenous IL-10. Importantly, only one of two Th1 clones could be inhibited by IL-10, indicating a differential effect by IL-10 on Th1 cell proliferation. These findings support the view that the cytokine network that regulates Th1 and Th2 cells described for inbred stains of mice may not be generally applicable to all mammals (11). Although bovine T-cell responses are in many ways more like those described for human than for murine T cells, characteristics unique to the bovine cellular immune response are also apparent from these studies.

MATERIALS AND METHODS

Bovine T-cell clones. Three concanavalin A (ConA; Sigma Chemical Co., St. Louis, Mo.)-induced T-cell clones were obtained from normal donor cattle C99 and G2. PBMC were stimulated for 3 to 4 days with ConA (5 μg/ml) in complete RPMI 1640 medium (4), and lymphoblasts were cloned by distributing a statistical average of 0.3 or 1 cell per well of 96-well round-bottomed plates as described before (3). The cloning frequencies ranged from 19 to 25%, and clones were maintained in medium containing 10% bovine T-cell growth factor (TCGF) (3) in the absence of irradiated PBMC as a source of AC. Two of the clones (99.2G7 and 99.2H5) expressed the cell surface phenotype $CD4^-$ $CD8^+$, and one clone (G2.1G8) expressed the Th cell phenotype $CD4^+$ $CD8^-$. The antigen specificity of these three clones is not known.

Antigen-specific $CD4^+$ Th cell clones were obtained from PBMC of cattle immune to either *B. bovis* or *F. hepatica*. The cloning procedures and characterization of the Th clones were described in detail in previous publications (2, 5, 7). The clones were cryopreserved and, upon thawing, were stimulated on a weekly basis with irradiated autologous PBMC as a source of AC, 10% bovine TCGF, and antigen. The majority of *B. bovis*-specific Th clones were stimulated with a crude membrane preparation of *B. bovis* merozoites (4), whereas one clone (C97.1C8) was stimulated with a recombinant form of the 77-kDa apical complex-associated protein expressed as a fusion protein with glutathione-*S*-transferase, designated Bb-1-GST (7). All *F. hepatica*-specific clones were stimulated with adult worm extract (2). The Th subtypes of the antigen-specific Th clones were characterized previously as Th0, Th1, or Th2 cells by the differential expression of IL-2, IL-4, and IFN-γ mRNA. Table 1 summarizes the characteristics of the T-cell clones used in this study.

Stimulation of T cells for analysis of cytokine mRNA. Antigen-specific T-cell clones were collected 6 or 7 days after the last stimulation with antigen and AC and washed and cultured for 8 or 24 h at a concentration of 1.3×10^6 cells per ml of complete medium containing ConA (2.5 μg/ml) in the absence of AC. Non-antigen-dependent TCGF-maintained clones were collected 3 or 4 days after TCGF stimulation and stimulated with ConA for 8 or 18 h. To determine the effects of human IL-10 on the induction of cytokine mRNA expression by the Th clones, selected clones were cultured at a concentration of 1.3×10^6 cells per ml for 13 h with 2×10^6 AC and 25 μg of *F. hepatica* antigen per ml in the absence or presence of 20 ng of human recombinant IL-10 (Genzyme Corp., Cambridge, Mass.) per ml (equivalent to 10 U/ml). As a positive control for cytokine mRNA expression, PBMC were stimulated at a density of 2×10^6 cells per ml with ConA for 15 or 18 h. Negative control cultures consisted of AC plus antigen or murine WEHI-164 fibroblasts. Total cellular RNA was prepared from the cells by using 2 ml of RNAzol B (Biotecx Laboratories, Inc., Houston, Tex.) per 10^7 cells, as specified by the manufacturer.

Northern (RNA) blot analysis. Northern blotting was performed to identify the expression of IL-2, IL-4, IFN-γ, and IL-10 mRNAs as detailed previously (2, 5, 7, 19). Briefly, equal amounts of RNA (20 μg per lane unless otherwise indicated)

were size-fractionated in formaldehyde–morpholinepropanesulfonic acid (MOPS)–1.6% agarose gels. Ethidium bromide-stained gels were visualized to confirm the integrity of the RNA and to verify that equal amounts of total RNA were loaded in each lane. RNA transferred to GeneScreen nylon membranes (Dupont NEN, Boston, Mass.) was then hybridized to ^{32}P-labeled cDNA probes for IL-2 (28), provided by Ray Reeves, Washington State University; IL-4 (20), provided by Dirk Dobbelaere, University of Bern, Bern, Switzerland; IFN-γ, provided by Arjun Singh, Genentech, San Francisco, Calif.; IL-10 (19); and actin (10), provided by Angelika Ehrfeld, Max Planck Institute for Immunology, Freiburg, Germany. The cDNAs were labeled with ^{32}P by the random priming method with a kit from Boehringher Mannheim, yielding probes with a specific activity of 1×10^9 to 2×10^9 cpm/μg. The membranes were then exposed to Hyperfilm (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.

To quantify the effect of IL-10 on cytokine mRNA expression, Northern blots of RNA prepared from Th clones cultured with antigen and AC with or without IL-10 were scanned with a Betascope model 603 Blot Analyzer (Betagen Corp., Waltham, Mass.), and the ^{32}P signals were counted for 30 min and recorded as cpm. The cpm obtained for the cytokine signals were compared with those obtained by hybridization with the bovine actin probe and corrected according to the relative intensity of the actin hybridization.

RT-PCR. cDNA was produced from 0.5 μg of total RNA, isolated as described above from antigen-stimulated Th clones cultured with or without IL-10, with a GeneAmp RNA PCR kit (Perkin-Elmer Cetus, Norwalk, Conn.) and following the manufacturer's protocol, with the addition of 2 μCi of [^{32}P]ATP per sample. The bovine IL-2-specific (28) and IL-4-specific (20) primers were provided by Dirk Dobbelaere, and actin-specific primers were selected from the published sequence (10) by using the MacVector DNA analysis software (IBI, New Haven, Conn.). First-strand cDNA synthesis was performed with a GTC thermal cycler (Precision Scientific, Chicago, Ill.), employing reverse transcriptase and bovine actin (5′-ACG TAGCAGAGCTTCTCCTTGATG-3′), IL-2 (5′-GAGAGGC ACTTAGTGATC-3′), or IL-4 (5′-GTCTTTCAGCGTACTT GT-3′) antisense primers (20 to 34 pmol), with an initial 15-min incubation at 48°C followed by a 5-min incubation at 99°C. Actin (5′-CCTTTTACAACGAGCTGCGTGTG-3′), IL-2 (5′-ACATTTGACTTTTACGCGCCCAAGGT-3′), or IL-4 (5′-TGCATTGTTAGCGTCTCCT-3′) sense primers (20 to 32 pmol) were then added to the reaction mixtures, and the mixtures were incubated at 95°C for 4 min. *Taq* polymerase was added, and the mixtures were further amplified for 35 cycles (94°C, 1 min; 50°C, 1 min; 72°C, 2 min). Fourteen microliters of each PCR product was then electrophoresed on a 1% agarose gel and stained with ethidium bromide. The gels were photographed and exposed to autoradiograph film for 4 h to visualize the [^{32}P]ATP incorporation. Cytokine-specific bands were then cut from the gels and quantified through Cerenkov radiation. The specificity of the primers and identity of the product bands were previously verified by Southern blotting with reverse transcription (RT)-PCR products obtained from ConA-stimulated PBMC and radiolabeled IL-2, IL-4, and actin cDNA probes.

IFN-γ assay. IFN-γ activity in the supernatants of Th clones cultured with antigen and AC with or without IL-10 was measured in serially diluted samples with a bovine IFN-γ-specific enzyme-linked immunosorbent assay (ELISA) kit (IDEXX Corp., Westbrook, Maine) according to the manufacturer's instructions. The IFN-γ activity in the culture supernatants was determined by comparison with a standard curve obtained with a supernatant from a *B. bovis*-specific Th clone that contained 400 U of IFN-γ per ml (previously determined by the neutralization of vesicular stomatitis virus [6]).

Preparation and IL-10 treatment of bovine Mϕ. PBMC were cultured in 100-mm petri dishes at a density of 5×10^6 cells per ml for 1 to 2 h. The nonadherent cells were removed by gentle washing. The medium was replaced, and the adherent cells were then cultured for 20 h with medium in the presence or absence of 20 ng of IL-10 per ml (1-day Mϕ). Alternatively, plastic-adherent cells were cultured for 6 days in complete RPMI medium and then treated overnight with IL-10 (7-day Mϕ). The medium and any nonadherent cells were removed, and adherent cells were removed following treatment with warm (37°C) Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (GIBCO, Grand Island, N.Y.) containing 0.1% disodium EDTA. The surface phenotypes of the Mϕ populations were characterized by flow cytometry after staining the cells with a bovine monocyte-specific monoclonal antibody (MAb), IL-A24 (16), and a bovine MHC class II-specific MAb, IL-A21. Both antibodies were obtained from John Ellis, University of Saskatchewan, Saskatoon, Canada. Macrophages were washed twice before use in proliferation assays.

Lymphocyte proliferation assays and neutralization of IL-10. Proliferation assays were carried out in duplicate wells of half-area 96-well plates (Costar, Cambridge, Mass.) at 37°C, in a humidified atmosphere of 5% CO_2 in air for 3 days, as described before (2, 5, 7). Each well (100 μl total volume) contained complete medium, responder T cells added at a final concentration of 3×10^5 cells per ml, and the stimulating agent in the absence or presence of 2×10^6 autologous AC per ml. ConA was used at a final concentration of 2.5 μg/ml, recombinant human IL-2 (Boehringher Mannheim Biochemicals, Indianapolis, Ind.) was used at 10 U/ml, and specific antigen was used at 5 to 50 μg/ml of complete medium. IL-10 was included in some assay wells at a final concentration of 0.3 to 80 ng/ml. Results are presented as the mean cpm ± 1 standard deviation (SD) for duplicate cultures.

To neutralize IL-10 activity, goat anti-human IL-10 immunoglobulin G (IgG), with a reported dosage to neutralize 50% of biological activity (ND_{50}) of 5 to 10 μg/ml (R&D Systems, Minneapolis, Minn.), or control goat IgG (provided by D. Scott Linthicum, Texas A & M University) was incubated in the assay plates with an equal volume of IL-10 for 30 min at 37°C before the addition of T cells and AC, to yield final concentrations of 1.25 to 20 μg of IgG and 10 ng of IL-10 per ml.

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

RESULTS

IL-10 mRNA is expressed by Th0, Th1, and Th2 T-cell clones. Studies with murine Th-cell clones have shown that Th2 cells but not Th1 cells produce IL-10 (17), whereas both Th1- and Th2-like subsets of human Th-cell clones were shown to express this cytokine (11, 35). We therefore proposed to analyze the expression of IL-10 by different subsets of bovine T cells. Table 1 describes the characteristics of a panel of 14 T-cell clones examined in this study. Clones with unknown specificity, including two $CD8^+$ and one $CD4^+$ clone, were

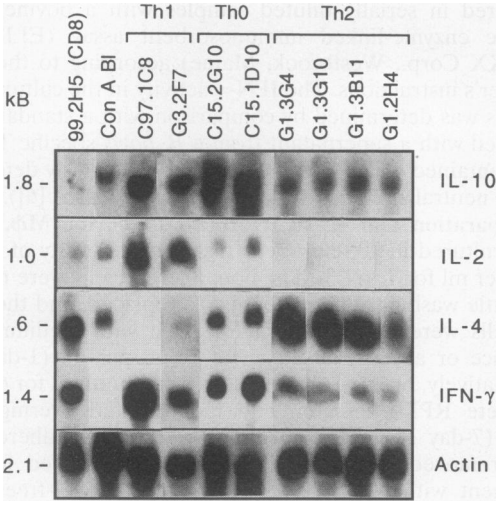

FIG. 1. Northern blot analysis of IL-10 mRNA expression by bovine Th1, Th0, and Th2 clones. RNA was prepared from *B. bovis*-specific Th1 and Th0 clones and *F. hepatica*-specific Th2 clones that were stimulated for 8 h with ConA. RNA was prepared from a $CD8^+$ clone, 99.2H5, stimulated for 8 h with ConA as a negative control and from PBMC stimulated for 18 h (ConA Bl) as a positive control for IL-10 mRNA expression. Total RNA (20 μg) was electrophoresed on agarose gels, transferred to nylon membranes, and hybridized successively with bovine IL-10 and actin probes or with IL-2, IL-4, or IFN-γ probes. The IL-10-hybridized membranes were exposed for 3 days (clone 99.2H5, ConA Bl, and clone C97.1C8) or 4 days (all other clones), and the actin-hybridized filters were exposed for 2 h. The results of hybridization with the other cytokine probes were obtained in separate experiments (2, 5, 7), and the membranes were exposed for the following time periods: IFN-γ, 2 to 5 h; IL-2 and IL-4, 2 days (99.2H5), 7 and 10 days (C97.1C8), 7 days (ConA Bl, C15.1D10, and C15.2G10), and 4 days (G1.3G4, G1.3G10, G1.3B11, and G1.2H4). The approximate sizes (in kilobases) of the indicated cytokine probes are shown on the left.

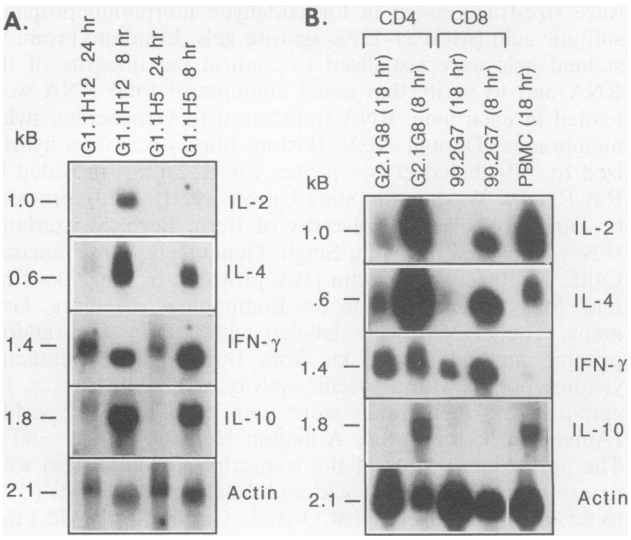

FIG. 2. Northern blot analysis of IL-10 mRNA expression in bovine T-cell clones stimulated for different time periods. *F. hepatica*-specific Th0 clones G1.1H5 and G1.1H12 (A) and ConA-induced $CD4^+$ clone G2.1G8 and $CD8^+$ clone 99.2G7 (B) were stimulated with ConA for 8, 18, or 24 h. RNA was prepared from PBMC stimulated with ConA for 18 h as a positive control. Total RNA (20 μg) was electrophoresed on agarose gels, transferred to nylon membranes, and probed with the indicated cytokine probes, including actin as a control for quantification of RNA. The filters were exposed for the following times: (A) IL-2, 3 days; IL-4, 1 day; IFN-γ, 4 h; IL-10, 7 days; actin, 1 h; (B) IL-2 and IL-4, 4 days; IFN-γ, 3 h; IL-10, 7 days; actin, 3 h. The approximate sizes (in kilobases) of the indicated cytokine probes are shown on the left.

derived from ConA-stimulated cultures of T cells from normal cattle. The remaining antigen-specific Th-cell clones were obtained from cattle immune to either *B. bovis* or *F. hepatica*. Previous studies have characterized the antigen-specific clones as belonging to either Th0, Th1, or Th2 subsets based on their expression of mRNA for IL-2, IL-4, and IFN-γ. Th0 clones expressed either all three cytokines or IL-4 and IFN-γ, whereas Th1 clones expressed IL-2 and IFN-γ and no IL-4, and Th2 clones expressed IL-4 and little or no IFN-γ or IL-2 (Fig. 1 and 2A) (2, 5, 7). When the panel of T-cell clones was examined for IL-10 mRNA expression following stimulation with ConA, $CD4^+$ T-cell clones belonging to all of the Th-cell subsets expressed this cytokine, whereas neither $CD8^+$ clone expressed IL-10 (Fig. 1 and 2B). There were no consistent differences in the levels of IL-10 mRNA expression among the different Th-cell subsets; if anything, Th1 clones appeared to express more cytokine message than Th2 clones (Fig. 1).

Comparison of mRNA obtained at 8 or 18 to 24 h following ConA stimulation revealed maximal expression of IL-10 mRNA at the earlier time point in *F. hepatica*-specific Th0 clones G1.1H5 and G1.1H12 (Fig. 2A) as well as in the ConA-induced Th0 clone G2.1G8 (Fig. 2B). In fact, the IL-10 transcripts were barely detectable by 18 to 24 h, in contrast to IFN-γ transcripts, which were still present at the later time points. $CD8^+$ clone 99.2G7 did not express IL-10 mRNA at either time point examined (Fig. 2B). Two $CD4^-$ $CD8^-$ $CD3^+$ γ/δ T-cell-receptor-positive (TcR^+) clones obtained from *F. hepatica*-stimulated T-cell lines and freshly prepared antigen-presenting cells did not express IL-10 mRNA (data not presented), ruling out potential contamination of the T-cell mRNA with mRNA from residual antigen-presenting cells. These results show that IL-10 expression by bovine T-cell subsets is similar to that described for human Th-cell clones (11, 35) and is not restricted to the Th2 subset, as described for mice (17). However, the kinetics of expression of bovine IL-10 mRNA in the Th clones differ from those for human T-cell clones, in which it was reported that maximal IL-10 mRNA expression occurred 24 h following activation (35).

Human IL-10 inhibits antigen-induced proliferation of Th0, Th1, and Th2 clones. The finding that IL-10 mRNA is expressed by Th0 and Th1 as well as Th2 cells derived from parasite-infected cattle prompted us to determine if all Th-cell subsets can be downregulated by exogenous IL-10, as recently reported for human Th1 and Th2 clones (11, 12). Table 2 presents the baseline levels of antigen-induced proliferation by representative T-cell clones used for the IL-10 inhibition studies presented in Fig. 3. All of the antigen-specific Th-cell clones proliferated to antigen in the absence of IL-2, although IL-2 potentiated the response of all clones. When human IL-10 was added to the T cells and AC at the initiation of the proliferation assay, a dose-dependent inhibition of proliferation of five of six $CD4^+$ clones in response to antigen was observed (Fig. 3). Maximal levels of inhibition usually occurred with 20 ng of IL-10 per ml, although in some experiments 80 ng of IL-10 per ml was more effective. IL-10 was not selectively inhibitory for a given Th-cell subset but inhibited antigen-driven proliferation of Th0, Th1, and Th2 clones. As illustrated in Fig. 3, 20 ng of IL-10 per ml inhibited proliferation of Th2

TABLE 2. Proliferative responses of Th-cell clones against antigen or antigen and IL-2

T-cell clone (Th subtype)	Radioactivity (mean cpm ± SD) incorporated by Th clones stimulated with[a]:		
	Medium	Antigen	Antigen + IL-2
C97.1C8 (Th1)	24 ± 5	12,907 ± 288	156,778 ± 3,303
G3.2F7 (Th1)	169 ± 48	18,448 ± 2,152	71,061 ± 542
G1.1H5 (Th0)	158 ± 22	18,977 ± 126	36,648 ± 3,315
G1.1H12 (Th0)	635 ± 24	6,001 ± 1,700	15,074 ± 1,191
G1.3B11 (Th2)	175 ± 6	3,566 ± 137	12,339 ± 852
G1.3G10 (Th2)	663 ± 75	13,856 ± 648	24,820 ± 662

[a] Th-cell clones (3×10^4 cells) were cultured for 3 days with 2×10^5 irradiated PBMC as AC and antigen, which consisted of Bb-1-GST (25 μg/ml) for clone C97.1C8, the crude membrane fraction of *B. bovis* (Texas isolate) merozoites (25 μg/ml) for clone G3.2F7, or *F. hepatica* adult worm extract (50 μg/ml) for clones G1.1H5, G1.1H12, G1.3G10, and G1.3B11, in the absence or presence of 10 U of IL-2 per ml. Incorporation of radioactivity is expressed as the mean cpm of [^{125}I]deoxyuridine in duplicate samples ± 1 SD. Proliferative responses of the T-cell clones cultured with antigen or antigen plus IL-2 were shown to be significantly different from proliferative responses of T-cell clones cultured with medium alone ($P < 0.05$) by the Student one-tailed *t* test.

clones G1.3G10 and G1.3B11 against 50 μg of *F. hepatica* adult worm antigen per ml by 76 and 87%, respectively. The responses of Th0 clones G1.1H5, G1.1H12, and G1.3E11 (not shown) were similarly inhibited by 59, 61, and 38%, respectively, and antigen-specific proliferation of Th1 clone C97.1C8 was inhibited by 61% in the presence of 80 ng of IL-10 per ml. More effective inhibition of T-cell proliferation was generally obtained when a suboptimal antigen concentration was used. For example, the response of Th2 clone G1.3G10 against 10 μg of *F. hepatica* antigen per ml (6,356 ± 114 cpm) was completely inhibited by 20 ng of IL-10 per ml (375 ± 26 cpm). Interestingly, in three experiments, Th1 cell clone G3.2F7 could not be inhibited by any concentration of IL-10 in the presence of either a low (5 μg/ml) or high (25 μg/ml) concentration of *B. bovis* antigen. In fact, in two of three experiments, IL-10 enhanced the proliferative response of this clone to antigen (Fig. 3). It thus appears that human IL-10 can both inhibit and costimulate bovine T cells, as reported previously for murine IL-10 in studies demonstrating costimulatory activity for thymocytes and peripheral T cells (23).

Inhibition of human T-cell proliferation by IL-10 is associated with a reduction in endogenous IL-2 and IL-4 production (11, 13, 31), and suppressed proliferation could be restored by the addition of IL-2 (13, 31) or IL-4 (31) to the cultures. Human IL-2 similarly reversed the suppressed proliferative responses of the five bovine Th-cell clones cultured with IL-10. As shown in Fig. 3, nearly complete (96%) restoration of the response was achieved with Th1 clone C97.1C8 and Th0 clone G1.1H12, both of which expressed IL-2 mRNA upon ConA stimulation (Fig. 1 and 2A) (5, 7), whereas only partial restoration (69 to 79%) of the response was achieved with Th2 clones G1.3G10 and G1.3B11 and Th0 clone G1.1H5 that did not express IL-2 mRNA (Fig. 1 and 2A) (2). These results are consistent with the hypothesis that IL-10 acts, either directly or indirectly, to inhibit bovine T-cell proliferation against antigen by inhibiting the synthesis of autocrine T-cell growth factors.

Inhibitory effect of IL-10 is neutralized by goat anti-human IL-10 antibody. Purified IgG fractions of goat anti-human IL-10 neutralizing antibody and control goat IgG were employed in blocking studies to verify the inhibitory activity of human recombinant IL-10 for bovine Th cells. When anti-IL-10 IgG or control goat IgG ranging from 1 to 20 μg/ml was incubated with IL-10 (10 ng/ml) for 1 h before the addition of Th cells, antigen, and AC to the assay mix, the inhibitory effect of IL-10 was completely reversed by anti-IL-10 antibody in a dose-dependent manner (Fig. 4). For four of five clones tested, the ND_{50} of the anti-IL-10 IgG neutralizing 10 ng of IL-10 per ml ranged from approximately 6 to 7.5 μg/ml and was approximately 15 μg/ml for the fifth clone, G1.1H12. For some clones, the levels of antigen-induced proliferation were enhanced in the presence of 20 μg of anti-IL-10 IgG per ml whether exogenous IL-10 was present in the assay (as shown for clones G1.3G10 and G1.1H5 in Fig. 4) or absent (Table 3). As presented in Table 3, the antigen-specific responses of four clones were augmented in the presence of anti-IL-10 antibody but not control goat IgG. The fifth clone, G3.2F7, which was not suppressible by IL-10, did not express enhanced proliferation in the presence of specific antibody. Together, these results demonstrate that the inhibitory activity of human IL-10 for bovine T cells is specifically mediated by the cytokine and suggest that anti-IL-10 antibody may neutralize endogenous bovine IL-10 produced by the T cells and/or AC, as indicated by others using a mouse model (15).

IL-10 inhibits the expression of cytokine mRNA by antigen-stimulated Th0 clones. Since IL-10 was originally described as cytokine synthesis inhibitory factor (17), we wished to determine whether human IL-10 was able to affect cytokine synthesis by antigen-stimulated bovine T cells. Preliminary studies with two Th0 clones revealed a suppressive effect of IL-10 on transcription of both IFN-γ and IL-4 mRNAs. Analysis of IFN-γ mRNA in Th0 clones by Northern blotting (Fig. 5A) and quantification of the cytokine mRNA signals analyzed with the Betascope Blot Analyzer revealed 72 and 45% reduction in the IFN-γ signals of clones G1.3E11 and G1.1H12, respectively, when normalized to the actin signal. IFN-γ levels in the supernatants of clone G1.1H12 were low (≤10 U/ml), so it was not possible to detect significant differences in IFN-γ levels in supernatants of IL-10-treated or untreated cells. However, IL-10 inhibited the level of IFN-γ produced by clone G1.3E11 by 38%, which fell from 61 to 38 U/ml. Because the signals for IL-2 and IL-4 mRNA were too weak to quantify on the Northern blots (data not shown), RT-PCR was performed with IL-2-, IL-4-, or actin-specific primers to evaluate the effects of IL-10 on IL-2 and IL-4 mRNA expression by these Th0 clones (Fig. 5B). As controls, RNA prepared from murine WEHI-164 fibroblasts (negative control for IL-2 and IL-4) or bovine PBMC activated for 15 h with ConA (positive control) was used for identical RT-PCRs. Compared with the control ConA-stimulated PBMC PCR product, which yielded a single band of the expected 306-bp size, several bands of inappropriate size were observed on the ethidium bromide-stained gels of amplified PCR products from the Th0 clones. Similar patterns of nonspecific reactivity were observed with the murine cell line, indicating a nonspecific reaction due to insufficient mRNA in the starting material of the Th0 clones. However, the IL-4 PCR products obtained from the Th0 clones yielded the expected 421-bp fragment observed with the positive control, with visibly less product from the IL-10-treated cells. The radiolabeled IL-4 PCR products were excised from the gels and quantified in a scintillation counter. When the cpm present in the IL-4 PCR products for the two clones were normalized to the cpm present in the actin PCR products, there was 80 and 75% less IL-4 product from IL-10-treated clones G1.3E11 and G1.1H12, respectively, compared with PCR products from untreated cells. Because of the low levels of expression of these cytokines following stimulation with antigen and AC (5), we were unable to detect IL-2 or

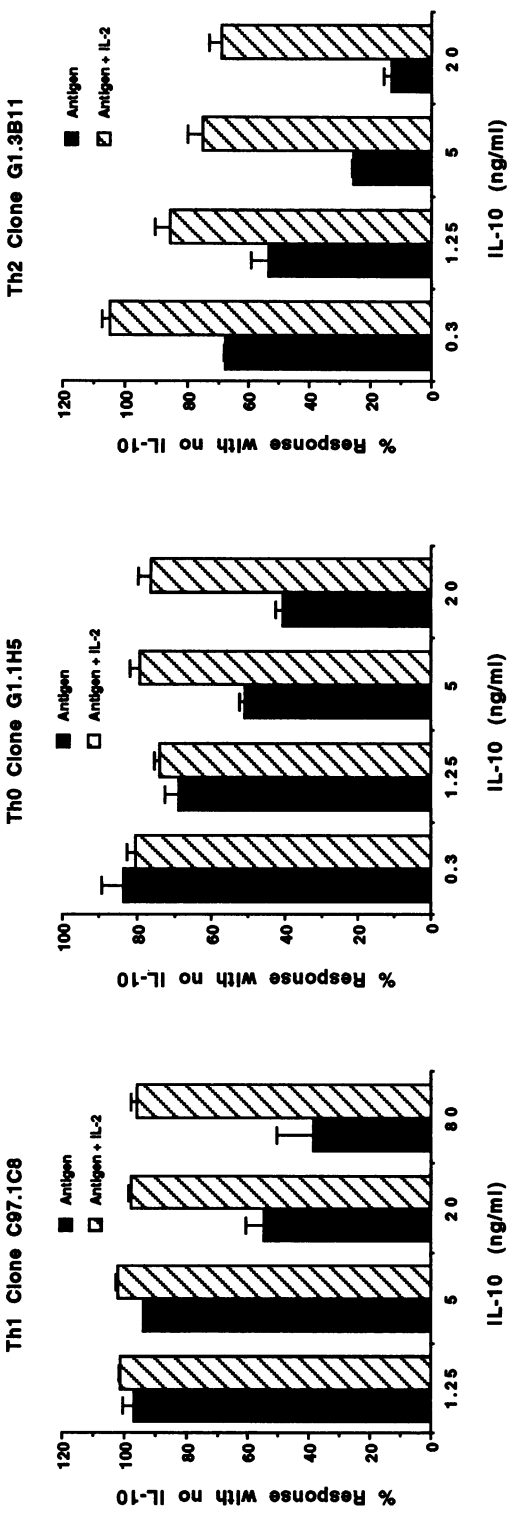

FIG. 3. Effects of IL-10 on antigen-induced proliferative responses of Th1, Th0, and Th2 clones and reversal of the effects by IL-2. A 3-day proliferation assay was performed with 3×10^4 T cells, 2×10^5 AC, and antigen in the absence of IL-10 or in the presence of 0.3 to 80 ng of IL-10 per ml alone (solid bars) or IL-10 plus 10 U of IL-2 per ml (hatched bars). The results are presented as the percentage of the proliferative response of the Th clone against antigen in the absence of IL-10 (the baseline data used in this experiment are presented in Table 2).

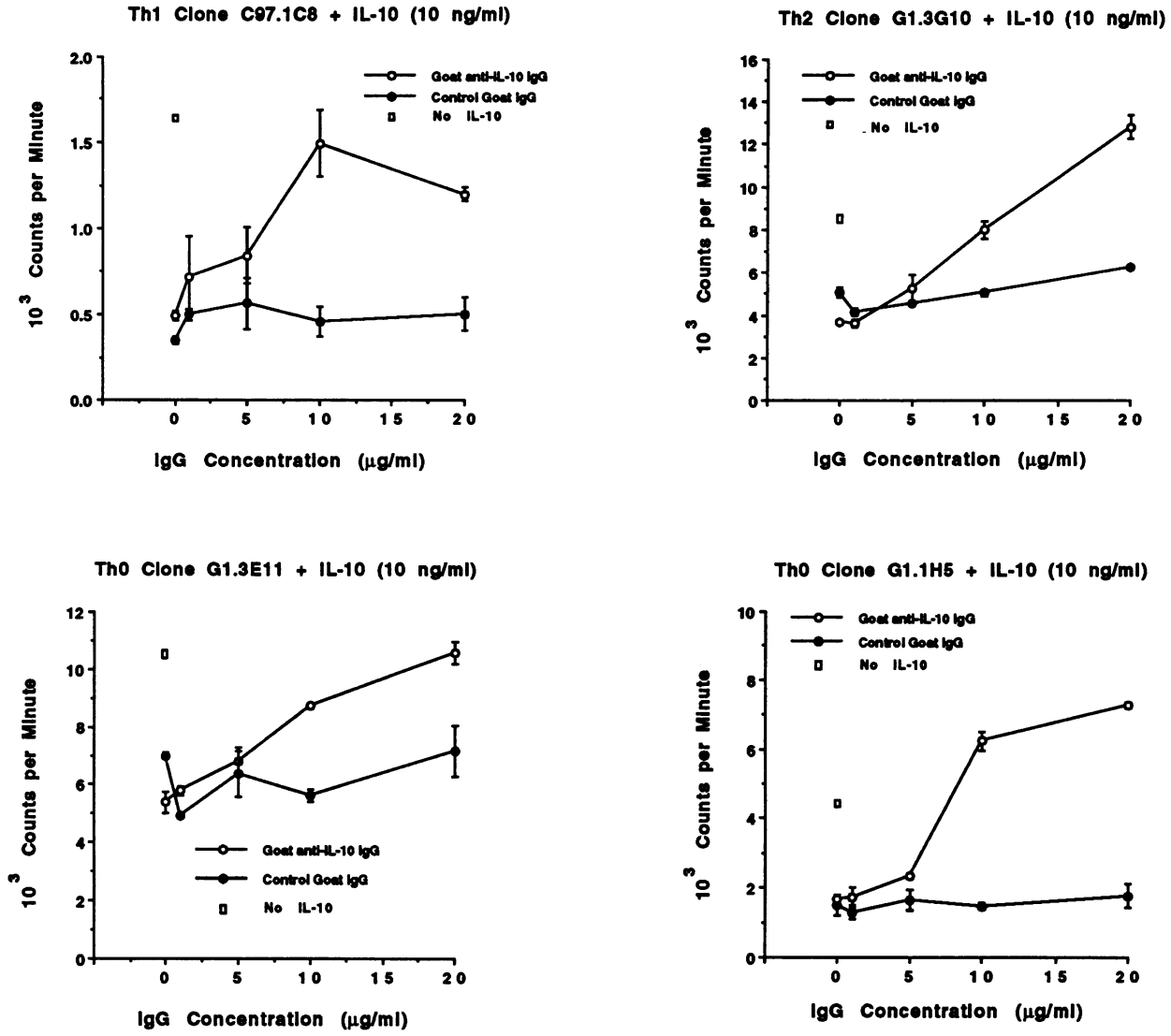

FIG. 4. Effects of anti-human IL-10 antibody on IL-10-mediated suppression of antigen-specific proliferative responses of bovine Th-cell clones. Selected Th clones were stimulated in a 3-day proliferation assay with antigen and AC in the absence (open squares) or presence of 10 ng of human IL-10 per ml and 0 to 20 μg of either goat anti-human IL-10 IgG (open circles) or control goat IgG (solid circles) per ml. Results are presented as the mean cpm of duplicate cultures ± 1 SD.

IL-4 activity in the supernatants of antigen-stimulated Th0 clones (data not shown).

IL-10 directly inhibits a CD8$^+$ T-cell clone but not antigen-specific CD4$^+$ Th clones stimulated with ConA or IL-2. To determine whether T-cell proliferation could be inhibited directly by IL-10, as recently reported for human Th-cell clones (13), T-cell clones were stimulated with ConA in the presence or absence of AC and IL-10 (Table 4). A dose-dependent inhibition of the proliferative response to ConA by CD8$^+$ clone 99.2G10 was obtained with IL-10, and the degree of inhibition (51%) by 20 ng of IL-10 per ml was nearly identical whether AC were absent or present. This result provides evidence that this CD8$^+$ T-cell clone can be directly inhibited by IL-10. In contrast, a direct effect of IL-10 on CD4$^+$ T-cell proliferation induced by ConA was not observed. Th1 clone C97.1C8 and Th0 clone G1.1H12 responded to ConA in the absence of AC, but the response was not inhibited by IL-10 in either the absence or presence of AC. In contrast, Th cell

TABLE 3. Effect of anti-human IL-10 antibody on Th-cell proliferation against specific antigen

T-cell clone (Th subtype)	Radioactivity (mean cpm ± SD) incorporated by Th clones cultured with[a]:			Response[b] (% of control)
	Medium	Antigen + control IgG	Antigen + anti-IL-10 IgG	
C97.1C8 (Th1)	65 ± 10	571 ± 16	1,317 ± 335	231
G3.2F7 (Th1)	602 ± 38	7,313 ± 782	7,360 ± 871	101
G1.1H5 (Th0)	26 ± 5	5,073 ± 13	9,426 ± 561	186**
G1.1H12 (Th0)	30 ± 9	6,888 ± 300	10,246 ± 624	149*
G1.3G10 (Th2)	98 ± 21	10,577 ± 109	13,039 ± 1,249	123

[a] Th-cell clones were stimulated with specific antigen as described in Table 2, footnote *a*. Control goat IgG or goat anti-IL-10 IgG was added to identical cultures at a final concentration of 20 μg of protein per ml. Incorporation of radioactivity is expressed as the mean cpm of [^{125}I]deoxyuridine in duplicate samples ± 1 SD.

[b] Data are expressed as the percentage of the response of the Th clone against antigen with control IgG. The level of significance determined by the one-tailed Student *t* test is indicated: *, $P < 0.05$; **, $P < 0.01$.

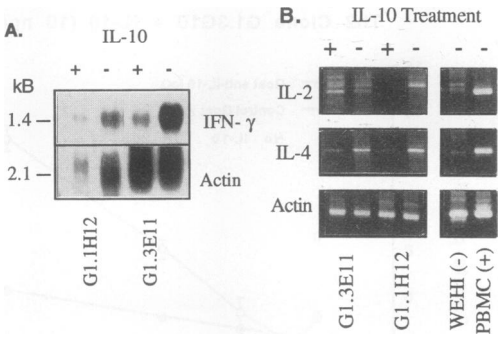

FIG. 5. Effects of IL-10 on the expression of IFN-γ and IL-4 mRNA in bovine Th0 clones. T-cell clones G1.H12 and G1.3E11 were activated for 13 h with antigen and AC in the absence (−) or presence (+) of IL-10. IFN-γ mRNA expression was determined by Northern blotting (A), and IL-4 mRNA expression was determined by semiquantitative RT-PCR (B). (A) Total cellular RNA (10 μg for clone G1.1H12 and 15 μg for clone G1.3E11) was electrophoresed on the gels, transferred to nylon membranes, and probed successively with IFN-γ and actin cDNA probes. Membranes were exposed for the following time periods: clone G1.1H12: IFN-γ, 48 h; actin, 9 h; clone G1.3E11: IFN-γ, 5 h; actin, 9 h. RNA was quantified by Betascope scanning of the membranes, and the values obtained for IFN-γ were corrected according to the relative intensities of actin hybridization. (B) RT-PCR was performed with 0.5 μg of RNA prepared from IL-10-treated (+) or untreated (−) cells of clones G1.3E11 and G1.1H12 and untreated control cells, employing IL-2-, IL-4-, or actin-specific primers and trace amounts of ^{32}P-labeled ATP. Control RNA was obtained from unstimulated murine WEHI-164 cells (negative control) or ConA-stimulated bovine PBMC (positive control). The cytokine or actin PCR products, indicated on the left, were visualized on ethidium bromide-stained gels. To semiquantify the mRNA, radiolabeled IL-4 and actin PCR products were excised from the gels and counted in a scintillation counter, and the cpm in the IL-4 PCR products of the two clones were corrected according to the cpm in the respective actin PCR products.

TABLE 4. Effect of IL-10 on ConA-induced proliferation of T-cell clones with and without AC

T-cell clone (Th subtype)	IL-2 mRNA[a]	AC	Radioactivity (mean cpm ± SD) incorporated by Th clones cultured with[b]: ConA	ConA + IL-10	% Inhibition[c]
CD8$^+$ clone 99.2G7 (T0)	+	−	13,873 ± 1,871	6,760 ± 385	51*
		+	38,050 ± 543	18,714 ± 233	51**
CD4$^+$ clones					
C97.1C8 (Th1)	+	−	13,987 ± 3,208	25,329 ± 1,806	0
		+	55,586 ± 22,151	69,478 ± 1,318	0
G3.2F7 (Th1)	+	−	46 ± 1	84 ± 4	NR
		+	8,171 ± 305	7,428 ± 576	10
G1.1H12 (Th0)	+	−	2,258 ± 33	2,667 ± 101	0
		+	2,834 ± 25	2,636 ± 26	7*
G1.1H5 (Th0)	−	−	192 ± 11	327 ± 60	NR
		+	5,246 ± 786	1,050 ± 1	80*
G1.3B11 (Th2)	−	−	401 ± 40	370 ± 35	NR
		+	3,507 ± 194	1,748 ± 68	50**
G1.3G10 (Th2)	−	−	146 ± 37	36 ± 18	NR
		+	47,707 ± 1,082	3,036 ± 42	94**

[a] Expression of IL-2 mRNA by ConA-stimulated clones.
[b] T cells (3×10^4) were stimulated for 3 days with ConA (2.5 μg/ml) or ConA plus 2×10^5 irradiated autologous PBMC as a source of AC in the absence or presence of human IL-10 (20 ng/ml). Results are expressed as the mean cpm ± 1 SD of duplicate cultures.
[c] Percent inhibition of the response against ConA plus IL-10 compared with the baseline response against ConA without IL-10. Underlined values were shown to be ≤50% and significantly lower than the response of T cells cultured with ConA alone (*, $P < 0.05$; **, $P < 0.01$) by the Student one-tailed t test. NR, negative response to ConA; the difference in the mean cpm between cells cultured with ConA and cells cultured with medium was <400 cpm.

clones that required costimulatory AC to proliferate to ConA (clones G1.1H5, G1.3B11, and G1.3G10) were inhibited by IL-10 in the presence of AC. ConA did not induce IL-2 mRNA expression in these clones (Fig. 1 and 2A) (2).

Additional evidence for the lack of a direct inhibitory effect of IL-10 on the Th clones was shown by the inability of IL-10 to inhibit IL-2-induced proliferation of Th0, Th1, and Th2 clones in the absence of AC (Table 5). As before, clone G3.2F7 was not inhibited by IL-10. However, when AC were present, 20 ng of IL-10 per ml did reduce IL-2-induced proliferation of the remaining clones by 28 to 62%. The apparently greater inhibitory effect of IL-10 on Th cell proliferation against IL-2 in the absence of antigen compared with the effect on Th cells cultured with antigen and IL-2 (i.e., 4 to 31% inhibition; Fig. 3) could be explained by the more potent stimulation achieved by the combination of antigen and IL-2 that would be less easily suppressed. In support of this hypothesis, greater suppressive effects of IL-10 were achieved with suboptimal antigen concentrations. In the absence of AC, IL-10 enhanced the IL-2-induced proliferation of five of the six clones, again reflecting the differential effects of IL-10 on bovine T-cell proliferation.

The reason for the lower proliferative response of clones G1.1H12 and G1.3B11 to IL-2 when AC were included in the assay, as shown in Table 5, is not known. One possibility is that inhibitory molecules, such as prostaglandin E_2 released by Mϕ in the AC population, could inhibit bovine T-cell proliferation (4).

IL-10 inhibits antigen-dependent Th-cell proliferation by indirectly inhibiting Mϕ. Several studies have reported that murine IL-10 can inhibit T-cell responses by indirectly inhibiting the AC function of Mϕ (14, 15, 18), independent of MHC class II–T-cell receptor interactions (15). Human IL-10 was similarly shown to inhibit Mϕ-dependent antigen-specific T-cell proliferation (12). However, the studies with human T cells showed that the effect on Mϕ was strongly associated with decreased MHC class II expression. In our studies, the inability of IL-10 to inhibit ConA- or IL-2-mediated Th-cell proliferation in the absence of AC also suggested that IL-10 inhibited antigen-dependent proliferation indirectly via the Mϕ. To test this hypothesis, Mϕ cultured from bovine peripheral blood for 1 to 2 h or 6 days were treated for 20 h with IL-10, washed, and compared with untreated Mϕ for the capacity to serve as AC for the Th clones in the absence or presence of additional IL-10 in the assay (Table 6). The purity of the Mϕ population was assessed by flow cytometry. Plastic-adherent mononuclear cells examined after 1 day of culture contained 89 to 90% MHC class II$^+$ cells, and 64 to 72% of the cells expressed the Mϕ determinant recognized by MAb IL-A24 (data not shown), indicating that the majority of the AC were Mϕ. By 6 to 7 days of culture, the percentage of Mϕ in the cultures increased to 95% (data not shown). Mϕ cultured for 6 to 7 days were usually as effective as irradiated PBMC in presenting antigen to both Th1 and Th2 clones. However, Th2 clone G1.3G10 and Th0 clone G1.1H5 were not inhibited when stimulated with

TABLE 5. IL-10 inhibits IL-2-induced Th-cell proliferation only in the presence of AC

T-cell clone (Th subtype)	AC	Radioactivity (mean cpm ± SD) incorporated by Th clones cultured with[a]:		% Inhibition[b]
		IL-2	IL-2 + IL-10	
C97.1C8 (Th1)	−	4,594 ± 327	11,582 ± 1,694	0
	+	38,038 ± 3,499	14,443 ± 1,386	62**
G3.2F7 (Th1)	−	2,118 ± 129	2,045 ± 14	3
	+	27,244 ± 16	28,199 ± 1,575	0
G1.1H12 (Th0)	−	9,617 ± 508	12,137 ± 188	0
	+	4,205 ± 116	2,969 ± 552	36
G1.1H5 (Th0)	−	24,982 ± 1,139	29,601 ± 27	0
	+	66,723 ± 3,897	31,582 ± 783	53**
G1.3B11 (Th2)	−	6,842 ± 258	8,415 ± 251	0
	+	3,554 ± 119	2,574 ± 275	28*
G1.3G10 (Th2)	−	2,583 ± 1,336	3,954 ± 195	0
	+	3,809 ± 73	2,323 ± 61	39**

[a] T cells (3×10^4) were cultured for 3 days with human IL-2 (10 U/ml) in the presence or absence of 0.3 to 80 ng of IL-10 per ml and 2×10^5 autologous, irradiated PBMC as AC. Experiments performed with IL-10 at 20 ng/ml are presented. Data are presented as the mean cpm ± 1 SD of duplicate cultures.
[b] Percent inhibition of the response against IL-2 in the presence of IL-10 compared with the baseline response against IL-2 without IL-10. The level of significance, determined by a one-tailed Student *t* test, is indicated: *, $P < 0.05$; **, $P < 0.01$.

antigen and autologous, IL-10-treated, 7-day Mϕ (data not shown). In contrast, inhibition was observed with Th1 clone C97.1C8, as shown in Table 6. Treatment of autologous Mϕ with IL-10 resulted in a 43% reduction in proliferation of clone C97.1C8 compared with T cells stimulated with untreated Mϕ (experiment II). Furthermore, when IL-10 was continuously present during the assay, proliferation of clone C97.1C8 cultured with IL-10-pretreated Mϕ was further reduced (experiment II). Similar results were achieved when 1-day Mϕ were used to stimulate clone G1.3G10. In two experiments (III and IV), the response of the T cells to *F. hepatica* antigen was reduced by 37 and 67%, respectively, when IL-10-pretreated Mϕ were used as AC, compared with untreated Mϕ. The inclusion of IL-10 in the assay with IL-10-pretreated Mϕ again resulted in further inhibition of T-cell responses. Together, these experiments show that under certain conditions, IL-10 can significantly inhibit antigen-dependent Th-cell proliferation by acting on the AC, which is presumably a Mϕ. In the case of clone G1.3G10, the inhibitory effect on the Mϕ was only detected when the Mϕ were treated early after their selection from PBMC by adherence, suggesting that by 6 days, these cells can be refractory to the inhibitory action of IL-10. The levels of expression of MHC class II on the surface of either 1- or 6-day Mϕ was not significantly reduced by treatment with IL-10 (data not shown), indicating that an impairment in MHC class II expression does not explain the reduced T-cell proliferation in these studies.

DISCUSSION

IL-10 is a pluripotent immunoregulatory cytokine that has not been previously characterized in T-cell clones of cattle. Bovine IL-10 is not only genetically and biochemically more similar to human than murine IL-10 (19); the results presented here also demonstrate that bovine T cells behave more like human T cells in their unrestricted IL-10 expression patterns and regulation by this cytokine. The recent production of a bovine IL-10 cDNA clone has enabled the characterization of IL-10 gene transcription in different T-cell subsets. Analysis of mRNA expression by several Th0, Th1, and Th2 clones specific for parasite antigens provides the first evidence that IL-10 is expressed by all three subsets of Th clones in cattle, independent of antigenic specificity or expression of other cytokines. Although we are unable to measure IL-10 protein expression because of the lack of specific reagents, a comparison of IL-10 and actin signals on RNA blots exposed for the same time

TABLE 6. Inhibition of antigen-induced Th-cell proliferation by Mϕ pretreated with IL-10

Expt no. and Mϕ	T-cell clone (Th phenotype)	Mϕ pretreatment[a]	Radioactivity (mean cpm ± SD) incorporated by Th clones cultured with[b]:		% Inhibition[c]
			Antigen (% inhibition)	Antigen + IL-10 (% inhibition)	
I (7-day Mϕ)	C97.1C8 (Th1)	Medium	ND[d]	ND	
		IL-10	2,800 ± 837	560 ± 126	80
II (7-day Mϕ)	C97.1C8 (Th1)	Medium	2,237 ± 27	1,773 ± 171	21
		IL-10	1,281 ± 5 (43**)	676 ± 161 (62*)	47*
III (1-day Mϕ)	G1.3G10 (Th2)	Medium	1,963 ± 108	918 ± 140	53*
		IL-10	1,235 ± 11 (37**)	584 ± 396 (33)	62
IV (1-day Mϕ)	G1.3G10 (Th2)	Medium	3,136 ± 70	1,445 ± 464	54*
		IL-10	1,038 ± 443 (67*)	232 ± 40 (84)	78

[a] PBMC were allowed to adhere to plastic dishes for 1 to 2 h, and the adherent cells were cultured for an additional 20 h with either medium or IL-10 (20 ng/ml) (1-day Mϕ), or plastic-adherent PBMC were cultured for 6 days and then treated for an additional 20 h with medium or IL-10 (7-day Mϕ).
[b] Purified Mϕ (5×10^3) and Th cells (3×10^4) were cultured in the presence of specific antigen (5 μg of Bb-1 per ml for experiment I and 25 μg of Bb-1 or 50 μg of *F. hepatica* per ml for experiments II to IV) and in the absence or presence of IL-10 (80 ng/ml). [^{125}I]deoxyuridine incorporation was determined as described in Table 2, footnote *a*. Background proliferative responses of the clones cultured in the absence of antigen were ≤70 cpm for C97.1C8 and ≤105 cpm for G1.3G10. The percent inhibition of the response of T cells and antigen cultured with IL-10-pretreated Mϕ compared with the response of T cells and antigen cultured with untreated Mϕ is shown in parentheses. The level of significance, determined by the one-tailed Student *t* test, is indicated: *, $P < 0.05$; **, $P < 0.01$.
[c] Percent inhibition of the response of T cells cultured with Mϕ and antigen with IL-10 in the assay mix compared with the response of cells cultured without IL-10. The level of significance, determined by the one-tailed Student *t* test, is indicated.
[d] ND, not determined.

periods indicates that IL-10 mRNA expression by Th1 clones is equal to, if not stronger than, that by Th2 or Th0 clones. These results are comparable to those of Yssel and coworkers (35) and Del Prete et al. (11), who similarly demonstrated IL-10 mRNA expression by both Th1- and Th2-like clones of human T cells stimulated with antigen, ConA, or phorbol myristate acetate plus anti-CD3 antibody, and at variance with the results of Fiorentino et al. (17), who reported that IL-10 was selectively expressed by Th2 cells in mice. IL-10 mRNA was not detected in either of two bovine $CD8^+$ clones, and although the number of clones is small, this finding supports the view that $CD8^+$ T cells are poor producers of IL-10 (36).

Time course studies suggested that, following activation, IL-10 is expressed earlier in bovine T cells than in human T cells. We previously showed that IL-10 mRNA expression was elevated by 8 h following activation of resting PBMC with ConA (19), whereas IL-10 mRNA expression in mitogen-activated human PBMC peaked after 24 h (35). Similarly, the kinetics of expression of IL-10 in activated bovine T-cell clones revealed higher levels of mRNA at 8 h than at later time points, once again contrasting with the results of Yssel et al., who found that IL-10 expression in human T cell clones was maximal at 24 h following stimulation (35). The reasons for these differences are not clear, but one possibility is that the mode of T-cell activation differed in the two studies. The studies here used ConA to mimic antigen-induced T-cell activation, whereas those reported by Yssel and coworkers used a phorbol ester and anti-CD3 antibody. Other bovine cytokine transcripts, including IL-4 (20) and IL-2 (34), were maximally expressed from 2 to 4 h after stimulation, like the human cytokines (35). Thus, the relatively later expression of bovine IL-10 than of IL-2 and IL-4 is consistent with the hypothesis that IL-10 serves to dampen an ongoing T-cell proliferative response (11).

The ability of human IL-10 to inhibit proliferation of all Th-cell subsets is also similar to results reported by others for human Th clones (11, 12). In experiments performed with bovine and human Th-cell clones, human IL-10 strongly suppressed antigen-induced proliferation of both Th1 and Th2 cells, and IL-2 reversed the suppressed proliferation. These results indicate that for bovine as well as human T cells, the immunoregulatory effects of IL-10 are not restricted to type 1 or delayed-type hypersensitivity responses.

The majority of studies with mouse and human T-cell clones have indicated that IL-10 exerts its suppressive effects on Th cells by indirectly affecting AC function (12, 14, 15, 17, 18). Furthermore, the AC functions of Mϕ and not B cells were inhibited by IL-10 (12, 15, 18). Studies with murine T cells showed that even though the inhibitory effects of IL-10-pretreated Mϕ on T-cell function were variable, when IL-10 was added to cultures of T cells, antigen, and Mϕ, T-cell proliferation and cytokine production were inhibited (15, 18). In the mouse studies, the mechanism of Mϕ inhibition by IL-10 was not due to downregulation of MHC class II expression (14, 15, 18), but IL-10 was shown to prevent the induction on Mϕ membranes of another molecule, B7, the ligand for CD28 required for T-cell activation (14). The results of our experiments with bovine T cells are also consistent with the view that IL-10 suppresses Th-cell proliferation by indirectly affecting a Mϕ costimulatory function(s), which is supported by the following observations: (i) the source of AC used in the IL-10 inhibition studies was irradiated (3,000 rads) PBMC, and since B cells are radiosensitive (1), the functional AC is likely to be a monocyte or Mϕ; (ii) only AC-dependent proliferative responses of $CD4^+$ T-cell clones against ConA were inhibited by IL-10; ConA responses that were not augmented in the presence of AC were not inhibited by IL-10; (iii) IL-2-induced proliferation was inhibited only in the presence of AC; and (iv) pretreatment of Mϕ with IL-10 partially inhibited antigen-driven proliferation of both Th1 and Th2 clones; however, as reported for murine Th1 cells (18), inhibition was further augmented when IL-10 was continuously present during the proliferation assay. Mϕ isolated by adherence to plastic were less sensitive to the effects of IL-10 than freshly isolated irradiated PBMC and could not be reproducibly suppressed. One explanation for this is that activation of bovine Mϕ by adherence to plastic could result in the expression of B7 or other costimulatory molecules, rendering the cells resistant to the effects of IL-10. In mice, B7 expression was induced on Mϕ by overnight culture of the cells, and once upregulated, could not be inhibited by IL-10 (14).

The effects of exogenous IL-10 on bovine T-cell clones were variable, as seen by others with murine T cells (17, 22). In contrast to the Th clones, one $CD8^+$ clone was inhibited by IL-10 independent of AC. In addition, *B. bovis*-specific Th1 clone G3.2F7 could not be inhibited by IL-10 in the absence or presence of either irradiated PBMC or cultured Mϕ, suggesting that this T-cell clone has unique requirements for costimulation. The inability of IL-10 to inhibit this clone cannot be due to selective antigen presentation by B cells, since the clone responded strongly to antigen presented by 7-day Mϕ (38,980 ± 3,426 cpm, compared with a background response of 672 ± 12 cpm). Previous studies with clone G3.2F7 demonstrated low levels of IL-2 in the supernatant of activated cells (5), suggesting that the lack of downregulation of proliferation by IL-10 is not due to overriding effects of high levels of endogenous IL-2. In contrast to the results obtained with the other Th clones, IL-10 augmented antigen-induced proliferation of clone G3.2F7. Furthermore, in the absence of AC, IL-10 also enhanced proliferation of other Th0, Th1, and Th2 clones stimulated by a low concentration of IL-2. MacNeil et al. similarly reported that IL-10 acted as a costimulatory factor for purified murine splenic $CD4^+$ T cells in the presence of saturating levels of IL-2 and IL-4 (23).

A comparison of the effects of IL-10 on bovine Th cells that expressed IL-2 with the effects on clones that did not express IL-2 revealed additional differences. IL-2 more effectively restored the antigen-specific proliferative responses of clones that expressed IL-2 mRNA than those Th0 and Th2 clones that did not. In addition, T-cell clones that did not express IL-2 were dependent on AC for a proliferative response to ConA, and only these IL-2-negative clones were suppressed by IL-10 when stimulated with the mitogen and AC. Together, our results with bovine Th clones support the hypothesis that the suppressive or costimulatory effects of IL-10 are not predicated by the subset of responder T cell per se, but by the nature of the cytokines expressed by and the costimulatory requirements of these T cells (14, 15). Studies are in progress to determine whether expression of a B7 homolog on bovine Mϕ is similarly regulated by IL-10 and if the differential effects of IL-10 on the T-cell clones are related to differential requirements for B7 as a costimulatory ligand. Studies examining the ability of B cells to serve as antigen-presenting cells for selected Th clones are also planned.

In addition to inhibiting potential costimulatory molecules on AC, IL-10 may suppress T-cell proliferation through reduced transcription of autocrine growth factor genes. Although the effects of IL-10 on IL-2 expression have not been demonstrated, IL-4 mRNA expression was inhibited in two IL-10-treated Th0 clones. IL-10 also downregulated expression of IFN-γ in the two Th0 clones at both the protein and mRNA levels, as shown for murine and human Th1 clones (11, 17).

Additional experiments are needed to more clearly define the relationships between the inhibitory or costimulatory effects of IL-10 on Th-cell proliferation and regulation of cytokine gene and protein expression. Studies are in progress to compare the effects of exogenous IL-10 on IL-2, IL-4, IFN-γ, and IL-10 mRNA expression by bovine Th0, Th1, and Th2 clones that we have characterized as having differential responses to IL-10.

In summary, this study shows that in cattle, as in humans, IL-10 does not discriminate between Th1 and Th2 cells, but downregulates AC-dependent proliferation by different subtypes of T cells, independent of other cytokines expressed. Thus, the cytokine network that regulates Th1 and Th2 cells that has been defined for inbred strains of mice may not be generally applicable to all mammals (11). Our studies further show that the bovine host provides an additional system with which to study T-cell responses against selected pathogens in outbred species.

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