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Antigen-Specific Lymphocyte Transformation Induced by Oocyst Antigens of *Eimeria bovis*†

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Lymphoproliferative responses against a preparation of *Eimeria bovis* antigens (EBAg) were measured in *E. bovis*-immune and naive animals. Optimal lymphocyte responsiveness could be measured after 7 days of culture in the presence of antigen at a cell concentration of 2×10^5 cells per well. The specificity of the reaction was confirmed by limiting dilution analysis. Whereas immune peripheral blood mononuclear cells responded to EBAg ($f = 1/18,824$), naive cells did not ($f = 0$). The helper function of cells proliferating in response to EBAg was investigated by raising T-cell lines and a clonal population derived from a line. The T-cell line showed an enhanced reactivity to EBAg by limiting dilution analysis ($f = 1/256$) and was interleukin-2 dependent. Limiting dilution analyses indicated at least two populations of cells: one that was interleukin-2 restricted and antigen dependent and another that was antigen independent. Supernatants from T-cell lines and the clone were analyzed for the production of lymphokines after antigen stimulation. Minimal amounts of interleukin-2 were produced. The T-cell line produced both gamma interferon (IFN-γ) (750 U) and IFN-α (1,250 U), whereas the clone produced IFN-γ (1,250 U) only. Short-term (4-day) stimulation of immune cells by EBAg induced the production of IFN-γ (600 U) and a non-IFN macrophage-activating lymphokine. We conclude that this macrophage-activating lymphokine is only produced after short-term culture and that further culture of T cells results in the proliferation of other clones producing other factors (such as IFN).

Infection of animals or humans with coccidian parasites results in immunity against subsequent lethal challenge with the same species. Resistance to coccidian parasites, including *Eimeria* species and *Toxoplasma gondii*, is predominantly cell mediated, and specifically committed T lymphocytes or soluble mediators (lymphokines) appear to play an essential role in the control of infection (1, 11). Recently, we described the existence of a lymphokine, produced by concanavalin A (ConA)-stimulated T lymphocytes from *Eimeria bovis*-immune cattle (9, 19), that is capable of inducing microbistatic function in naive murine macrophages (9) or a putative bovine monocyte cell line (9, 19). This macrophage-activating lymphokine does not seem to share the same biological properties as gamma interferon (IFN-γ) or granulocyte-macrophage colony-stimulating factor (9), both of which have microbistatic effects against protozoan parasites (13, 17).

The kinetics of antibody responses in calves infected with *Eimeria* species have been partially characterized (14) (W. M. Whitmire, personal communication), and the antigenic profiles of *E. bovis* sporozoites and merozoites have been at least partially characterized (18). However, nothing is known regarding the T-cell response of bovine lymphocytes or their resultant function. This study was designed to assess how animals reacted after primary infection with *E. bovis* and whether any cellular response resulted in helper T-cell function. We have started by analyzing a crude preparation of *E. bovis* sporulated oocysts. Later reports will define further those antigens that elicit a cellular response.

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MATERIALS AND METHODS

Parasites and antigens. *E. bovis* oocysts were separated from feces by sugar flotation, sporulated in aqueous 2.5% $K_2Cr_2O_7$ (9, 18), and stored at 4°C for not more than 5 months before use. Soluble antigen from *E. bovis* oocysts (EBAg) was prepared essentially as described before (6, 7). Oocysts were vigorously mixed with glass beads for 20 min until all oocysts, sporocysts, and sporozoites were destroyed. The homogenate was centrifuged at $600 \times g$ (10 min), and the supernatant was aspirated and further centrifuged at $11,000 \times g$ (10 min). The resulting supernatant was sterile filtered (0.22-μm-pore-size filter) and dialyzed against RPMI 1640 medium. Concentrations of EBAg are defined in terms of the number of sporozoites used to make the solution; i.e., a concentration of 10^3 ml^{-1} would be equivalent to having 10^3 sporozoites per ml; 2×10^7 EBAg units gave a protein concentration of approximately 1 mg ml^{-1} (12).

T. gondii (RH strain; originally obtained from Milford N. Lunde, National Institutes of Health) was grown in continuous culture by using a bovine monocyte cell line as the host (8).

Lymphocyte preparation. Two-month-old Holstein-Friesian bull calves were infected with *E. bovis* oocysts as described previously (9, 19). Preliminary work showed that they were refractory to reinfection 28 days after shedding oocysts. These animals were therefore defined as immune and were used as a source of antigen-reactive lymphocytes. Controls comprised age- and sex-matched naive animals with no evidence of infection. Blood was taken aseptically into heparinized Vacutainer tubes and diluted 1:2 in Hanks balanced salt solution, and 25 ml was overlaid on 15 ml of histopaque (Sigma Chemical Co). After centrifugation ($600 \times g$, 35 min), peripheral blood mononuclear cells (PBMC) were aspirated from the interface, washed twice in Hanks balanced salt solution, and suspended at the desired concen-

tration in RPMI 1640 medium supplemented with glutamine (20 mmol), penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}), and fetal bovine serum to a concentration of 10% (RPMI-FBS). PBMC were T cell enriched by passage down a column of nylon wool (10). In some experiments, interleukin-2 (IL-2)-dependent, activated T cells were purified by centrifugation over a discontinuous Percoll gradient. The following concentrations (in RPMI-FBS) of Percoll were overlaid in a 15-ml centrifuge tube: 40% (5 ml), 35% (1.5 ml), 31.3% (2.5 ml), 26% (2.5 ml). PBMC in RPMI-FBS (1.5 ml) were added to the gradient. After centrifugation at 600 × *g* for 30 min, activated (i.e., IL-2-dependent) lymphocytes could be drawn off from the interface of the second and third layers. Feeder cells were prepared from isolated PBMC (above) and then incubated at 2×10^6 ml^{-1} with 25 μg of mitomycin C per ml. After cells were washed four times in RPMI-FBS, they were then plated out at 2×10^5 to 4×10^5 ml^{-1}.

Lymphocyte transformation. Lymphocyte transformation experiments were carried out essentially as described before (6, 7). PBMC were prepared and incubated at 1×10^6 or 2×10^6 ml^{-1} in the presence of EBAg or mitogen (ConA). Triplicate cultures of 1×10^5 or 2×10^5 cells per well were prepared in U-form microwell trays by the addition of 100 μl of cell suspension and 100 μl of antigen (EBAg), mitogen (ConA), or RPMI-FBS. EBAg was used at concentrations from 10^7 to 10^5 units, and ConA was used at concentrations from 20 to 1 μg ml^{-1}. After 120 h, 168 h (with antigen), or 72 h (with mitogen) of culture at 37°C in a humidified atmosphere of 5% CO_2 in air, the mitogenic response to antigen and mitogen stimulation was assessed by the addition of 0.5 μCi of [*methyl*-^{3}H]thymidine ([^{3}H]TdR; specific activity, 2.0 Ci $mmol^{-1}$; New England Nuclear Corp., Beverly, Mass.) in 20 μl of RPMI. After a further 4 h at 37°C, cells were harvested onto glass fiber filters and prepared for β-spectroscopy. Results are expressed either as increased counts per minute over unstimulated cultures or as counts per minute.

IL-2. IL-2 came from three sources. Bovine recombinant IL-2 was a kind gift from Paul E. Baker, Immunex Corp., Seattle, Wash. Gibbon IL-2 was produced by the gibbon thymona cell line MLA 144 (16). Crude bovine IL-2 was prepared from PBMC incubated at 10^7 cells ml^{-1} in RPMI-FBS containing 3 μg of ConA per ml at 37°C for 24 h. Supernatants were aspirated, and excess ConA activity was neutralized with 20 mmol of α-methyl mannoside. All IL-2 preparations were sterile filtered (0.22-μm-pore-size filter) before use.

IL-2 was assayed by using ConA-activated T cells. PBMC were isolated and stimulated at high density with ConA, and activated cells were purified over discontinuous Percoll. Cells were then cultured at 2×10^5 cells per well in 24-well cluster trays in RPMI-FBS containing either ≈3.3 U of recombinant IL-2 per ml or 10% MLA 144 supernatant and 15% crude bovine IL-2. Cells were incubated at 37°C for 3 to 4 days and subcultured at 2×10^5 cells per well. After at least 2 weeks of culture, cells were used for a standard IL-2 assay (15). Cells (2×10^4) were placed in triplicate wells of a microdilution tray in a total volume of 200 μl of RPMI-FBS containing either recombinant IL-2 at a known concentration, an unknown concentration of IL-2, or RPMI-FBS alone. After incubation for 24 h, cultures were pulsed with 0.5 μCi of [^{3}H]TdR for a further 24 h, harvested onto glass fiber filters, and prepared for β-spectroscopy. Results are expressed as increased counts per minute, and units of IL-2 were calculated from the regression curve of increased counts per minute versus IL-2 concentration.

Production of microbistatic lymphokines. Lymphokines capable of activating macrophages to kill or inhibit the growth of intracellular parasites were present in the supernatants of bovine PBMC (2×10^6 cells ml^{-1}) cultures after 3 days of incubation with ConA (5 μg ml^{-1}) (19). These supernatants were used as a source of crude activating factor, against which other preparations from antigen-activated cultures were tested.

Limiting dilution analysis. Limiting dilution analysis was carried out essentially as described by others (4, 21). Responder cells (10×10^5 to 2×10^5 $well^{-1}$) were plated into 96-well U-form culture plates in 100 μl of RPMI-FBS with 12 to 96 replicates per responder number. Spontaneous IL-2- or antigen-induced proliferation was assessed in wells containing 2.5×10^4 autologous mitomycin-C-treated PBMC in a total volume of 200 μl. Plates were cultured for 3 days to determine IL-2-induced proliferative precursor frequencies and for 10 days to determine antigen-induced proliferative precursor frequencies. Previous studies had established that extension of incubation time did not influence estimates of frequencies but increased quantitative differences between positive and negative wells. Microcultures of responder cells or feeder cells cultured alone served as background controls. Proliferation was assessed after an 18-h pulse with [^{3}H]TdR (0.5 μCi $well^{-1}$) at the end of the 3- or 10-day incubation period. Cultures were harvested onto glass fiber filters, and radioactivity was measured by β-spectroscopy.

Assessment of results. Test wells were considered positive when values exceeded the mean control value by three standard deviations. Linear regression analysis of the number of responders plated against log percent wells negative yielded the line equation from which the minimal estimate of frequencies could be calculated as that number of cells plated corresponding to 37% negative wells. The significance of the difference between the lines for the same experiment was calculated as described by Taswell (20).

Microbicidal assays. The bovine monocyte cell line M617 (9, 19) was used as a source of host cells. M617 cells were routinely maintained in RPMI-FBS containing 10^{-5} M 2-mercaptoethanol (9, 19). Inhibition of parasite development or multiplication in M617 was assessed visually and by the uptake of [^{3}H]uracil by *T. gondii*. This is proven as a good model for microbistatic activity (9). M617 monolayers in 96-well cluster trays were treated with supernatants known to contain activating factors or unknown lymphokine preparations from T-cell lines (TCL) or clones. After inoculation with 10^4 RH strain tachyzoites of *T. gondii* in 100 μl of RPMI-FBS, plates were incubated for 24 h at 37°C, pulsed for 18 h with 0.5 μCi of [^{3}H]uracil (specific activity, 41 Ci/mmol; New England Nuclear Corp.), and harvested, and the uptake of uracil was assessed by β-spectroscopy. Growth of parasites was also assessed visually (9) and in all cases agreed with uptake of [^{3}H]uracil, unless otherwise stated. Growth of *E. bovis* was assessed by using Giemsa-stained, sporozoite-infected M617 monolayers and by the uptake of [^{3}H]uracil (9, 19). Control cultures comprised parasites cultured in the absence of cells, cells in the absence of parasites, and parasites with cells that had been preincubated for 24 h in the presence of human recombinant IFN-γ.

IFN assay. The production of acid-labile and acid-stable IFN by TCL and clones was assessed as described previously (2). All samples were divided in half; one half was dialyzed at pH 2 against a 0.1 mM glycine hydrochloride buffer in saline for 24 h at 4°C and then exposed to further

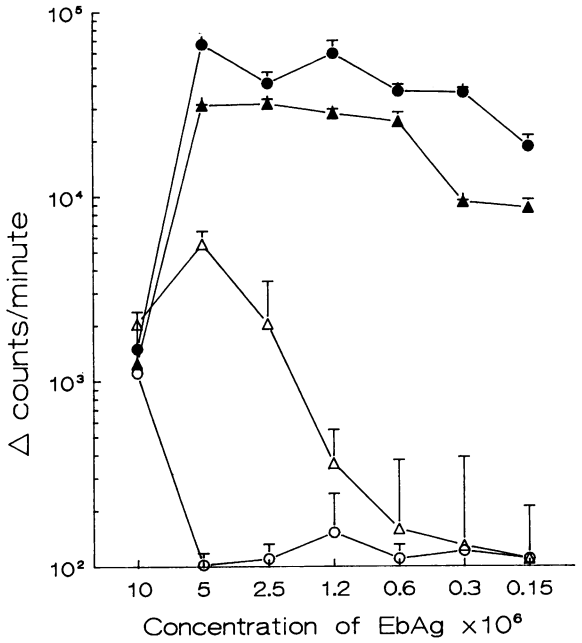

FIG. 1. Uptake of [^{3}H]TdR by lymphocytes from immune and naive calves. Symbols: (○, △) responses by naive animals; (●, ▲) responses by immune animals; (○, ●) 5 days of incubation; (△, ▲) 7 days of incubation. Enhanced responses with greater specificity occurred after 7 days.

dialysis against phosphate-buffered saline (twice) and RPMI 1640. After dialysis, all samples were sterile filtered (0.22-μm-pore-size filter). The plaque reduction assay for IFN was carried out exactly as described previously (2). Briefly, Madin-Darby bovine kidney (MDBK) cells were plated in flat-bottomed, 96-well cluster trays at 3×10^4 cells per well. Serial dilutions of test supernatant or human IFN-γ standard were added to each well. After 24 h of incubation at 37°C, medium was removed, and a thawed sample of vesicular stomatitis virus (previously quantified) was diluted so that each well would receive 3 to 3.5 infectious particles in 100 μl. After 1 h of incubation at 37°C, unattached virus was removed, and each well was overlaid with 100 μl of 0.25% agarose in medium. After further incubation (up to 40 h), agarose was removed, wells were stained with crystal violet, stain was removed, and plaques were counted. IFN activity was calculated by established formulae (2).

Bovine TCL and clones. Bovine TCLs and clones were initiated and cultured essentially as described previously for the establishment of human TCL or clones (3). PBMC were isolated from the peripheral blood of a bull calf that was known to have excellent T-cell responses to EBAg in vitro. PBMC were cultured in RPMI-FBS containing 2-mercaptoethanol and the optimal concentration of EBAg in 24-well cluster trays (1 ml; 2×10^6 cells per well with 1.25×10^6 U of EBAg). After 4 days of incubation, 750 μl was aspirated from each well and replaced with RPMI-FBS containing IL-2. After 5 days of growth, it appeared that most cells had undergone mitogenesis, and they were subcultured into 24-well plates at 10^5 ml^{-1} in RPMI-FBS with IL-2. TCL were subcultured in medium containing IL-2 every 3 to 4 days, with passage onto feeder cells with EBAg (as above) at approximate 10-day intervals.

After the initial EBAg stimulation, cells were cloned by limiting dilution into U-form microplates containing autologous feeder cells (10^5 ml^{-1}) in medium with IL-2 and EBAg. After a further 4 to 5 days, medium was replaced with fresh medium containing IL-2. When visible growth occurred with cells plated at 0.3 or 1 cell per well, cultures were expanded into 24-well plates and recloned by limiting dilution. A single colony arising from a recloned culture was defined as a clone. Antigen stimulation was carried out whenever T cells were incubated with feeders. TCL or clones were periodically frozen in dimethyl sulfoxide containing medium after either antigen or IL-2 stimulation.

After at least 1 month of culture, TCL were tested for their ability to respond to antigen by using a lymphocyte transformation test. Of the TCL tested, one gave a particularly good response and was further analyzed. This consisted of limiting dilution analysis of the proliferative frequency of response to IL-2 and EBAg and the ability of TCL to secrete lymphokines such as IFN, IL-2, and macrophage-activating factors.

RESULTS

Lymphocyte transformation. Initial studies were carried out to assess the optimal conditions for the stimulation of immune bovine lymphocytes with EBAg. Initial studies showed that the optimal concentration of PBMC for mitogen or antigen stimulation was 2×10^6 ml^{-1}. This cell concentration was therefore used throughout the study for all antigen stimulation of T lymphocytes. In a series of experiments, PBMC were incubated with various concentrations of antigen for different lengths of time. The results for 5 or 7 days of incubation are shown in Fig. 1 and Table 1. After 5 or 7 days of incubation, there was adequate lymphocyte responsiveness by PBMC from immune animals, but lymphocytes from nonimmune animals also incorporated significant amounts of [^{3}H]TdR at 5 days (Fig. 1). After 7 days, however, there was no evidence of any increased uptake of [^{3}H]TdR by naive donors (Fig. 1). Seven days was therefore used as the optimum time period for lymphocyte stimulation. The concentration of EBAg used for further studies was between 1×10^6 and 2×10^6, based on the curves generated in Fig. 1.

Proliferative frequency of response to EBAg. Lymphocytes from immune or naive PBMC or TCL were plated out under limiting dilution conditions in the presence of optimal concentrations of EBAg. Lymphocytes from TCL showed an increasing number of responders compared with either autologous (immune) PBMC or cells from an allogeneic individual. The maximum incorporation of [^{3}H]TdR was either the

TABLE 1. Responses of lymphocytes from PBMC and TCL to EBAg and ConA[a]

Lymphocytes	Increased cpm with stimulation by:		
	ConA	EBAg (5 days)	EBAg (7 days)
PBMC[b]	47,827 ± 4,306	31,772 ± 2,749	41,030 ± 6,217
Unstimulated	946 ± 331	668 ± 505	557 ± 268
TCL		77,175 ± 6,745	69,165 ± 3,425
Control	44,118 ± 18,252	5,525 ± 2,015	191 ± 119

[a] EBAg responses in both TCL and PBMC are either optimal or supraoptimal when compared with responses from PBMC. Decreased reaction to EBAg by control cells is due to the specificity of the reaction rather than lack of responsiveness by control PBMC. As negative cells gave little or no reaction after 7 days compared with that after 5 days of incubation, this time period was chosen.

[b] PBMC were from the donor calf for the TCL. Unstimulated lymphocytes were used to determine background counts per minute.

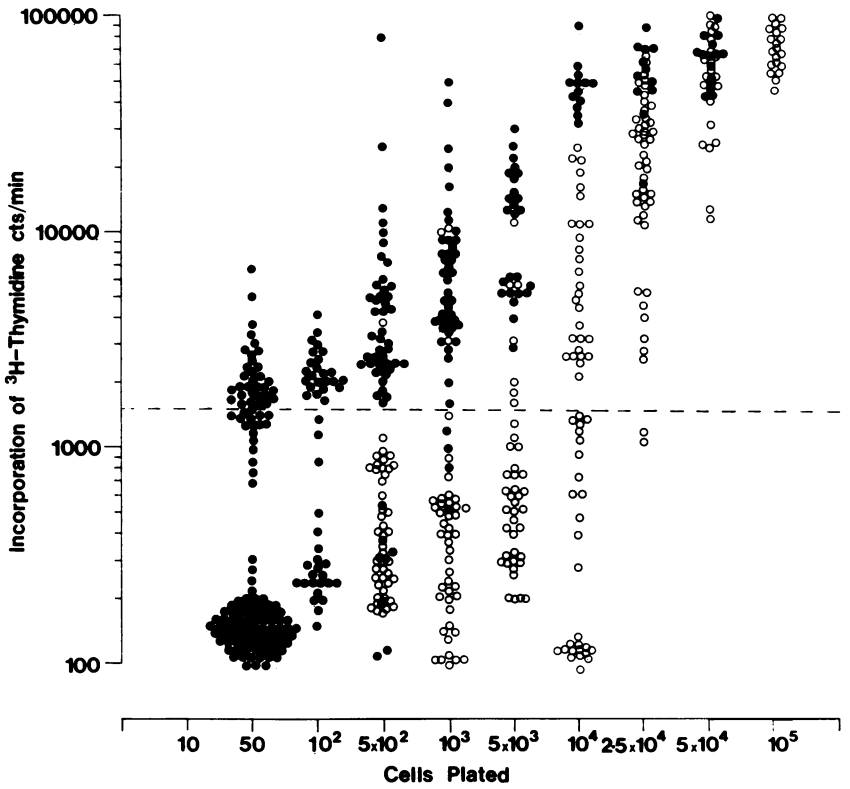

FIG. 2. Limiting dilution analysis of the frequency of cells responding to EBAg. Increasing numbers of responders were plated, and uptake of [^{3}H]TdR was measured on day 10. Wells were considered positive when the uptake exceeded the mean of feeders alone by 3 standard deviations (broken line). Symbols: (○) response by PBMC; (●) response by TCL. No cells from a naive animal responded (data not shown).

same or better in the TCL compared with that in immune lymphocytes (Fig. 2). This indicates that optimal responses were occurring in both instances, particularly since the mean [^{3}H]TdR incorporation was as good as or better than that of cells stimulated by ConA (Table 1). These data were transformed into straight-line plots of log percent wells negative against the number of cells plated. Lines intercepted the *y* axis at approximately 100, and minimal estimates of limiting frequency of EBAg-responsive cells were 1/249 in TCL and 1/18,824 in PBMC (Fig. 3). There was no measurable response by allogeneic (negative) PBMC (Fig. 3) (full data not shown). There was no significant difference between the control groups ($P > 0.05$), and the mean counts per minute of the three pooled groups was 452 ± 348, which gave a positive threshold of 1,496 cpm (mean + 3 standard deviations).

Proliferative frequency of response to IL-2. Lymphocytes from TCL or PBMC were plated out as described above in medium containing optimal amounts of IL-2 with α-methyl mannoside to block any exogenous ConA activity. Increasing the number of responders resulted in a greater proportion of positive cultures (Fig. 4). At high density, cells from TCL showed a decreased incorporation of [^{3}H]TdR, presumably due to overcrowding and utilization of available IL-2 before the assay had come to completion. Control cultures were not significantly different from each other ($P > 0.05$) and gave a positive threshold (mean + 3 standard deviations) of 1,506 cpm (Fig. 4). After transformation of data to linear plots (Fig. 5), the frequency of cells from TCL that responded to IL-2 was estimated as 1/50, whereas from PBMC it was estimated that only 1 of 273,000 cells responded.

Production of lymphokines by TCL and clones. TCL and clones were analyzed for their production of lymphokines. The production of IL-2 after antigen stimulation was assessed and compared with the activity of recombinant IL-2. Previous studies have shown that optimal concentrations of

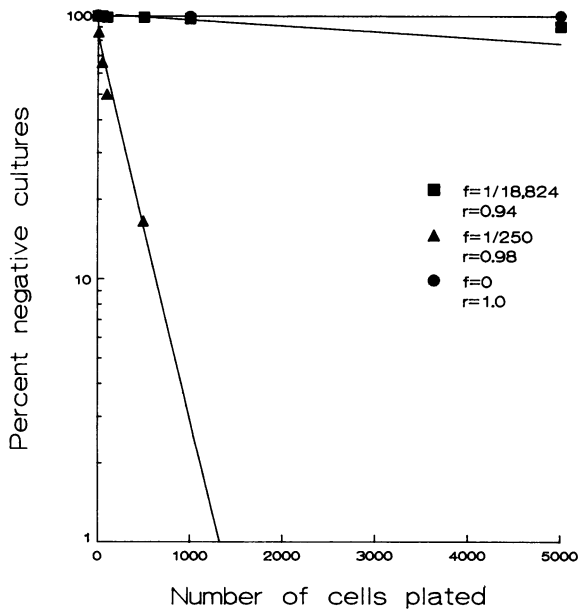

FIG. 3. Frequency determination of cells responding to EBAg from a plot of number of cells plated against log percentage of wells that were negative.

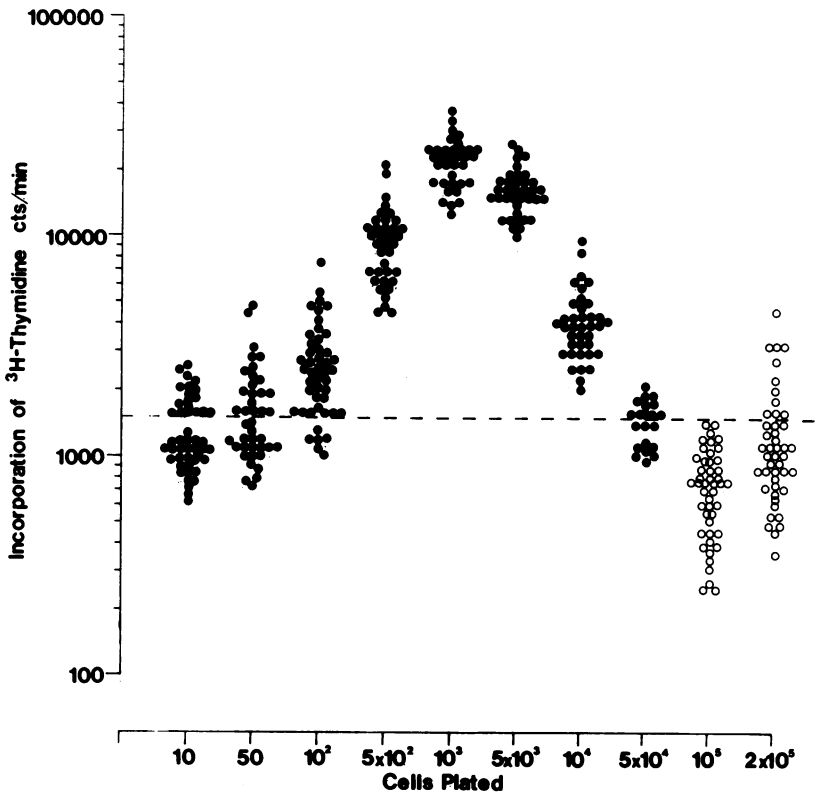

FIG. 4. Limiting dilution analysis of the frequency of cells responding to IL-2. Increasing numbers of responders were plated, and uptake of [^{3}H]TdR was measured on day 3. Wells were considered positive when the uptake exceeded the mean of feeders alone by 3 standard deviations. Symbols: (○) response by PBMC; (●) response by a TCL.

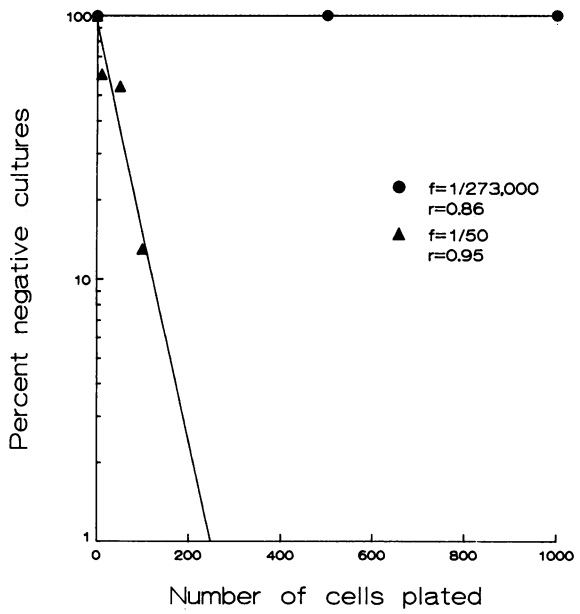

FIG. 5. Frequency determination of cells responding to IL-2 from a plot of the number of cells plated against log percentage of wells that were negative.

recombinant IL-2 for the maintenance of bovine T lymphocytes is approximately 3 U ml^{-1} (P. E. Baker, personal communication). When stimulated with EBAg, PBMC produced 0.6 U ml^{-1}, cloned T lymphocytes produced 0.3 U ml^{-1}, and TCL did not produce any detectable IL-2. ConA stimulation of PBMC used to provide IL-2 for growth of TCL and clonal populations resulted in the production of 5 U of IL-2 ml^{-1} (Table 2).

Lymphocytes were tested for their ability to produce macrophage-activating lymphokines (Fig. 6). Incubation in the absence of lymphokine resulted in the proliferation of *T. gondii*, with concurrent increased uptake of [^{3}H]uracil (Fig. 6). When supernatants from bovine lymphocytes stimulated with ConA were used, inhibition of parasite growth occurred, as indicated by the decreased uptake of [^{3}H]uracil (Fig. 6). Similarly, stimulation of lymphocytes with EBAg

TABLE 2. Production of IL-2 by PBMC, TCL, and a clone after EBAg stimulation

Supernatant	Increased cpm	IL-2 (U ml^{-1})
Recombinant IL-2[a]	58,260	10
ConA (3 days)[b]	25,464	4
EBAg (4 days)[c]	7,848	<0.1
TCL[d]	4,399	<0.1
T-cell clone[e]	3,563	<0.1

[a] Bovine recombinant IL-2 was a kind gift from P. E. Baker.
[b] PBMC were incubated in the presence of IL-2 for 3 days.
[c] PBMC were stimulated for 4 days with EBAg.
[d,e] Supernatants were taken from the TCL or clone during antigen stimulation.

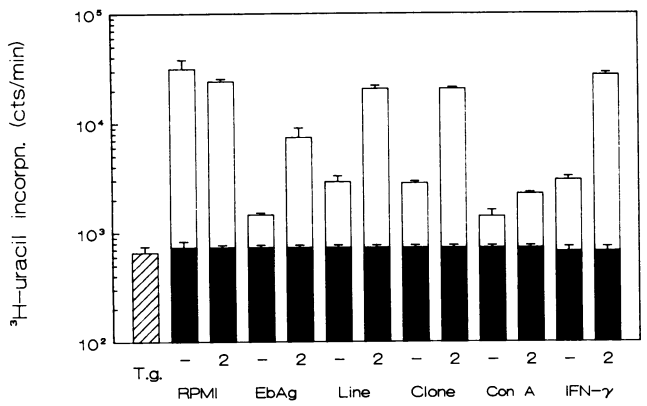

FIG. 6. Inhibition of *Toxoplasma* multiplication within M617 cells. M617 cells were incubated in different dilutions of lymphokine preparation for 24 h, infected with 10^4 *Toxoplasma* tachyzoites (24 h), and pulsed with [^{3}H]uracil (18 h). Bars: (▨) uptake by *T. gondii* alone, (■) uptake by host cells alone, and (□) increased uptake of uracil by parasitized cells. The effects of pretreating supernatants with glycine hydrochloride (bars 2) are shown. −, Supernatants that remained untreated. Supernatants from EBAg- or ConA-stimulated cells and from the TCL (Line) and clone were prepared as described in Materials and Methods. Human recombinant IFN-γ was used.

also resulted in decreased uptake of uracil to levels similar to that of ConA-stimulated cells (Fig. 6). Significantly, TCL and a cloned population (Fig. 6) both induced killing, although not to the same extent as either ConA or EBAg. After dialysis against a glycine hydrochloride buffer (pH 2), microbicidal lymphokines produced by ConA-stimulated lymphocytes were stable, consistent with data previously reported (9). Lymphokines produced from a 4-day EBAg stimulation of PBMC were only partially stable at pH 2, and those from the TCL and clone appeared to be totally labile at pH 2 (Fig. 6). When *E. bovis* was used as the infective agent in the microbicidal assay, similar results were obtained. Supernatants from both ConA-stimulated and EBAg (4 days)-stimulated lymphocytes both induced effective inhibition of meront development. Those from the TCL and clone only inhibited growth to a degree, and only supernatants from ConA- or EBAg (4 days)-stimulated cultures were stable at pH 2 to any degree. Human recombinant IFN-γ stimulated M617 cells to inhibit the growth of *E. bovis* and *T. gondii* to a lesser extent (Table 3).

TABLE 3. Ability of different sources of lymphokines to inhibit *E. bovis* meront development

Supernatant[a]	No. of meronts[b] ± SD		% Inhibition	
	pH 7[c]	pH 2[d]	pH 7	pH 2
Recombinant IFN-γ	0 ± 0	16 ± 3	100	0
ConA (3 days)	0 ± 0	0.3 ± 0.8	100	98
EBAg (4 days)	0 ± 0	1.4 ± 2	100	90
TCL	0 ± 0	13.2 ± 0.9	100	6
T-cell clone	0 ± 0	7 ± 1.58	100	50
RPMI	14 ± 1.6		0	

[a] See footnotes to Table 2.
[b] Assessed 10 to 14 days after inoculation of M617 monolayers with *E. bovis* sporozoites.
[c] Sham-treated lymphokine preparations.
[d] Preparations were dialyzed against glycine hydrochloride.

TABLE 4. Production of IFNs by immune lymphocytes after stimulation by *E. bovis* antigens[a]

T lymphocyte	IFN production (U ml^{-1})	
	pH 7.4[b]	pH 2.0[c]
EBAg (4 days)[d]	600	0
TCL[e]	2,000	1,250
T-cell clone[f]	1,250	0
RPMI 1640[g]	0	0

[a] IFN was assessed by using a standard vesicular stomatitis virus inhibition assay with MDBK as host cells.
[b] Supernatants were kept at 4°C for 48 h.
[c] Supernatants were dialyzed against glycine hydrochloride for 24 h and then against RPMI 1640 for 24 h.
[d] See footnote *c* of Table 2.
[e,f] Cells from the TCL or clone were stimulated with antigen after 2 months of culture for 5 days between passages with IL-2.
[g] MDBK in culture medium alone.

Production of IFNs. The ability of supernatants to inhibit viral growth in MDBK cells was assessed by using human IFN as a standard (Table 4). The TCL produced the most IFN (2,000 U ml^{-1}), the majority of which appeared to be acid stable. The clone produced acid-labile IFN, and the 4-day stimulation with EBAg produced relatively little IFN of either type (Table 4).

DISCUSSION

In these preliminary studies, we have outlined some of the cellular events after stimulation of bovine lymphocytes from calves immune to challenge by *E. bovis*. The rationale for using the crude EBAg preparation was as follows. (i) The definition of immunity against the invasive stage of the parasite is arguably more important than immunity against other stages (merozoite, gamete). Any applied goals (e.g., a new serodiagnostic test or a vaccine) should relate to the stage of the parasite that the host first encounters; if antigenic variation occurs, then false results may be encountered in tests or any vaccine may be ineffective until there has been extensive damage to host cells during invasion. (ii) EBAg can be produced very simply from flotations from infected feces. Sporozoites can be purified from excysted sporozoites, but usually this results in a considerable loss of yield (18). (iii) EBAg contains antigens that would be released by oocysts during rupture in the gut; the use of EBAg means that responses to oocyst and sporozoite antigens are being measured.

The response of PBMC to EBAg was remarkably specific; the results presented herein have been repeated in 10 more animals. Furthermore, four different batches of EBAg have been prepared, and there has been no significant difference between batches (data not shown). Immune and naive animals reacted equally well to ConA, indicating that any differences in the reaction to EBAg were due to differences in specificity rather than individual differences in T-cell reactivity. Background counts per minute of unstimulated cells were essentially the same (Table 1). Increasing the incubation time from 5 to 7 days appeared to reduce nonspecific reaction by naive PBMC to EBAg while increasing the reaction by immune lymphocytes (Fig. 1), perhaps because of the death of lymphocytes resulting from the greater incubation time.

The reactivity of immune lymphocytes and subsequent function was further investigated with TCL. First, limiting

TABLE 5. Summary of lymphokine release by EBAg-stimulated cells

Supernatant	Lymphokine release		
	IL-2	IFN	MAF[a]
ConA[b]	+++	++	+++
EBAg (4 day)[c]	±	+	+
TCL[d]	±	+++	−
T-cell clone[e]	±	++	−

[a] MAF, Acid-stable lymphokine.
[b] PBMC were stimulated for 3 days with 5 μg of ConA per ml.
[c] Immune PBMC were stimulated with EBAg for 4 days.
[d,e] Supernatants from a TCL and clone after EBAg stimulation.

dilution analysis confirmed the specificity of the EBAg reactivity; the minimal estimate of EBAg responsiveness was 0 for naive animals, compared with 1/18,824 for immune PBMC. These experiments have been repeated on two separate occasions, with similar results. As expected, the TCL had a greater number of cells responding than PBMC, presumably due to further antigen selection of the reactive clones from the original mixed cell population. The specificity of this reaction is noteworthy; similar preparations from other coccidian parasites (e.g., *T. gondii*) are generally not specific in lymphocyte transformation tests (6, 7, 22) and can induce nonspecific cellular immune reactions (5). Limiting dilution analysis of IL-2-responsive cells showed that the TCL was largely IL-2 dependent, with a minimal estimate of 1/50.

The results from limiting dilution analysis indicate that, whereas the line was almost totally IL-2 dependent, it did not uniformly respond to antigen. This is consistent with studies in other systems, which have shown the activation or proliferation of unrestricted cells after stimulation of PBMC (e.g., NK cells) (1, 5). It is possible that similar events are happening after EBAg stimulation, although while there is neither an adequate NK assay nor phenotypic markers for bovine lymphocytes, this hypothesis cannot adequately be tested.

Previously, we described the possible existence of a non-IFN, non-granulocyte-macrophage colony-stimulating factor microbicide-inducing activating factor from supernatants of ConA-stimulated T lymphocytes with an adapted *T. gondii* killing assay (9, 19). The TCL and clone under investigation were tested for their ability to release lymphokines with macrophage-activating potential as well as IL-2 (Table 5). In all cases, EBAg stimulation did not induce the production of significant amounts of IL-2. IFN (as measured with a vesicular stomatitis virus plaque inhibition assay) was produced in large quantities by the TCL and clone but in relatively small quantities by a short-term incubation period with EBAg. The TCL produced both acid-labile and acid-stable IFN, whereas the clone only produced acid-labile IFN (IFN-γ). When assayed for microbistatic function in a *Toxoplasma* killing assay, it appeared that only ConA induced production of large amounts of acid-stable (non-IFN) microbistatic lymphokine (9). Short-term (4-day) incubation of PBMC appeared to produce only a limited amount of this acid-stable lymphokine. Lymphokines from the TCL and clone both had similar microbistatic activities. Native lymphokine appeared to have some microbistatic function, although this was reduced to zero after pH 2 treatment. These results indicate that this activity is due to the action of IFN-γ rather than any non-IFN lymphokine, particularly since IFN-γ does not totally inhibit *Toxoplasma* replication (Fig. 6).

It appears that EBAg induces the proliferation of T lymphocytes producing non-IFN microbistatic lymphokines but only in short-term cultures. After this period, the generation of other factors occurs, including increased production of IFNs. We are investigating the production of lymphokines by preparations of EBAg antigens separated by chromatography and how the outgrowth of clones producing non-IFN lymphokines can be prevented by either cloning early or presenting only those antigens that induce the required function. These results do demonstrate that the non-IFN lymphokine can be produced after antigen stimulation and that its production is not limited to mitogen-driven systems (9, 19).

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