INFECTION AND IMMUNITY, May 1990, p. 1146–1152
0019-9567/90/051146-07$02.00/0

Vol. 58, No. 5

T-Cell Hybridomas Reveal Two Distinct Mechanisms of Antileishmanial Defense

JOSEPH P. SYPEK* AND DAVID J. WYLER

Division of Geographic Medicine and Infectious Diseases, Department of Medicine, The New England Medical Center Hospitals, and Tufts University School of Medicine, Boston, Massachusetts 02111

Received 6 September 1989/Accepted 25 January 1990

Using lymph node lymphocytes of *Leishmania major*-infected mice, we constructed and cloned two T-cell hybridomas that could activate macrophages to exert antileishmanial defense in vitro. One clone, 1D5, produced lymphokines (including gamma interferon) that induced these effects. Production of the macrophage-activating lymphokines and the protective effect of 1D5 were suppressed by the addition of cyclosporine A to cultures. The other clone, 1B6, produced no detectable macrophage-activating lymphokines, and its protective ability was not suppressed by cyclosporine A. Granulocyte-macrophage colony-stimulating factor (a lymphokine also known to induce antileishmanial effects in macrophages) was not detectable in culture supernatants of either clone. Furthermore, neither clone was cytotoxic to infected macrophages. Antileishmanial defense induced by 1B6 was genetically restricted; that is, infected macrophages and hybridoma cells had to be syngeneic for an antileishmanial effect to occur. In contrast, such restriction was not a property of clone 1D5, a clone that was responsive to alloantigens as well as leishmanial antigens. When incubated at a temperature (34°C) at which lymphokines are relatively ineffective for antileishmanial defense, 1B6 but not 1D5 retained its antileishmanial properties. These observations provide clear evidence for the existence of two distinct mechanisms of macrophage activation: one that is lymphokine dependent, and one that is apparently lymphokine independent. The expression of these two mechanisms by cloned cells strongly suggests that they are properties of different T-cell subpopulations, extending our prior conclusions based on studies of heterogeneous T-cell populations. We hypothesize that the latter macrophage activation process involves a cell contact-dependent mechanism which might involve the interaction of a lymphocyte membrane-associated macrophage-activating factor (such as tumor necrosis factor) with its receptor on the macrophage, resulting in activation of antileishmanial effects but not host cell cytotoxicity.

The essential role of T cells in defense against intracellular microbes was recognized more than two decades ago (13). In vitro studies revealed that specifically sensitized lymphocytes, when incubated with infected macrophages, could induce the expression of antimicrobial defense. Soluble factors contained in supernatants of these cocultures (or ones present in supernatants of lymphocytes stimulated with specific antigens or lectin mitogens) could also activate macrophages to exert such defenses (9, 23). These early studies inspired subsequent efforts at identifying the soluble T-cell-derived molecules (lymphokines) involved in macrophage activation. Recently, gamma interferon (IFN-γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF) have been identified as two such lymphokines (15, 25, 26, 46). In some of the earlier in vitro studies it was observed that antimicrobial defenses (against *Listeria monocytogenes*) could be induced in macrophages in the presence of intact lymphocytes but not by their supernatants (37, 38, 49). The observations suggested that lymphocyte-associated signals that induced macrophage activation might not be the sole property of lymphokines. Conceivably, activation signals might also be imparted by molecules present on the lymphocyte plasma membrane during direct cell-to-cell contact.

We recently obtained in vitro evidence that murine $CD4^+$ lymphocytes can activate antimicrobial defense in *Leishmania*-infected macrophages by an apparently lymphokine-independent mechanism that is not toxic to the host cell (30, 40). Specifically, we observed that heterogeneous populations of draining lymph node lymphocytes from *Leishmania*-infected mice, when incubated with *Leishmania*-infected macrophages, interfere with the parasite's intracellular growth (30). These antimicrobial effects occur only when the lymphocytes and macrophages are permitted direct contact with each other (30), when the lymphocytes and macrophages are syngeneic (42), and when the lymphocytes have specificity for antigens exposed on the infected macrophages (41). Moreover, we found that the addition of cyclosporine A (CsA) to our cultures does not interfere with the induction of the antimicrobial effects even though this drug inhibits the production of all detectable lymphokines capable of activating antileishmanial effects in macrophages (48). On the other hand, we could not ascertain whether the apparently lymphokine-independent (CsA-resistant) defense was the property of a unique subpopulation of lymphocytes (or also of lymphokine-secreting cells), since we used heterogeneous lymphocyte populations in these studies. We therefore prepared and cloned T-cell hybridomas from lymph node lymphocytes of *L. major*-infected mice and tested their ability to induce antileishmanial effects in vitro. The results of our observations provide evidence that the lymphokine-dependent and lymphokine-independent mechanisms of macrophage activation are the properties of distinct subpopulations of lymphocytes.

MATERIALS AND METHODS

Parasites. *L. major*, NIH Seidman strain (World Health Organization strain designation MHOM/SN/74 SEIDMAN), was originally isolated by one of us (D.J.W.) from a Peace Corps volunteer in Senegal (29).

Animals and infection. Female BALB/cAnNTacfBR

* Corresponding author.

(amastigote donor), C57BL/6NTacfBR, C3H/HeNTacfBR, and B6C3F1 (macrophage and lymphocyte donors) mice (18 to 20 g) were obtained from Taconic (Germantown, N.Y.). B10.D2/n SnJ (macrophage and lymphocyte donors) mice were obtained from Jackson Laboratories (Bar Harbor, Maine). Mice were infected subcutaneously into each hind footpad with 0.05 ml of a suspension containing 2×10^5 tissue-derived amastigotes (30) in 0.15 M NaCl.

Effector lymphocytes. T lymphocytes were derived from draining popliteal lymph nodes excised from C57BL/6 mice infected for 5 weeks with *L. major* (30). Hybridomas were produced directly from lymph node lymphocyte suspensions without prior in vitro expansion and were cloned by customary methods. The rationale for this protocol was based on our concern that conditions required for in vitro T-cell expansion (antigen and interleukin-2 [IL-2] stimulation) might selectively promote the overgrowth of cells that lacked the biological properties we sought to study. Following nylon wool column enrichment (19) for T cells, lymphocytes were fused with cells of the 8-azaguanine-resistant thymoma line BW5147, as described before (14). T-cell hybrids were cloned and subcloned twice by limiting dilution and expanded in continuous culture. Hybrids were assayed for their ability to induce antileishmanial activity in the in vitro assay we have described previously (30).

Lymphokine preparations. Lymphokine-containing culture supernatants were prepared by published methods (27). Briefly, suspension cultures of splenic lymphocytes from C3H/HeN mice infected with *Mycobacterium bovis* BCG (Connaught Laboratories, Willowdale, Ontario, Canada) were stimulated with purified protein derivative of tuberculin (PPD; 50 to 100 μg/ml; Connaught). The BCG-PPD conditioned medium was harvested after 24 h of incubation of splenocytes, filter sterilized (0.22-μm filter; Millipore Corp., Bedford, Mass.), and stored at −70°C.

Colony-stimulating factor 1 (CSF-1)-enriched supernatants were obtained from cultures of murine L929 cells (CCL 1; American Type Culture Collection, Rockville, Md.) as described before (39). Conditioned medium from cultures of mouse lung tissue provided a source of natural GM-CSF (36). All culture supernatants were harvested after 24 h of incubation, filter sterilized (0.22 μm), and stored at −70°C.

Recombinant murine GM-CSF (rGM-CSF; 5×10^7 CFU/mg) from *Saccharomyces cerevisiae* was obtained from Genzyme Corp. (Boston, Mass.).

Assay for antileishmanial activity. The ability of hybrid cells, T-cell clones, lymph node lymphocytes, and culture supernatants to activate *Leishmania*-infected macrophages was assessed by methods we previously described (30). Resident macrophages were harvested by peritoneal lavage with Hanks balanced salt solution without Ca^{2+} and Mg^{2+}. Peritoneal cells were washed and suspended in supplemented medium containing RPMI 1640, 10% heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories, Logan, Utah), 5×10^{-5} M 2-mercaptoethanol, penicillin (100 U/ml), streptomycin (100 μg/ml), and 4 mM L-glutamine. Two hundred thousand cells were combined with 2×10^5 to 4×10^5 *Leishmania* amastigotes in polypropylene tubes (12 by 75 mm; Falcon Labware, Becton Dickinson, Lincoln, N.J.) in a total volume of 0.8 ml. Lymphocytes were added to infected macrophages 24 h later (1 to 10 hybrid cells per macrophage); suspension cultures were incubated for an additional 48 h at 37°C either with or without 1 μg of CsA (Sandimmune I.V.; Sandoz, Basel, Switzerland; stock solution was provided as 50 mg of CsA per ml dissolved in a 32.9% solution of 650-mg/ml polyoxyethylated castor oil in alcohol and was subsequently diluted in supplemented medium) per ml.

We used our previously described methods (27) to assess lymphokine effects on infected macrophages in suspension cultures. Supernatants were retrieved after 24 h of coincubation of infected macrophages and T-cell hybridomas. The supernatants (conditioned medium) were subsequently added at 20% (vol/vol) final concentration (in fresh culture medium) to fresh suspension cultures of resident macrophages that had been infected 4 h previously; incubation at 37°C was continued for a total of 72 h.

The percentage of infected macrophages and mean number of amastigotes per infected macrophage were determined microscopically. One hundred macrophages in remote fields were scored in Wright-Giemsa-stained (Diff-Quick, Dade Diagnostics, Aquada, P.R.) cytocentrifuge preparations (Shandon-Southern, Sewickley, Pa.) of individual cultures. Mean ± standard error of the mean (SEM) of amastigotes per 100 macrophages in cultures with or without the addition of lymphocytes or conditioned medium was compared by a one-tailed Student's *t* test. Antileishmanial effect was expressed as the percent reduction in parasites per 100 macrophages cultured with lymphocytes (or conditioned medium) compared with parasites in ones cultured without.

Bioassay by uptake of tritiated thymidine. The proliferative response of T-cell hybridomas (1×10^4 to 10×10^4) cultured in the presence or absence of 1×10^5 to 2×10^5 irradiated (2,000 rad) uninfected or *L. major*-infected macrophages was assayed by measuring the magnitude of [^{3}H]thymidine incorporation. Cells were assayed in triplicate wells of 96-well polystyrene flat-bottomed microtiter plates in which each well contained a volume of 200 μl. Plates were incubated for 48 h at 37°C in 5% CO_2 and 95% air. Ten microcuries of [^{3}H]thymidine (6.7 Ci/mmol specific activity; New England Nuclear Corp., Boston, Mass.) were added to each well for the final 18 h of incubation. The cells were harvested onto glass fiber filters, which were then counted in 2.5 ml of scintillation cocktail (Beckman Instruments Inc., Fullerton, Calif.) in a scintillation counter (Beckman LS 3801).

Immunoassay for IFN-γ. The concentration of IFN-γ present in supernatants of cocultures of infected macrophages and T-cell hybridomas was determined with an enzyme-linked immunosorbent assay (ELISA; Amgen Biologicals, Thousand Oaks, Calif.) that employs a monoclonal antibody specific for murine IFN-γ (45). A standard curve for each assay was generated with murine IFN-γ provided by the kit manufacturer. The unit of interferon activity was defined relative to the activity in NIH standard no. Gg 02-901-533.

Bone marrow colony-forming cell assay. Stimulation of granulocyte or macrophage colonies in bone marrow cultures was performed as described before (18). Murine femoral bone marrow cells were plated at 2×10^5 to 4×10^5 cells per ml in 1% methycellulose (viscosity, 4,000 cps; Methocel; Dow Chemical Company, Camden, N.J.) diluted in Dulbecco medium supplemented with essential and nonessential amino acids, L-glutamine (4 mM), 2-mercaptoethanol (5×10^{-5} M), penicillin (100 U/ml), streptomycin (100 μg/ml), and HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (25 mM) with 30% heat-inactivated FBS. Lymphocyte culture supernatants (at final concentration of 5% [vol/vol]) or preparations of natural CSF-1, GM-CSF or rGM-CSF were added to bone marrow cultures, which were incubated at 37°C for 6 to 7 days unless otherwise noted. Colony formation (≥25 cells per aggregate) was enumerated microscopically.

TABLE 1. Antileishmanial effects induced by T hybridoma cells

T hybridoma cells added	Mean % infected macrophages ± SEM	No. of amastigotes		% Antileishmanial effect[c]
		A/Mϕ[a]	A/100 Mϕ[b]	
None	30.7 ± 4.26	6.95 ± 0.29	212 ± 26	
1D5	21.0 ± 2.00	3.96 ± 0.08	83 ± 9	61
1B6	23.0 ± 1.00	4.35 ± 0.26	100 ± 10	53
3C5	12.0 ± 2.00	4.40 ± 0.6	54 ± 6	75
1B2	23.5 ± 0.05	4.48 ± 0.09	105 ± 4	51

[a] Amastigotes per infected macrophage (mean ± SEM; three determinations).
[b] Amastigotes per 100 macrophages (derived as the product of infected macrophages times the mean amastigotes per macrophage).
[c] Antileishmanial effect is percent reduction in A/100 Mϕ in the presence of T hybridoma cells.

RESULTS

T-cell hybridomas activate antileishmanial effects in infected macrophages. From more than 120 cloned T-cell hybridomas screened, we selected 4 for their superior ability to induce antileishmanial effects in macrophages (Table 1). The magnitude of antileishmanial effects imparted by the T-cell hybridomas was comparable to those imparted by our crude lymphokine preparations (antileishmanial effect, 45 to 55% [43]) and rIFN-γ (100 U/ml; antileishmanial effect, 54% [43]). We selected two of these clones, 1B6 and 1D5, for further analysis.

Two T-cell hybridomas differ in their production of macrophage-activating lymphokines. Conditioned cell-free medium harvested from cocultures of 1D5 cells and infected macrophages was able to activate antimicrobial effects in fresh cultures of infected macrophages incubated without hybridoma cells (25% antileishmanial effect; $P < 0.03$, comparison of amastigotes per 100 macrophages in the presence and absence of conditioned medium). In contrast, macrophage-stimulating activity was not detected in the supernatants derived from cocultures of infected macrophages and 1B6 cells (<2% antileishmanial effect; $P > 0.13$).

These observations suggest that 1D5 and 1B6 cells function by distinct mechanisms, the former by secreting macrophage-activating lymphokines, the latter by another mechanism. To assess this further, we analyzed the ability of these clones to activate antileishmanial effects in infected macrophages in the presence and absence of CsA, an agent that we previously demonstrated interferes with the production of lymphokines that activate antileishmanial effects in macrophages (48). The magnitude of the antileishmanial effect induced by 1B6 cells was similar in the presence and absence of CsA (Table 2). In contrast, CsA significantly diminished the antileishmanial effects induced by 1D5 cells as well the production of macrophage-activating lymphokines by 1D5 cells ($P > 0.03$; comparison of antileishmanial effects in CsA-treated and untreated cultures). These findings suggest that 1B6 cells activate macrophages in a lymphokine-independent manner and that 1D5 cells activate macrophages via CsA-suppressible lymphokine secretion.

IFN-γ has been specifically identified as a lymphokine that can induce antileishmanial defense in infected macrophages (25, 26). Accordingly, we tested for the presence of IFN-γ in conditioned medium of T-cell hybridomas cocultured with infected macrophages. We did not detect IFN-γ in the supernatants from cultures containing 1B6 cells, while supernatants from 1D5 cultures contained 5 to 20 U of IFN-γ per ml.

Because GM-CSF recently has also been reported to induce antileishmanial effects in vitro (15, 46), we also assayed for the presence of GM-CSF in conditioned medium of hybridomas cocultured with infected macrophages, by examining the capacity of the conditioned medium to induce clonal expansion of individual bone marrow progenitor cells (18). We did not detect colony-stimulating activity for granulocytes and macrophages in the supernatants derived from either 1D5 or 1B6 cocultures (Table 3), although activity was detected in crude lymphokine preparations that could activate antileishmanial effects (27, 43). It therefore appears that neither 1B6 or 1D5 produces GM-CSF under the culture conditions we examined.

Hybridoma cells are not cytotoxic to infected macrophages. The antileishmanial effects of neither 1B6 nor 1D5 cells were a result of cytotoxic effects on infected macrophages; the infected macrophages retained their functional integrity when cocultured with either hybridoma clone. Macrophage suspensions cultured for 48 h with and without hybridomas were incubated for 2 h at 37°C with sheep erythrocytes sensitized with rabbit anti-sheep erythrocyte immunoglobulin G (IgG; Cordis Laboratories, Inc., Miami, Fla.) (30). Erythrocyte uptake was assessed microscopically in fixed and stained specimens. In the absence of hybridoma cells, the percentage of infected macrophages that ingested 1 or more IgG-coated sheep erythrocytes (EA) was 42.67 ± 7.84% (mean ± SEM; three determinations). When EA were added 48 h after coculture of macrophages with 1B6 or 1D5 cells, the percentage of macrophages that ingested EA was 48.00 ± 1.00% and 43.00 ± 0.58%, respectively. Furthermore, the addition of T hybridoma cells to macrophage

TABLE 2. Effects of CsA on ability of T hybridoma cells to induce antileishmanial defense in vitro

Treatment of infected macrophages[a]	Mean no. of amastigotes/100 macrophages ± SEM	% Antileishmanial effect[b]	*P* value
None	360 ± 61	0	0
CsA	224 ± 60	0	0
1B6	107 ± 20	70	0.0352[c]
1B6 plus CsA	84 ± 4	62	0.0779[d]
1D5	114 ± 26	68	0.0516[c]
1D5 plus CsA	258 ± 11	0	0.3267[d]

[a] Hybridomas were added to 24-h infected macrophages at a 2:1 target-to-effector cell ratio. CsA (1 μg/ml) was added to macrophage cultures simultaneously with the addition of hybridoma cells where indicated.
[b] See Table 1, footnote *c*.
[c] Comparison by one-tailed Student *t* test of cultures tested with and without hybridoma cells.
[d] Comparison by one-tailed Student *t* test of CsA-containing cultures with and without the addition of hybridomas.

TABLE 3. Granulocyte and macrophage colony formation in vitro in response to various culture supernatants and recombinant CSFs

Supernatant[a]	Amt (U)	No. of colonies/ml of culture[b]
Exp 1		
Medium alone		1.0 ± 0.6
1B6		1.0 ± 0.6
1B6 + MφI		1.0 ± 0.6
1D5 + MφI		1.3 ± 0.3
BCG/PPD		19.67 ± 1.3
mCSF-1 (L929)		44.3 ± 0.9
mGM-CSF (lung tissue)		36.3 ± 1.4
rmGM-CSF	100	32.3 ± 1.4
Exp 2		
MφI + Medium		1.3 ± 0.9
MφI + 1B6		1.0 ± 0.00
MφI + 1D5		0.7 ± 0.3
rGM-CSF	50	59.0 ± 4.7
	10	30.7 ± 2.8
	2.5	13.3 ± 2.8

[a] Test at a final dilution of 5% (vol/vol). mCSF-1, Native murine colony-stimulating factor 1; mGM-CSF, native murine granulocyte-macrophage colony-stimulating factor; rmGM-CSF, recombinant murine GM-CSF; MφI, *Leishmania*-infected macrophages; BCG/PPD, culture supernatant from BCG-sensitized lymphocytes incubated with PPD (50 μg/ml).

[b] Femoral bone marrow cells were cultured at 2×10^5 to 4×10^5 cells per ml in Dulbecco medium supplemented with 1% methylcellulose and 30% FBS. Granulocyte and macrophage colonies (>25 cells per aggregate) were scored after 6 to 7 days of incubation. Values are means ± SEM, three determinations.

cultures consistently failed to cause a decrease in the number of macrophages (30).

Activation of antileishmanial effects by 1B6 cells is genetically restricted. 1B6 cells activate antileishmanial effects in infected macrophages by a mechanism that appears to be lymphokine independent and not cytotoxic to the infected host cell. To exert this effect, 1B6 and the infected macrophage must be syngeneic. 1B6 cells induced significant antileishmanial effects ($P < 0.05$; comparison of treated and untreated cultures; Fig. 1) in syngeneic infected macrophages from $B6C3F_1$ mice ($H\text{-}2^{b/k}$ haplotype and therefore syngeneic with the 1B6 cells derived by fusion of C57BL/6 [$H\text{-}2^b$] lymphocytes and AKR [$H\text{-}2^k$] thymoma cells) but not in infected allogeneic B10.D2 ($H\text{-}2^d$) macrophages ($P > 0.30$; Fig. 1). In contrast, heterogeneous populations of *Leishmania*-specific lymph node lymphocytes from B10.D2 mice induced marked antileishmanial effects in homologous (syngeneic) as well as allogeneic macrophages ($P < 0.005$ and $P < 0.05$, respectively; comparison of amastigotes per 100 macrophages in cultures with and without hybridoma cells; Fig. 1). 1D5 cells exerted antileishmanial effects in syngeneic as well as allogeneic cells (≤20% antileishmanial effect). The antiparasitic effect induced in allogeneic (but not syngeneic) cocultures was abolished in the presence of CsA ($P > 0.40$; Fig. 1), suggesting that it resulted from lymphokines released in response to stimulation by alloantigens (32).

These experiments could not distinguish whether the genetic restriction applied to antigen presentation or the T-hybridoma effect on macrophages. We therefore assessed independently the proliferative response of the hybridomas to syngeneic and allogeneic antigen presentation. We observed that 1B6 cells proliferated only in response to syngeneic infected macrophages, whereas 1D5 cells responded to both syngeneic and allogeneic macrophages (uninfected and infected). In the absence of hybridoma cells, [^{3}H]thymidine uptake in infected macrophages was 1,437 ± 51 cpm ($B6C3F_1$ [F1; $H\text{-}2^{b/k}$]; mean ± SEM, three determinations) and 806 ± 68 cpm (BALB/c [$H\text{-}2^d$]). 1B6 cells proliferated in the presence of infected syngeneic (F1) macrophages (4,222 ± 2,414 cpm) but not in response to infected allogeneic (BALB/c) macrophages (1,039 ± 385 cpm), indicating that the 1B6 hybridoma was neither autoreactive nor alloreactive. In contrast, when infected F1 and BALB/c macrophages were added to 1D5 cells, [^{3}H]thymidine incorporation was 3,914 ± 668 and 4,852 ± 1,204 cpm, respectively. When uninfected BALB/c macrophages were added, incorporation was 7,667 ± 2,903 cpm. Thus, 1D5 may actually be an alloreactive T-cell clone.

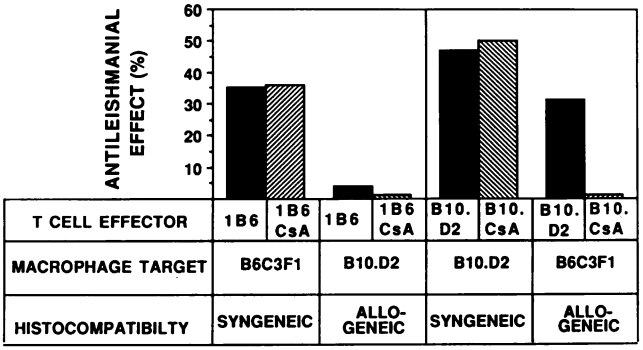

FIG. 1. Genetic restriction of hybridoma cell-mediated macrophage activation. 1B6 ($H\text{-}2^{b/k}$) cells were added to *Leishmania*-infected syngeneic ($B6C3F1$ [$H\text{-}2^{b/k}$]) or allogeneic (B10.D2 [$H\text{-}2^d$]) resident peritoneal macrophages at a 2:1 hybridoma-to-infected macrophage ratio. Popliteal lymph node lymphocytes from B10.D2/nSnJ mice ($H\text{-}2^d$) obtained at 5 weeks of *L. major* infection were added to infected macrophages at a ratio of 100:1. Macrophages were infected 24 h prior to the addition of effector T cells. Suspension cultures were then maintained for an additional 48 h with or without the addition of CsA (1 μg/ml). Data were derived from triplicate determinations in representative experiments.

Temperature affects T-cell hybridoma-mediated macrophage activation. Activation by lymphokines of antileishmanial defense in macrophages in vitro has been shown to occur optimally at 37°C; at lower temperatures (34°C), the lymphokines are substantially less effective (34). We compared the influence of temperature on the capacity of 1B6 and 1D5 cells to induce antileishmanial effects. The clones were tested in the in vitro assay at 34 and 37°C. At both 34 and 37°C, 1B6 induced antileishmanial effects in macrophages (Fig. 2). In marked contrast, 1D5 cells only activated these effects when cultured at 37°C; their antileishmanial effects at 34°C were minimal (Fig. 2). These observations reinforce our foregoing evidence that 1B6 and 1D5 cells activate antileishmanial defense by distinct mechanisms.

DISCUSSION

In the present study, we attempted to determine whether the two distinct T-cell mechanisms for activation of antimicrobial effects in *L. major*-infected murine macrophages that we previously observed with heterogeneous lymphocyte populations were likely to be properties of different T-cell subpopulations. For these studies, we prepared T-T hybridomas directly from lymph node lymphocyte suspensions in order to avoid the selective overgrowth of or functional alterations in lymphocyte populations that might result from prior in vitro stimulation with antigen and IL-2 (10, 16). The evidence we obtained in our analysis which supports the

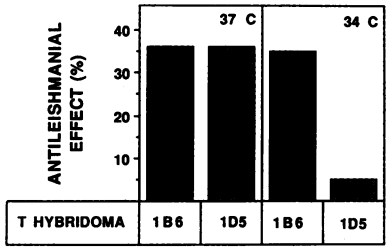

FIG. 2. Temperature sensitivity of effects of 1B6 and 1D5 on macrophage activation. 1B6 and 1D5 cells were added to syngeneic *Leishmania*-infected resident peritoneal macrophages at a 2:1 ratio. Cultures were maintained for an additional 48 h at 34 and 37°C. Control cultures were maintained at these temperatures without hybridoma cells. Data were derived from triplicate determinations in representative experiments.

existence of distinct mechanisms includes the following: (i) of the two cloned T-cell hybridoma lines that induced antileishmanial effects in vitro, only one (1D5) secreted soluble factors (including IFN-γ) that activated macrophages; (ii) the ability of the macrophage-activating factor (MAF)-producing hybridoma (1D5) to exert its antileishmanial effects was abrogated in the presence of CsA (a drug which inhibits the production of MAFs that induce antileishmanial effects in vitro [48]), whereas the non-lymphokine-producing hybridoma (1B6) was resistant to the effects of CsA (Table 2); and (iii) 1B6 cells can exert antileishmanial effects at 37 and 34°C, whereas 1D5 cells are effective only at 37°C (Fig. 2). IFN-γ (25, 26) and GM-CSF (15, 46) have been specifically identified as lymphokines capable of inducing in vitro antileishmanial effects in infected macrophages. IFN-γ production is blocked by CsA (12), whereas GM-CSF synthesis is not (3). We therefore could not exclude that hybridoma 1B6 made sufficient GM-CSF to induce antileishmanial effects despite the presence of CsA. However, since we failed to identify GM-CSF in a sensitive ELISA for this lymphokine (an assay that we found could detect <1 ng of GM-CSF per ml [Table 3]), and in view of the fact that relatively high concentrations of GM-CSF (>4 ng/ml) are required to induce a detectable antileishmanial effect in vitro (46), we consider it highly unlikely that GM-CSF production by the hybridoma is the explanation for the ability of 1B6 cells to induce an antileishmanial effect. Recently, tumor necrosis factor (TNF) has been shown to be capable of activating antileishmanial defense in macrophages (44). We have not detected TNF activity (L929 cell cytotoxicity) in supernatants of stimulated 1B6 cells (unpublished data), suggesting that these cells do not secrete this MAF. We have not excluded, however, that 1B6 might possess cell-associated TNF (8, 20).

1D5 as well as lymphocytes contained in a heterogeneous population of lymph node cells can respond to both allogeneic stimulation and specific leishmanial antigen stimulation, whereas 1B6 responds only to the parasite antigens presented by syngeneic cells (Fig. 1 and data in text). The antileishmanial effect resulting from allogeneic stimulation is apparently lymphokine mediated; it was inhibited by CsA. 1B6 is insusceptible to such allogeneic stimulation, raising the possibility that both the afferent (antigen presentation) and efferent (macrophage activation) aspects of the lymphocyte-macrophage interactions might be genetically restricted. In any case, the differences in requirement for inducing the effector function of the two hybridomas are emphasized by these findings.

Two recent observations may shed light on how the apparently lymphokine-independent macrophage activation mechanism might function. Poo et al. (31) examined the release of a B-cell growth factor by D10.G4.1 cells wedged into the pores of polycarbonate filters. The cells were stimulated to release this lymphokine by cross-linking (with monoclonal antibody) the T-cell receptor (TCR). Secretion of the B-cell growth factor was observed to occur only on that side of the filter membrane to which the anti-TCR antibody was added. The investigators postulated that lymphokine secretion in a polar manner perhaps also occurs in the localized region of interaction between T cells and other relevant cells (B cells, macrophages). Based on this model, we could interpret our observations to suggest that 1B6 cells release a macrophage-activating lymphokine only at the site of contact with *Leishmania*-infected macrophages, whereas 1D5 cells release such lymphokines in a diffuse fashion. Against this interpretation, however, is our observation that CsA did not interfere with the action of 1B6 but did interfere with the effects of 1D5. Furthermore, no IFN-γ activity was detected in 1B6 supernatants in a sensitive ELISA (an assay that we found that could detect <2.5 U of IFN-γ per ml). In preliminary studies we were unable to detect by Northern (RNA) blot analysis steady-state levels of mRNA for IFN-γ in 1B6 cells which had been stimulated with infected macrophages or lectin mitogens; we did detect this mRNA species in 1D5 cells cultured with infected macrophages (Sypek, Heinzel, and Wyler, unpublished results). We must therefore conclude that if 1B6 cells work by the localized release of lymphokine, this lymphokine is not IFN-γ. On the other hand, our observations do not exclude the possibility that another lymphokine secreted in such a restricted manner might account for our observations. This being the case, we would then conclude that there are subpopulations of T cells that secrete MAFs only in this topographically restricted manner, while others (such as that represented by 1D5) can also secrete lymphokines in a nonpolar manner.

An alternative explanation for our observations derives from recent observations suggesting that certain cytokines (IL-1 and TNF) may be biologically active when they are associated with the plasma membranes of the cells which produce them (8, 20, 21). Paraformaldehyde-fixed macrophages and planar membrane preparations derived from macrophages can stimulate T-lymphocyte proliferation and killing of L929 or WEHI 164 tumor targets. These effects are blocked with anti-IL-1 (21) and anti-TNF antibody (20), respectively. The results of similar types of experiments suggest that IL-4, IL-5, and GM-CSF may also function in a membrane-associated compartment (5, 11). By analogy, T cells such as those represented by 1B6 hybrids might activate antimicrobial effects in macrophages with which they make specific contact through the binding of membrane-associated MAF and a corresponding receptor on the macrophage. In this model, the genetic restriction and antigenic specificity we observed (Fig. 1) may represent concomitant membrane ligand-receptor interactions that enhance the affinity or stabilize the binding of T cells and the infected macrophages. A final possibility is that ligation of the TCR with leishmanial antigen-Ia complexes expressed on the surface of infected macrophages (33) (perhaps in conjunction with other molecules such as CD4 [47], lymphocyte function-associated antigen 1 [7], intracellular adhesion molecule 1 [4], or as yet undefined receptor-ligand systems) is sufficient to provide the induction of events that initiate the antimicrobial effects associated with macrophage activation.

1B6 cells function equally well at 34 and 37°C, whereas

1D5 cells and lymphokines that induce antileishmanial effects function optimally only at 37°C (Fig. 2). These observations suggest that there may be important physiological distinctions between the conventional lymphokine-mediated activation mechanism (exerted by 1D5 cells) and the apparently unique one we have observed with 1B6 (and other) cells. We uncovered additional distinctions in our studies with heterogeneous lymph node populations. We found that contact-mediated macrophage activation can result in antimicrobial effects on strains of *Leishmania* which are resistant to the effects of lymphokine-mediated macrophage activation (35, 43). Furthermore, both resident and elicited (inflammatory) peritoneal macrophages can be activated by the contact mechanism to inhibit growth of intracellular *Leishmania* organisms (30), whereas lymphokine is effective only on the former population (17). Since the elicited macrophage population includes many recently recruited and relatively immature mononuclear phagocytes (1), our findings suggest that direct cell contact is an effective activation signal for macrophage populations of different maturity.

We have determined in previous studies that the effector lymphocytes from heterogeneous lymph node populations that activate macrophages in a contact-dependent manner possess the $CD4^+$ phenotype (40). 1B6 and 1D5 also possess this helper T-cell phenotype (unpublished data). Recent studies indicate that T cells of the $CD4^+$ phenotype can be separated into at least two subsets (designated Th1 and Th2), based on their unique patterns of lymphokine secretion following stimulation (22, 24). Th1 cells produce IL-2 and IFN-γ; Th2 cells secrete several B-cell growth and stimulatory factors, including IL-4 and IL-5 (6, 22, 24). Since we have been able to detect activity for IL-2 but not for IL-4 in supernatants collected from stimulated 1B6 cells (unpublished data), these T cells may be members of a Th1 subset that does not make IFN-γ.

T lymphocytes can provide signals that lead to the activation of macrophages for defense against certain intracellular pathogens. Classically, the activating signals have been ascribed to soluble products of the lymphocytes, the lymphokines (13, 28). We believe that the observations we report here for homogeneous populations of T cells provide support for the notion that activation of antimicrobial (antileishmanial) defense can also result from a distinctive interaction between T cells and macrophages, ones that appear to be cell contact dependent (30, 40–43, 48). Contact-mediated macrophage activation presumably represents a more specifically targeted mechanism of defense than does lymphokine-mediated activation and may also have unique physiological consequences. It will therefore be of interest to learn the relative importance of these two mechanisms at different times during infection (2).

ACKNOWLEDGMENTS

This work was supported by a First Independent Research and Transition Award R29 AI 24500 (to J.P.S.) and by Public Health Service grant RO1 AI 17151 (to D.J.W.) from the National Institutes of Health.

We thank Marion M. Matzilevich for her technical assistance and Paula Hochman for her contribution of the BW5147 cell line. We are grateful to John R. David, Sidney Leskowitz, Geoffrey Sunshine, Honorine Ward-Pillai, and Miercio E. A. Pereira for helpful discussions and their critical reading of the manuscript. We thank Priscilla Rogers for her administrative assistance and Irene Doucette and Ann Boyd for typing the manuscript.

LITERATURE CITED

1. **Adams, D. O., and T. A. Hamilton.** 1984. The cell biology of macrophage activation. Annu. Rev. Immunol. **2**:283–318.
2. **Belosevic, M., D. S. Finbloom, P. H. Van Der Meide, M. V. Slayter, and C. A. Nacy.** 1989. Administration of monoclonal anti-IFNγ antibodies *in vivo* abrogates natural resistance of C3H/HeN mice to infection with *Leishmania major*. J. Immunol. **143**:266–274.
3. **Bickel, M., H. Tsuda, P. Amstad, V. Evequoz, S. E. Mergenhagen, S. M. Wahl, and D. H. Pluznik.** 1987. Differential regulation of colony-stimulating factors and interleukin-2 production by cyclosporine A. Proc. Natl. Acad. Sci. USA **84**: 3274–3277.
4. **Boyd, A. W., S. O. Wawryk, G. F. Burns, and J. V. Fecondo.** 1988. Intracellular adhesion molecule 1 (ICAM-1) has a central role in cell-cell contact-mediated immune mechanisms. Proc. Natl. Acad. Sci. USA **85**:3095–3099.
5. **Brian, A. A.** 1988. Stimulation of B-cell proliferation by membrane-associated molecules from activated T cells. Proc. Natl. Acad. Sci. USA **85**:564–568.
6. **Cherwinski, H. M., J. H. Schumacher, K. D. Brown, and T. R. Mosmann.** 1987. Two types of helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays and monoclonal antibodies. J. Exp. Med. **166**: 1229–1244.
7. **Davigon, D., E. Martz, T. Reynolds, K. Kurzinger, and T. A. Springer.** 1981. Monoclonal antibody to a novel lymphocyte function-associated antigen (LFA-1): mechanism of blockage of T lymphocyte-mediated killing and effects on other T and B lymphocyte functions. J. Immunol. **127**:590–602.
8. **Decker, T., M. Lohmann-Matthes, and G. E. Gifford.** 1987. Cell-associated tumor necrosis factor (TNF) as a killing mechanism of activated cytotoxic macrophages. J. Immunol. **138**: 957–962.
9. **Fowles, R. E., I. M. Fajardo, J. L. Leibowitch, and J. R. David.** 1973. The enhancement of macrophage bacteriostasis by products of activated lymphocytes. J. Exp. Med. **138**:952–964.
10. **Gajewski, T. F., J. Joyce, and F. W. Fitch.** 1989. Antiproliferative effect of IFNγ in immune regulation. III. Differential selection of Th1 and Th2 murine helper T lymphocyte clones using recombinant IL-2 and recombinant IFNγ. J. Immunol. **143**:15–22.
11. **Gordon, M. Y., G. P. Riley, S. M. Watt, and M. F. Greaves.** 1987. Compartmentalisation of a hematopoietic growth factor (GM-CSF) by glycoaminoglycans in the bone marrow environment. Nature (London) **326**:403–405.
12. **Granelli-Piperno, A., K. Inaba, and R. M. Steinman.** 1984. Stimulation of lymphokine release from T lymphoblasts. Requirement for mRNA synthesis and inhibition by cyclosporin A. J. Exp. Med. **160**:1792–1802.
13. **Hahn, H., and S. H. E. Kaufman.** 1981. The role of cell-mediated immunity in bacterial infections. Rev. Infect. Dis. **3**:1221–1250.
14. **Hammerling, G. J.** 1977. T lymphocyte tissue culture lines produced by cell hybridization. Eur. J. Immunol. **7**:743–746.
15. **Handman, E., and A. W. Burgess.** 1979. Stimulation by granulocyte-macrophage colony-stimulating factor of *Leishmania tropica* killing by macrophages. J. Immunol. **122**:1134–1137.
16. **Hayakawa, K., and R. R. Hardy.** 1989. Phenotypic and functional alteration of $CD4^+$ T cells after antigen stimulation. Resolution of two populations of memory T cells that both secrete interleukin 4. J. Exp. Med. **169**:2245–2250.
17. **Hoover, D. L., and C. A. Nacy.** 1984. Macrophage activation to kill *Leishmania tropica*: defective intracellular killing of amastigotes by macrophages elicited with sterile inflammatory agents. J. Immunol. **132**:1487–1493.
18. **Iscove, N. N., and F. Sieber.** 1975. Erythroid progenitors in mouse bone marrow detected by microscopic colony formation in culture. Exp. Hematol. **3**:32–43.
19. **Julius, M. H., E. Simpson, and L. A. Herzenberg.** 1973. A rapid method for isolation of functional thymus-derived murine lymphocytes. Eur. J. Immunol. **3**:645–649.

20. **Kriegler, M., C. Perez, K. DeFay, I. Albert, and S. D. Lu.** 1988. A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramifications for complex physiology of TNF. Cell **53**:45–53.
21. **Kurt-Jones, E. A., D. I. Beller, S. B. Mizel, and E. R. Unanue.** 1985. Identification of a membrane-associated interleukin-1 in macrophages. Proc. Natl. Acad. Sci. USA **82**:1204–1208.
22. **Kurt-Jones, E. A., S. Hamberg, J. O'Hara, W. E. Paul, and A. K. Abbas.** 1987. Heterogeneity of helper/inducer T lymphocytes. I. Lymphokine production and lymphokine responsiveness. J. Exp. Med. **166**:1774–1787.
23. **Mackaness, G. B.** 1969. The influence of immunologically committed lymphoid cells on macrophage activity *in vivo*. J. Exp. Med. **129**:973–992.
24. **Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman.** 1986. Two types of murine helper T cell clones. I. Definition according to profiles of lymphokine activities and secreted proteins. J. Immunol. **136**:2348–2357.
25. **Murray, H. W., G. L. Spitalny, and C. F. Nathan.** 1985. Activation of mouse peritoneal macrophages *in vitro* and *in vivo* by interferonγ. J. Immunol. **134**:1619–1622.
26. **Nacy, C. A., A. H. Fortier, M. S. Meltzer, N. A. Buchmeir, and R. S. Schreiber.** 1985. Macrophage activation to kill *Leishmania major*: activation of macrophages for intracellular destruction of amastigotes can be induced by both recombinant interferon$_\gamma$ and non-interferon lymphokines. J. Immunol. **135**:3505–3511.
27. **Nacy, C. A., M. S. Meltzer, E. J. Leonard, and D. J. Wyler.** 1981. Intracellular replication and lymphokine-induced destruction of *Leishmania tropica* in C3H/HeN mouse macrophages. J. Immunol. **127**:2381–2386.
28. **Nacy, C. A., C. N. Oster, S. J. James, and M. S. Meltzer.** 1984. Activation of macrophages to kill rickettsiae and *Leishmania*: dissociation of intracellular microbicidal activities and extracellular destruction of neoplastic and helminth targets. Cont. Top. Immunol. **13**:147–170.
29. **Neva, F. A., D. J. Wyler, and T. Nash.** 1979. Cutaneous leishmaniasis—a case with persistent organisms after each treatment in presence of normal immune response. Am. J. Trop. Med. Hyg. **28**:467–471.
30. **Panosian, C. B., J. P. Sypek, and D. J. Wyler.** 1984. Cell contact-mediated macrophage activation for antileishmanial defense. I. Lymphocyte effector mechanism that is contact-dependent and noncytotoxic. J. Immunol. **133**:3358–3365.
31. **Poo, W. J., L. Conrad, and C. A. Janeway, Jr.** 1988. Receptor-directed focusing of lymphokine release by helper T cells. Nature (London) **332**:378–380.
32. **Pure, E., K. Inaba, and J. Metlay.** 1988. Lymphokine production by murine T cells in the mixed leukocyte reaction. J. Exp. Med. **168**:795–800.
33. **Schwartz, R. H.** 1985. T lymphocyte recognition of antigen in association with gene products of the major histocompatibility complex. Annu. Rev. Immunol. **3**:237–261.
34. **Scott, P.** 1985. Impaired macrophage leishmanicidal activity at cutaneous temperatures. Parasite Immunol. **7**:277–288.
35. **Scott, P., D. Sacks, and A. Sher.** 1983. Resistance to macrophage-mediated killing as a factor influencing the pathogenesis of chronic cutaneous leishmaniasis. J. Immunol. **131**:966–971.
36. **Sheridan, J. W., and D. Metcalf.** 1973. A low molecular weight factor in lung conditioned medium stimulating granulocyte and macrophage colony formation *in vitro*. J. Cell Physiol. **81**:11–24.
37. **Simon, H. B., and J. N. Sheagren.** 1971. Cellular immunity *in vitro*. I. Immunologically mediated enhancement of macrophage bactericidal capacity. J. Exp. Med. **133**:1377–1389.
38. **Simon, H. B., and J. N. Sheagren.** 1972. Enhancement of macrophage capacity by antigenically stimulated immune lymphocytes. Cell. Immunol. **4**:163–174.
39. **Stanley, E. R., and P. M. Heard.** 1977. Factors regulating macrophage production and growth: purification and some properties of the colony stimulating factor from medium conditioned by mouse L cells. J. Biol. Chem. **252**:4305–4312.
40. **Sypek, J. P., C. B. Panosian, and D. J. Wyler.** 1984. Cell contact-mediated macrophage activation for antileishmanial defense. II. Identification of effector cell phenotype and genetic restriction. J. Immunol. **133**:3351–3357.
41. **Sypek, J. P., C. B. Panosian, and D. J. Wyler.** 1985. Antigen recognition by effector T cells in antileishmanial defense. J. Infect. Dis. **152**:1057–1063.
42. **Sypek, J. P., and D. J. Wyler.** 1985. Cell contact-mediated macrophage activation for antileishmanial defense: a mapping of the genetic restriction to the I region of the MHC. Clin. Exp. Immunol. **62**:449–457.
43. **Sypek, J. P., and D. J. Wyler.** 1988. Susceptibility of lymphokine-resistant *Leishmania* to cell contact-mediated macrophage activation. J. Infect. Dis. **158**:392–397.
44. **Titus, R. G., B. Sherry, and A. Cerami.** 1989. Tumor necrosis factor plays a protective role in experimental murine cutaneous leishmaniasis. J. Exp. Med. **170**:2097–2104.
45. **Van Der Meide, P. H., M. Dubbeld, K. Vijverberg, T. Kos, and H. Schellekens.** 1986. The purification and characterization of rat gamma interferon by use of two monoclonal antibodies. J. Gen. Virol. **67**:1059–1071.
46. **Weiser, W. Y., A. Van Niel, S. C. Niel, S. C. Clark, J. R. David, and H. G. Remold.** 1987. Recombinant human granulocyte/macrophage colony-stimulating factor activates intracellular killing of *Leishmania donovani* by human monocyte-derived macrophages. J. Exp. Med. **166**:1436–1446.
47. **Wilde, D. B., P. Marrack, J. Kappler, D. P. Dialynas, and F. W. Fitch.** 1983. Evidence implicating L3T4 in class II MHC antigen-specific proliferation, release of lymphokines, binding by cloned murine helper T lymphocyte lines. J. Immunol. **131**:2178–2183.
48. **Wyler, D. J., D. I. Beller, and J. P. Sypek.** 1987. Macrophage activation for antileishmanial defense by an apparently novel mechanism. J. Immunol. **138**:1246–1249.
49. **Zinkernagel, R. M., A. Altage, B. Adler, R. V. Blanden, W. F. Davidson, U. Kees, M. B. C. Dunlop, and D. C. Shreffler.** 1977. H-2 restriction of cell-mediated immunity to an intracellular bacterium. Effector T cells are specific for *Listeria* antigen in association with H-2I region-coded self markers. J. Exp. Med. **145**:1353–1367.