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Comparison of Gamma Interferon, Tumor Necrosis Factor, and Direct Cell Contact in Activation of Antimycobacterial Defense in Murine Macrophages

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We compared the abilities of gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), and sensitized murine lymph node lymphocytes to activate syngeneic murine peritoneal macrophages to inhibit the growth of intracellular *Mycobacterium bovis* BCG in vitro. IFN-γ could activate antimycobacterial defense only when added to macrophage cultures prior to their infection with BCG. TNF-α was without any effect. In contrast, BCG-sensitized lymphocytes could induce antimycobacterial defenses when added after macrophages had been infected with BCG. The cell-mediated effect required direct contact between effector lymphocytes and the targets (BCG-infected macrophages), as revealed in studies in which these cell populations were separated by a semipermeable membrane. Cyclosporin A, which inhibits the production of relevant macrophage-activating lymphokines, did not abrogate the ability of sensitized lymphocytes to activate antimycobacterial effects in infected macrophages. Furthermore, only BCG-sensitized lymphocytes, and not *Listeria*-sensitized lymphocytes, could activate the antimycobacterial effects. These lymphocytes were not cytotoxic to the infected macrophages. The presence of anti-TNF-α antibody in cocultures reduced the antimicrobial effects. We propose that the activation of antimycobacterial defense in macrophages can occur by direct physical contact with sensitized lymphocytes. This process may be due to lymphocyte membrane-associated TNF-α, as we previously demonstrated in our studies of antileishmanial defense.

Host defense in mycobacterial infections—as with that by other microbes that reside within macrophages—is largely mediated by T lymphocytes (7). Appropriately sensitized $CD4^+$ T cells establish physical interactions with infected macrophages and secrete soluble macrophage-activating factors that can induce antimycobacterial effects. These lymphokines include gamma interferon (IFN-γ) (4, 9, 13), tumor necrosis factor (TNF) (6, 20), interleukin-4 (6), and interleukin 6 (5). In addition, specifically sensitized $CD8^+$ T cells (3) and some γ/δ T lymphocytes are able to lyse a subpopulation of mycobacterium-infected macrophages (8). Thus, in vitro T cells are capable of limiting mycobacterial infection by at least two distinct mechanisms, one dependent on lymphokine secretion and the other dependent on contact-directed cell lysis.

In related studies of host defense to another intracellular pathogen, *Leishmania major*, we uncovered a novel mechanism of macrophage activation that involves direct cell contact between $CD4^+$ T lymphocytes and *Leishmania*-infected macrophages (12, 17, 20, 22). The mechanism is distinctive, since it involves neither the secretion of macrophage-activating lymphokines nor cytolysis of infected macrophages. This contact-dependent activation results from the interaction of a membrane-associated form of TNF on the surface of the effector T cells with the infected macrophages (21). Notably, the cell contact mechanism of macrophage activation, unlike lymphokine-mediated mechanisms, is antigen specific and genetically restricted (18–20).

We now report the results of our studies suggesting that specifically sensitized lymphocytes can activate antimycobacterial defenses (*Mycobacterium bovis* BCG) in murine macrophages by a mechanism that appears to be similar to the one we previously observed in our studies of *Leishmania* species. These observations indicate that *Leishmania* species are not uniquely susceptible to a contact-dependent mechanism of macrophage activation. Our findings also extend the potential mechanisms of antimycobacterial defenses that are worthy of further investigation.

MATERIALS AND METHODS

Bacteria. *M. bovis* (BCG, Pasteur strain, ATCC 35734; American Type Culture Collection, Rockville, Md.) was grown with agitation to mid-log phase (7 to 10 days of culture) at 37°C in Dubos Broth (Difco, Detroit, Mich.) supplemented with 10% Dubos Medium Albumin (Difco). Cultures were centrifuged (250 × *g*, 10 min, 15°C), washed in 0.15 M phosphate-buffered saline (pH 7.4), and stored at −70°C. To prepare BCG for infection of macrophages, 10- to 15-ml aliquots of mid-log-phase BCG cultures were filtered through 5-μm-pore-size filters (Acrodisc; Gelman Sciences, Ann Arbor, Mich.) to obtain unicellular suspensions. BCG organisms were enumerated in a Petroff-Hausser counting chamber.

Animals and infection. Female C57BL/6NTacFBR mice (18 to 20 g; macrophage and lymphocyte donors) were obtained from Taconic Farms (Germantown, N.Y.). Mice were infected subcutaneously at each hind footpad with 0.05

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ml of a suspension containing 10^6 viable BCG organisms in 0.15 M NaCl.

Effector lymphocytes. Draining popliteal lymph nodes from C57BL/6 mice infected for 2 to 10 weeks with *M. bovis* were a source of effector cell-containing populations of lymphocytes. Lymph nodes were excised aseptically, minced, and processed through sterile stainless-steel mesh into cold Ca^{2+}-Mg^{2+}-free Hanks' balanced salt solution (12). Lymph node lymphocytes were assayed for their ability to induce antimicrobial activity in an in vitro assay described below.

Lymphokines and antibody. Murine recombinant (r)IFN-γ (lot 42-96-2, specific activity of 6.8×10^6 U/mg; lot 22-71-68, specific activity of 5.2×10^6 U/mg) was obtained from Genentech, San Francisco, Calif., or from Genzyme Corp., Boston, Mass. (lot B1446, specific activity of 4.5×10^6 U/mg); murine rTNF alpha (TNF-α) (lot B1364, specific activity of 4×10^7 U/mg) was purchased from Genzyme. Anti-murine TNF-α antibody (rabbit hyperimmune serum) was obtained from Genzyme. This antibody can neutralize the antileishmanial effects of recombinant TNF (1), and it also specifically detects a membrane-associated form of TNF produced by CD4 lymphocytes (21).

Assay for mycobacterial growth and for antimicrobial activity. The abilities of lymph node effector cells and lymphokines to activate mycobacterium-infected macrophages were assessed by a modification of the methods of Rook and Rainbow (14). This assay assesses mycobacterial growth by measuring the magnitude of [^{3}H]uracil incorporation by BCG after various periods of intracellular growth following the release of the organisms from lysed macrophages.

Resident macrophages from uninfected C57BL/6 mice were harvested by peritoneal lavage with divalent cation-deficient Hank's balanced salt solution (12). Peritoneal cells were washed and suspended (10^6 cells per ml) in supplemented RPMI 1640 containing 10% fetal calf serum (Hyclone Laboratories, Logan, Utah), cefazolin (5 μg/ml), and 4 mM L-glutamine. Two hundred thousand peritoneal cells were combined with 6×10^4 to 60×10^4 mid-log-phase mycobacteria in a total volume of 0.5 ml in sterile 1.5-ml polypropylene Eppendorf tubes (Brinkman Instruments Co., Westbury, N.Y.). Effector lymphocytes (two to five lymphocytes per macrophage, which were enumerated by morphological assessment) were added 4 h after macrophages were infected. Lymphokines were added to macrophage cultures 4 h prior to infection or alternatively 4 h after infection. In additional experiments, suspension cultures were incubated either with or without 1 μg of cyclosporin A (CsA) per ml (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 of polyoxyethylated castor oil per ml in alcohol and was subsequently diluted in supplemented medium).

In selected experiments, effector lymphocytes and BCG-infected macrophages were cultured while separated by a semipermeable membrane (0.4-μm pore diameter, which permits diffusion of lymphokines). For this purpose, lymphocytes were placed in Transwell polycarbonate microporous cell culture inserts (no. 3401; Costar Corp., Cambridge, Mass.) with BCG-infected macrophages as antigen-presenting cells (one per 20 lymphocytes). Infected macrophages were placed in the wells of 24-well cluster plates (no. 3424; Costar). The Transwell units were then introduced into the cluster plate wells over the target cells and incubated as in the previously noted experiments.

After incubation at 37°C in a 5% CO_2–95% air atmosphere, representative cultures were harvested at 24 h (day 1) or 120 h (day 5). To harvest them, cultures in Eppendorf tubes were subjected to centrifugation at ~12,000 × *g* for 0.5 min in an Eppendorf 5415 Centrifuge (Eppendorf Geratebau; Hamburg, Germany). Supernatants were carefully removed, and pelleted cells were lysed by the addition of 1 ml of Dubos medium supplemented with 10% albumin and 0.1% saponin. Supernatants of cultures in cluster plates were also removed after centrifugation, and cells were treated in the same manner. Tritiated uracil (1.0 μCi/ml, specific activity of 38.6 Ci/mmol; NET-368 Uracil, [5,6-^{3}H]; New England Nuclear-Dupont, Boston, Mass.) was added, and the cultures were maintained at 37°C for an additional 24 h. Mycobacteria were then harvested onto glass microfiber filters (no. 934-AH; Whatman International, Ltd., Maidstone, England) and washed, and the magnitude of radionuclide incorporation was measured by standard liquid scintillation spectrometry with a Beckman LS 3801 Beta Scintillation counter (Beckman Instruments, Inc., Irvine, Calif.).

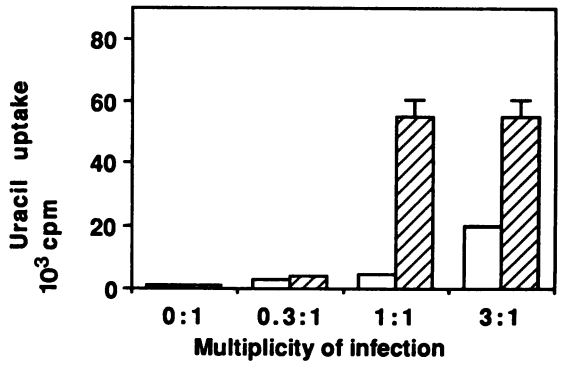

FIG. 1. Intracellular growth of BCG in peritoneal macrophages in vitro. Resident peritoneal macrophages were infected in vitro at various multiplicities of infection with *M. bovis* BCG from log-phase broth cultures. Their subsequent intracellular growth was assessed by comparing [^{3}H]uracil incorporation at day 1 (open bars) and day 5 (hatched bars) by the Rook and Rainbow method (14). The means (± standard errors) of triplicate determinations from a representative experiment (of three performed) are shown.

Statistical methods. The means and standard errors of triplicate determinations were compared by Student's *t* test (one tail). To standardize the results of experiments in which absolute counts per minute varied, the influence of specified treatments of cultures on BCG replication is expressed as percent uracil uptake relative to that of BCG in control cultures (no treatment = 100%).

RESULTS

Multiplication of mycobacteria. When suspension cultures of infected resident peritoneal macrophages were incubated in supplemented medium alone, mycobacteria replicated intracellularly over a 5-day period (Fig. 1). Optimum results were obtained when macrophages were infected with BCG at a ratio of 1:1 (11-fold increase in counts per minute). Heavier initial infections (3:1) increased the 24-h determination of counts per minute without proportionally increasing the determination at day 5. In selected parallel experiments, we compared this assay with a more cumbersome and time-consuming colony formation assay (2). Whereas CFU increased 5.0- ± 0.7-fold (mean ± standard error of the mean; three experiments), counts per minute increased 3.4- ± 0.2-fold between day 1 and day 5.

Lymphokine-mediated antimycobacterial effects. We con-

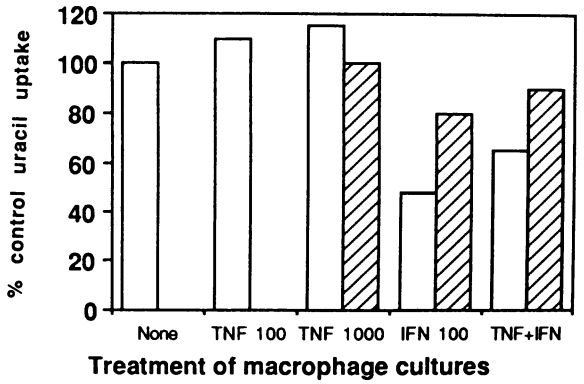

FIG. 2. Inhibition of BCG intracellular growth in peritoneal macrophages: comparison of IFN-γ and TNF-α treatment. The effect of preincubating macrophages for 4 h prior to BCG infection (open bars) with either TNF-α or IFN-γ alone or with TNF-α and IFN-γ in combination was compared with that of adding lymphokines after 4 h of infection (hatched bars). Percent replication is determined from counts per minute in lymphokine-treated cultures relative to counts per minute in cultures without additives (100%) at 5 days of incubation. The standard error of the mean of counts per minute determinations was ≤13% mean cpm. The results shown are from a representative experiment (of three performed).

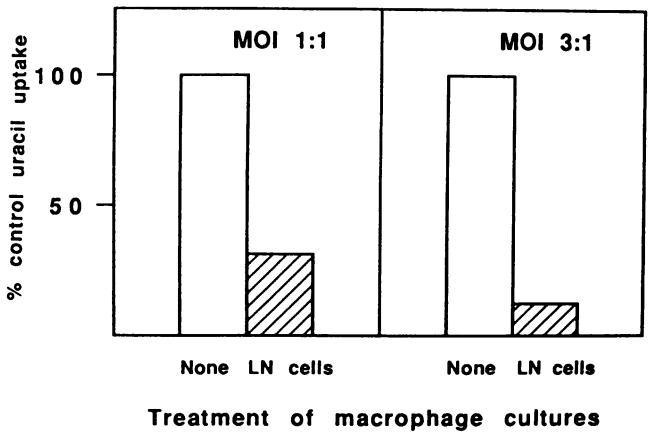

FIG. 3. Effect of popliteal lymph node lymphocytes from BCG-infected mice on the in vitro intracellular growth of BCG in resident peritoneal macrophages. Lymph node lymphocytes (LN cells) were obtained from mice infected in the hind footpads for 2 weeks and were added to peritoneal macrophages 4 h after infection. Mycobacterial growth was assessed at 5 days in macrophage populations infected with two different multiplicities of infection. Percent replication is derived from triplicate determinations of counts per minute. Data from a representative experiment (of more than three performed) are shown.

firmed in our assay that antimycobacterial defense can be induced with IFN-γ when this is added to macrophage cultures prior to infection. Macrophages were pretreated with recombinant TNF-α or IFN-γ 4 h prior to or 4 h following infection with *M. bovis* BCG. Treatment of macrophages prior to infection with rIFN-γ alone (100 U/ml) did not reduce mycobacterial uptake (no change in 24-h counts per minute) but subsequently inhibited mycobacterial growth (as estimated by uracil uptake; $P \leq 0.0125$ when compared with untreated cultures) (Fig. 2). In contrast, the addition of rIFN-γ after infection resulted in only a minimal, statistically insignificant antimicrobial effect (19.8%, $P > 0.15$) (Fig. 2).

Soluble TNF-α alone (100 and 1,000 U/ml) induced no antimycobacterial effect (Fig. 2) (P, >0.60 and >0.29, respectively, when compared with untreated cultures). When both TNF-α (100 U/ml) and IFN-γ (100 U/ml) were used in combination (pretreatment only), the antimycobacterial effects were no greater than those induced by IFN-γ alone (Fig. 2).

Lymphocyte-mediated antimycobacterial effects. The addition of effector lymphocytes derived from the draining popliteal lymph nodes of BCG-infected mice to macrophages infected 4 h previously resulted in significant antimycobacterial effects (81% reduction in mycobacterial growth; $P > 0.0015$) (Fig. 3). The ability to induce antimycobacterial effects postinfection and the greater magnitude of the effects distinguished the lymphocyte-mediated defense from the IFN-γ-mediated defense. When the effector lymphocytes used in this assay were obtained from mice that had been infected for 2 or 10 weeks (115 and 100% antimicrobial effect; P, <0.01 and <0.02, respectively) (Fig. 4), the antimycobacterial effects were greater than when the effector cells were taken from mice which had been infected for 15 weeks ($P > 0.2$). In no case did the coincubation of effector cells result in detectable cytolysis of the infected macrophages (12).

To assess whether the effects of the lymph node cells on intracellular mycobacterial growth are specific, we compared lymphocytes taken from BCG-sensitized mice with ones obtained from draining popliteal lymph nodes of *Listeria*-infected mice. On the basis of results of studies of antilisterial defense in mice (11), we chose to prepare lymphocytes from mice at weeks 1 to 2 of their infection with *Listeria* species. We previously observed (18) that these lymphocytes could exert antileishmanial effects in *Listeria* antigen-primed, *Leishmania*-infected macrophages but not in unprimed, *Leishmania*-infected macrophages. In contrast to BCG-sensitized lymphocytes, *Listeria*-sensitized lymphocytes failed to exert antimycobacterial effects in *M. bovis*-

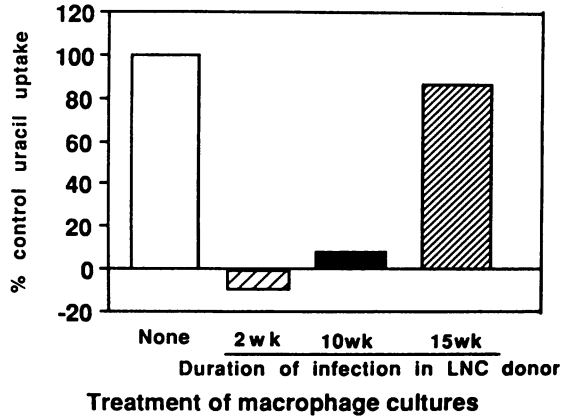

FIG. 4. Effect of lymph node lymphocytes (LNC) from BCG-infected mice on mycobacterial growth in macrophages: comparison of lymph node lymphocytes early and late in BCG infection. Lymph node lymphocytes were added to cultures of BCG-infected macrophages as previously described. The abilities of lymphocytes from mice infected for 2 and 10 weeks to activate antimycobacterial effects are compared. Percent replication was determined from 5-day cultures. Results from a representative experiment (of three performed) are shown.

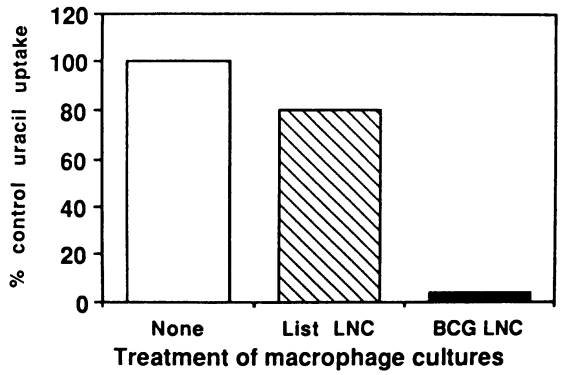

FIG. 5. Antigen specificity of activation of antimycobacterial defense in macrophages by lymph node lymphocytes (LNC). Macrophages were infected with BCG, and 4 h later lymph node lymphocytes were added to cultures. The abilities of lymph node lymphocytes from mice infected in the hind footpads for 4 weeks with BCG (BCG LNC) or for 2 weeks with *L. monocytogenes* (List LNC) to activate antimycobacterial effects are compared. Percent replication was determined from 5-day cultures. Two experiments with similar results were performed. Results from one experiment are shown.

infected macrophages in vitro ($P > 0.20$ when compared with untreated cultures) (Fig. 5).

The observation that BCG-sensitized lymphocytes but not lymphokine (IFN-γ) added after infection of macrophages can activate antimycobacterial effects (Fig. 2 and 3) suggests that these effects were unlikely to be a result of lymphokine secretions by the added cells. We confirmed this impression using two approaches. First, we cultured BCG-sensitized lymphocytes separated from infected macrophages by a semipermeable membrane. Under these culture conditions, no inhibition of mycobacterial growth was observed (Fig. 6). Second, when we inhibited macrophage-activating factor production by in vitro treatment of cocultures of lymphocytes and macrophages with CsA (22), antimycobacterial effects were not abolished (Fig. 6). CsA alone had no intrinsic antimycobacterial effects (data not shown).

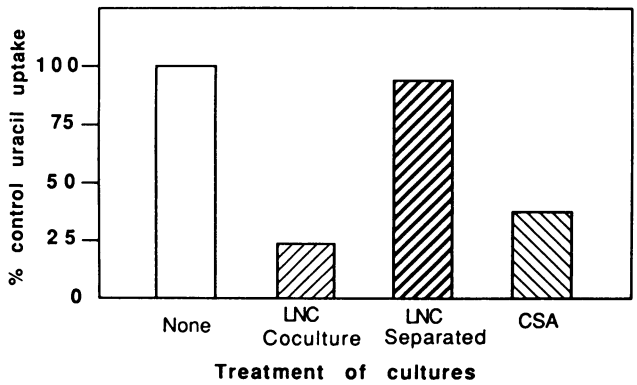

FIG. 6. Cell contact requirement for activation of antimycobacterial defense in macrophages by lymph node lymphocytes. Macrophages were infected with BCG for 4 h. BCG-sensitized lymph node lymphocytes (5-week footpad infection of BCG) were added to cultures directly (LNC Coculture) or were separated from macrophages by a semipermeable membrane (LNC Separated). BCG-infected macrophages (approximately one per 20 lymph node lymphocytes) were added as antigen-presenting cells to the lymph node lymphocytes placed in the upper compartment. Infected macrophages alone were placed in the lower compartment. In separate cultures in which cells were not separated, CsA was added at 1 μg/ml simultaneously with lymph node lymphocytes. The abilities of lymph node lymphocytes to activate antimycobacterial effects under these various treatment conditions are compared. Percent replication was determined from 5-day cultures. Two experiments with similar results were performed.

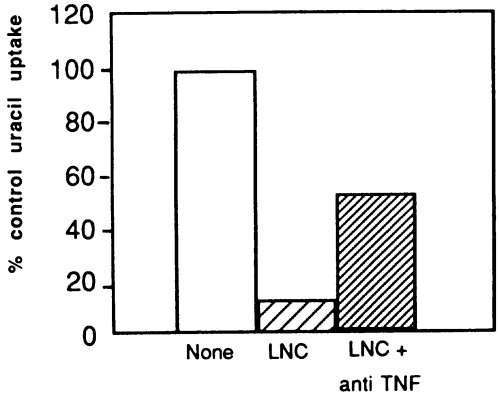

FIG. 7. Effects of anti-TNF on the ability of lymph node lymphocytes to induce antimycobacterial defense in vitro. Macrophage cultures were infected with BCG for 4 h. Lymph node lymphocytes (LNC) from mice infected with BCG were added to cultures without or with the addition of rabbit anti-mouse TNF polyclonal antibody (LNC + anti TNF) (1:100 dilution, a dilution sufficient to inactivate 1,000 U of recombinant TNF per ml). Percent replication was determined from 5-day cultures. Results of separate representative experiments (of three performed) are shown.

Activation of antimycobacterial effects by lymph node effector lymphocytes is inhibited by anti-TNF-α antibody. When we added polyclonal rabbit anti-murine TNF-α antibody (immunoglobulin G, 1:100 dilution of hyperimmune serum) to cocultures of BCG-sensitized lymphocytes and infected macrophages, the antimicrobial effects induced by the effector lymphocytes were markedly reduced (Fig. 7; Table 1); nonimmune rabbit immunoglobulin had no effect in this assay (data not shown).

DISCUSSION

Protective cell-mediated immunity in mycobacteriosis is presently thought to arise as a consequence of (i) certain sensitized $CD4^+$ T cells that secrete soluble macrophage-activating factors (lymphokines) which activate the macrophage to kill or inhibit the growth of intracellular mycobac-

TABLE 1. Reduction in lymphocyte-mediated macrophage activation by anti-TNF-α antibody

Additive to macrophage culture	Uracil uptake[a] by BCG at the following MOI:	
	1:1	3:1
None	6,102 ± 2,045	27,236 ± 1,245
Anti-TNF	NT[b]	21,135 ± 6,568
Lymphocytes	712 ± 411	1,974 ± 304
Lymphocytes + anti-TNF	2,491 ± 294	10,951 ± 1,599

[a] Results are means ± standard errors of the mean in counts per minute and are from three experiments.
[b] NT, not tested.

teria (1a, 9) or (ii) specific cytotoxic T lymphocytes that lyse mycobacterium-infected, bone-marrow-derived macrophages on direct cell contact, presumably contributing to the eventual elimination of the mycobacteria (1a, 3). In some earlier studies on T-cell-mediated defense against mycobacteria, as well as to another intracellular pathogen, *Listeria monocytogenes* (15, 16, 23), it was observed that sensitized T lymphocytes may also be capable of activating macrophages by a mechanism(s) that might not be mediated solely by soluble lymphokines. The possibility that activation signals might be imparted by T cells to macrophages by direct cell-to-cell contact was one that has only recently been investigated in detail (22). The results of the present studies suggest that such a process can occur in vitro against mycobacteria.

Using another model of intracellular infection caused by *L. major*, we recently observed that certain $CD4^+$ lymphocytes can activate macrophage antileishmanial defenses in vitro by cell-to-cell contact (12, 17, 20, 22). This mechanism is distinctive because it does not involve lymphokine secretion and induces no cytotoxic effects in the host cells (12, 23) and because its expression is antigen specific and genetically restricted (10, 19). We further demonstrated that these $CD4^+$ cells display TNF on their surface (mTNF) and provided evidence that the mTNF which $CD4^+$ cells expressed is involved in the activation of the antileishmanial defense (21).

In the present study, we observed that the addition of BCG-sensitized lymphocytes (but not that of *Listeria*-sensitized lymphocytes or of IFN-γ or TNF-α) to macrophages previously infected with BCG inhibited mycobacterial replication (Fig. 2–5). When the lymphocytes were separated from the infected macrophages by a semipermeable membrane that permitted diffusion of soluble mediators (12), no antimycobacterial effects were observed (Fig. 6). This suggested that direct cell contact between infected macrophages and effector lymphocytes was required in order to achieve this effect. The possibility that such contact was required solely for antigen presentation to lymphocytes with subsequent lymphokine release seems unlikely, since antigen-presenting cells (BCG-infected macrophages) were present in the compartment containing lymphocytes. Furthermore, as we and others have found, IFN-γ and other macrophage-activating factors induce antimycobacterial effects only when added prior to infection (Fig. 2, 4, and 6), whereas lymphocytes used in the present experiment were effective when added after infection. The addition of CsA to cultures at a concentration that inhibits IFN-γ production (22) did not abolish antimycobacterial effect (Fig. 6 and 7) imparted by the lymphocytes, further arguing against a role of IFN-γ in the effect.

Although soluble TNF alone, when added to suspension cultures of infected macrophages, exerted minimal or no antimycobacterial activity (Fig. 2), anti-TNF antibody substantially interfered with the effect imparted by the lymphocytes (Fig. 7). There are two possible explanations for this modifying effect of anti-TNF. One is that production of macrophage-derived TNF, in response to contact with effector lymphocytes, plays a key role in the antimicrobial effect. The other is that effector lymphocytes in this process possess membrane-anchored TNF through which the contact-dependent effect is exerted (21).

These observations extend those which we have previously made regarding leishmaniasis. We propose that activation of macrophages for antimicrobial events mediated by direct cell contact with $CD4^+$ T lymphocytes may be an important acquired host defense mechanism in intracellular infections and merits further analysis.

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