

Activation of Macrophages for Destruction of *Francisella tularensis*: Identification of Cytokines, Effector Cells, and Effector Molecules

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Francisella tularensis live vaccine strain (LVS) was grown in culture with nonadherent resident, starch-elicited, or Proteose Peptone-elicited peritoneal cells. Numbers of bacteria increased 4 logs over the input inoculum in 48 to 72 h. Growth rates were faster in inflammatory cells than in resident cells: generation times for the bacterium were 3 h in inflammatory cells and 6 h in resident macrophages. LVS-infected macrophage cultures treated with lymphokines did not support growth of the bacterium, although lymphokines alone had no inhibitory effects on replication of LVS in culture medium devoid of cells. Removal of gamma interferon (IFN- γ) by immunoaffinity precipitation rendered lymphokines ineffective for induction of macrophage anti-LVS activity, and recombinant IFN- γ stimulated both resident and inflammatory macrophage populations to inhibit LVS growth in vitro. Inflammatory macrophages were more sensitive to effects of IFN- γ : half-maximal activity was achieved at 5 U/ml for inflammatory macrophages and 20 U/ml for resident macrophages. IFN- γ -induced anti-LVS activity correlated with the production of nitrite (NO₂⁻), an oxidative end product of L-arginine-derived nitric oxide (NO). Anti-LVS activity and nitrite production were both completely inhibited by the addition of either the L-arginine analog N^G-monomethyl-L-arginine or anti-tumor necrosis factor antibodies to activated macrophage cultures. Thus, macrophages can be activated by IFN- γ to suppress the growth of *F. tularensis* by generation of toxic levels of NO, and inflammatory macrophages are substantially more sensitive to activation activities of IFN- γ for this effector reaction than are more differentiated resident cells.

Francisella tularensis live vaccine strain (LVS) is a facultatively intracellular bacterium which is pathogenic for several different strains of mice and produces infection in these experimental animals similar to that seen in humans (4, 16, 17, 22). Cell-mediated immunity is thought to play a prominent role in protection from and resolution of tularemia (3, 4, 22, 41, 42). The precise cells and mechanisms involved, however, are not known. From our experience with different intracellular pathogens, we recognize that it is not easy, nor is it particularly desirable, to generalize concepts of host defense to a particular pathogen from information derived with other intracellular pathogens. For example, inflammation per se is quite effective in prevention of lethal disease caused by *Listeria monocytogenes*: in vitro studies show that *L. monocytogenes* is killed efficiently by inflammatory macrophages and polymorphonuclear leukocytes (PMN) but not by differentiated tissue macrophages (13); induction of inflammation in vivo prior to challenge of mice with *L. monocytogenes* is protective (19); and mouse strains with defects in inflammatory responses are more innately susceptible to infection than mice with normal inflammatory responses (40). Other intracellular pathogens, however, are totally unaffected by inflammatory changes and are eliminated only after immune intervention. For these infections, the effector cells, signals that induce effector functions, and even effector mechanisms vary from one target organism to another.

Mice are protected from infection with the obligate intracellular protozoan parasite *Leishmania major* by treatment with the macrophage-activating agent *Mycobacterium bovis* BCG (21). This protection is not related to inflammation; in fact, inflammation exacerbates *L. major* disease (21). Inflam-

matory macrophages are more susceptible to infection with this parasite (20) and less responsive to activation in vitro for killing of *L. major* amastigotes than the more differentiated resident cells (29). BCG also protects mice against lethal *Rickettsia tsutsugamushi* infection (37), and this protection, again, is not due to inflammatory events: the 50% lethal dose (LD₅₀) for mice inoculated with an inflammatory stimulus prior to infection is the same as the LD₅₀ for untreated mice (37). Inflammation, while not beneficial, is not detrimental to the host. Inflammatory macrophages, in the case of the rickettsiae, cannot kill the bacteria spontaneously but are just as responsive as resident macrophages to activation signals that induce elimination of these pathogens (38).

Thus, with three different intracellular pathogens that can replicate within macrophages, we find the following: intracytoplasmic *L. monocytogenes* is spontaneously killed by inflammatory cells, not differentiated macrophages, and residual *L. monocytogenes* is cleared from either macrophage population after activation (7, 13); intraphagolysosomal *L. tropica* is not spontaneously killed by any macrophage population, and resident cells (but not inflammatory macrophages) can be activated to kill this parasite (20, 29); and intracytoplasmic rickettsiae, also not eliminated spontaneously, can be killed by activated inflammatory or differentiated macrophages with equivalent efficiency (38). While the macrophage is common to each of these systems, its capacity for effector activity is extremely versatile and clearly dependent upon its state of cellular differentiation, the intracellular compartment of the pathogen, exposure to the appropriate activation signal, and generation of the appropriate effector molecule(s).

That macrophages play a role in resolution of *Francisella* infections has not been documented, but the weight of evidence for other intracellular pathogens and the information that cell-mediated immunity is protective are highly

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suggestive of macrophage involvement. Macrophages can act as a natural barrier defense in early interactions with the pathogen or during inflammation or as immune effector cells after induction of pathogen-specific immunity. For *Francisella* infections, inflammation alone, as in the *Listeria* model, may be protective: indeed PMN can kill LVS (34). As a first step in examining the role of macrophages in *Francisella* infections, we describe the interaction between differentiated tissue macrophages and inflammatory macrophages and *F. tularensis* LVS. We analyze the capacity of these cells to support replication of the pathogen and to be activated for suppression of bacterial growth. We also identify cytokines involved in activation of macrophages and document that NO is the effector molecule for destruction *F. tularensis*.

MATERIALS AND METHODS

Animals. Specific-pathogen-free C3H/HeN male mice were purchased from Harlan Sprague Dawley (Frederick, Md.) and used at 5 to 7 weeks of age. Mice were housed in a barrier facility at the Walter Reed Army Institute of Research (WRAIR) and routinely tested for common animal room pathogens by a diagnostic service provided by the Division of Veterinary Medicine, WRAIR. In conducting the research in this report, the investigators adhered to the *Guide for Laboratory Animal Facilities and Care* as promulgated by the Committee of the Guide for Laboratory Animals and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences, National Research Council.

Bacteria. *F. tularensis* LVS (ATCC 29684; American Type Culture Collection, Rockville, Md.) (43) was cultured on cystine heart agar (CHA) plates with 5% defibrinated rabbit blood (Remel Labs, Lenexa, Kans.) for 4 days at 37°C in 5% CO₂ and 95% humidity. Colonies were selected for growth in modified Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) supplemented with ferric PP_i and IsoVitaleX (Becton Dickinson, Cockeysville, Md.) (6). Inoculated broth cultures were incubated for 24 to 36 h until the bacterial density reached 10⁸ to 10⁹ CFU/ml; they were then aliquoted and frozen at -70°C. One-milliliter aliquots were periodically thawed, and viable bacteria were quantified by plating serial dilutions in 0.1% gelatin-saline on CHA plates. The number of CFU after thawing varied less than 5% over a 4-month period.

PC. Peritoneal cells (PC) were collected from untreated mice (resident peritoneal macrophages) and from mice inoculated 5 days previously with 2% colloidal starch or 3 days previously with 3% Proteose Peptone (inflammatory macrophages) after intraperitoneal injection of 8 to 10 ml of culture medium (Dulbecco modified Eagle medium [DMEM, GIBCO, Grand Island, N.Y.] supplemented with 10% heat-inactivated fetal bovine serum [FBS; Sterile Systems, Inc., Logan, Utah]). Peritoneal fluid was drawn through the abdominal wall with a 19-gauge needle. Fluids from 3 to 10 mice were pooled, samples were removed for differential and total cell counts, and the remaining fluid was centrifuged at 250 × *g* for 10 min at 4°C. Differential cell counts were made on Wright-stained cell smears (Diff-Quick; Dade Diagnostics, Aquado, P.R.) prepared by cytocentrifugation (Cytospin centrifuge; Shandon Southern Instruments, Camberly, England). Washed PC suspensions were adjusted to 10⁶ macrophages per ml in culture medium and incubated as unfractionated PC cultures in polypropylene tubes (no. 2063;

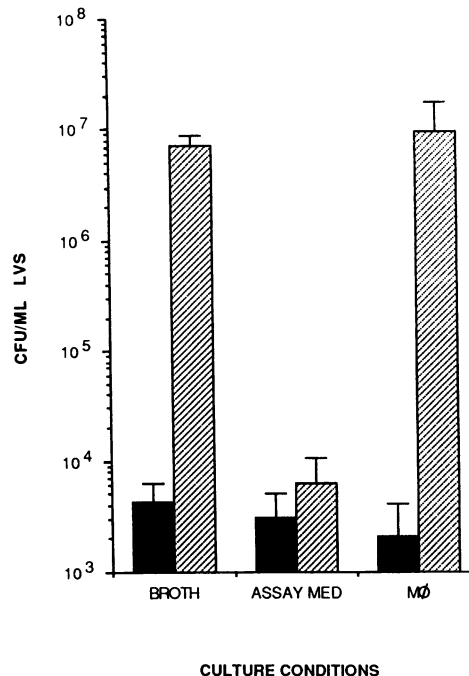


FIG. 1. LVS (10⁴ CFU) was added to either 1.0 ml of modified Mueller-Hinton broth with IsoVitaleX (BROTH), or 1.0 ml of DMEM plus 10% heat-inactivated FBS (ASSAY MED); LVS (10⁶ CFU) was added to 1.0 ml of DMEM plus 10% heat-inactivated FBS containing 10⁶ resident peritoneal macrophages (MΦ). All cultures were incubated at 37°C in 5% CO₂; all were analyzed for LVS CFU at 2 h (■) and at 72 h (▨) by serial dilutions in gelatin-saline and plating on CHA for quantification of CFU. Results are the means ± standard errors of the means from three separate experiments for BROTH cultures and two separate experiments for both ASSAY MED and MΦ.

Falcon Plastics, Oxnard, Calif.) in 5% CO₂ at 37°C before exposure to LVS.

Lymphokines. Spleen cells from untreated C3H/HeN mice were exposed to concanavalin A (ConA; Pharmacia LKB Biotechnology, Piscataway, N.J.) and used as a source of lymphokines. Spleens were aseptically removed and passed through 50-mesh stainless steel sieves into tissue culture medium (DMEM; GIBCO). Cells were treated with NH₄Cl lysis buffer, centrifuged at 250 × *g* for 10 min at 4°C, and resuspended to a concentration of 5 × 10⁶ viable cells per ml in medium with 5% heat-inactivated FBS (Sterile Systems). FBS contained <0.01 ng of lipopolysaccharide per ml by the *Limulus* amoebocyte lysate assay. Twenty milliliters of spleen cell suspension with 5 μg of ConA per ml was incubated in 75-cm² plastic tissue culture flasks (no. 3023; Falcon Plastics) for 48 h at 37°C. ConA was adsorbed by 10 mg of Sephadex G-10 (Pharmacia LKB Biotechnology) per ml before storage. Aliquots of cell supernatants were stored at 4°C until used. Control supernatants consisted of the culture medium from spleen cells, to which 5 μg of ConA per ml was added after the incubation period. In no experiments did control supernatants induce antimicrobial activity which was observed in macrophage cultures treated with lymphokines; bacterial growth in macrophage cultures treated with control spleen cell supernatants was similar to that in medium-treated controls. It should be noted that no antibiotics were included in the lymphokine preparations.

Infection and treatment of macrophages for induction of

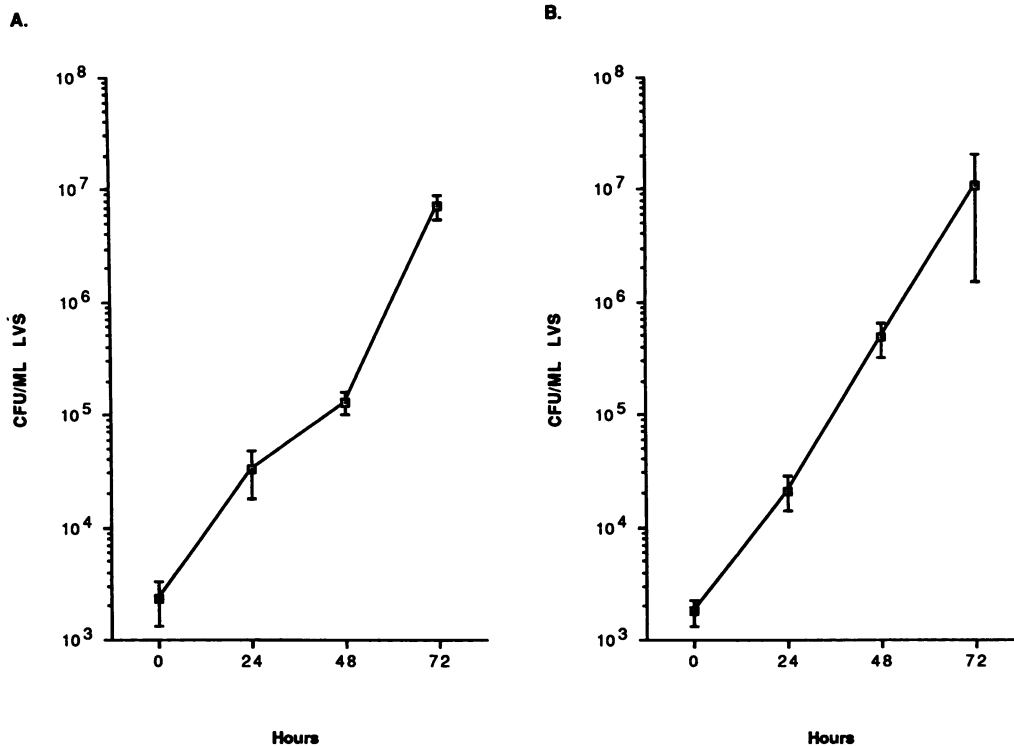


FIG. 2. Time course for growth of LVS. (A) LVS was inoculated into modified Mueller-Hinton broth and cultured at 37°C in 5% CO₂ for 72 h. Samples were taken directly from the broth culture and plated to determine CFU at each time point. Results are the means ± standard errors of the means from three separate, but similar, experiments. (B) LVS was inoculated into cultures of resident peritoneal macrophage (10⁶ macrophages per ml in DMEM plus 10% heat-inactivated FBS) at an MOI of 1 and cultured as described in Materials and Methods. At the indicated times macrophages were sonicated, serially diluted, and plated for estimation of CFU. Results are means ± standard errors of the means from three separate, but similar, experiments.

anti-LVS activity in vitro. Macrophages were exposed to LVS at a multiplicity of infection (MOI) of 1 for 2 h at 37°C in a humid environment. Higher MOIs (bacterium/cell ratios of 10:1 and 100:1) were also used to infect macrophages, resulting in increased bacterial outgrowth by 1 and 2 logs, respectively. Infected PC were washed with culture medium by low-speed centrifugation (70 × *g* for 7 min) to remove uningested bacteria, and cell pellets were resuspended in fresh culture medium. To determine infection at time zero, PC cultures were sonicated at 100 W for 30 s (Branson Sonifier; Branson Ultrasonics, Danbury, Conn.) on ice. Lysed cell suspensions were serially diluted in 1% gelatin-saline and plated on CHA to quantify bacteria. Time zero outgrowth was usually 1% of the input inoculum (5 × 10³ LVS CFU were recovered from macrophages infected for 2 h with 5 × 10⁵ LVS). Attempts to increase cell uptake by addition of fresh normal serum or LVS-immune serum (serum from mice inoculated intradermally with 10³ LVS CFU 28 days prior to blood collection) were unsuccessful. Remaining cultures were incubated in medium or treated with dilutions of lymphokines or recombinant murine gamma interferon (IFN-γ) (Genentech, South San Francisco, Calif.) for an additional 72 h at 37°C in 5% CO₂. Increased MOI or addition of sera did not alter the effects of IFN-γ on macrophage cultures. Some cultures were also exposed to N^G-monomethyl-L-arginine (N^G-MMLA; Calbiochem-Behring Corp., La Jolla, Calif.), a specific inhibitor of the nitrogen oxidation of L-arginine. Still other cultures were treated with either 40 μg of anti-tumor necrosis factor (TNF) antibody (TN3) per ml or control antibody of a similar

isotype (L9) (both generous gifts from Robert D. Schreiber, Washington University, St. Louis, Mo.). At other time points (up to 72 h), PC cultures were sonicated, diluted, and plated as described above to determine bacterial numbers.

Immunoaffinity depletion of IFN-γ from lymphokines. IFN-γ was precipitated with a hamster monoclonal antibody (MAb) prepared against recombinant murine IFN-γ (H22, a generous gift from Robert D. Schreiber) and protein A-Sepharose (Pharmacia LKB Biotechnology). Quantitation of IFN-γ in lymphokines was by enzyme-linked immunosorbent assay (ELISA) (36). Lymphokines routinely contained 40 to 80 U of IFN-γ per ml, which was reduced to levels undetectable by ELISA following immunoaffinity precipitation with H22.

Measurement of NO₂⁻ production by LVS-infected macrophages. Culture fluids were assayed for NO₂⁻ by the Griess reaction (24). Briefly, 50-μl aliquots of the conditioned medium were incubated with 200 μl of 1% sulfanilamide and 200 μl of 0.1% *N*-1-naphthylethylenediamine dihydrochloride in 2.5% H₃PO₄ (Sigma) at room temperature for 5 min. A₅₄₃ was measured. NO₂⁻ was quantified by comparison to NaNO₂ as a standard.

RESULTS

Growth of LVS in vitro. LVS is a facultative intracellular organism (41), and there is now direct proof of the intracellular nature of its association with cells (2). Indeed, the bacterium grows in murine peritoneal macrophages (Fig. 1). Bacterial numbers increased approximately 4 logs both in

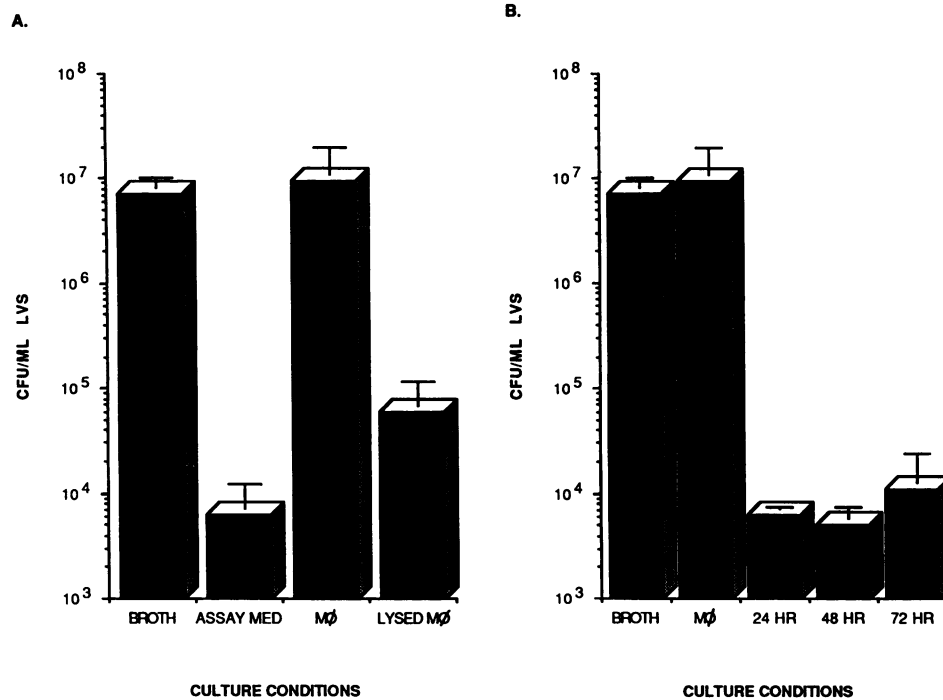


FIG. 3. Growth of LVS under different in vitro tissue culture conditions. (A) LVS (10^4 CFU) was cultured in *Francisella* growth medium (BROTH), macrophage assay medium (ASSAY MED), and macrophage assay medium supplemented with 10^6 resident peritoneal macrophages per ml sonicated at 150 W for 30 s (LYSED Mφ). Bacteria were cultured for 72 h at 37°C in 5% CO₂. Duplicate samples from each culture were serially diluted and inoculated onto CHA plates for estimation of CFU per milliliter. Results are means \pm standard errors of the means from two separate experiments. Growth of LVS in cultures with 10^6 live resident peritoneal macrophages per ml (Mφ) was estimated as before (see the legend to Fig. 1). (B) For macrophage conditioned medium, 10^6 resident peritoneal macrophages per ml were cultured for 24, 48, and 72 h, culture fluids were sterilized by filtration (0.2- μ m pore size; Gelman Sciences, Ann Arbor, Mich.). LVS was grown in this conditioned medium, and growth was compared with that in *Francisella* broth and live macrophage cultures. Culture conditions were the same as described for panel A; CFU were estimated after 72 h in culture. Results are means \pm standard errors of the means from two separate experiments.

broth medium designed to support LVS growth (modified Mueller-Hinton broth) and in peritoneal macrophage cultures. In contrast, tissue culture medium alone did not support bacterial growth at all. Growth curves for LVS in broth and macrophage cultures were different. While the overall growth in both cultures was similar (4-log increase over 72 h), there was a biphasic nature of the growth curve for broth that was not apparent in the growth curve for cells (Fig. 2). Generation time for the bacterium in broth between 0 and 24 h was similar to that observed at all times in resident peritoneal cells (5 to 6 h). Growth in broth slowed between 24 and 48 h and then entered another exponential phase between 48 and 72 h, with a generation time of 2 to 3 h.

To determine if live macrophages were essential for bacterial growth, we cultured LVS with lysed (by sonication) or fixed (by paraformaldehyde) macrophages in assay medium (Fig. 3A); neither of these preparations supported LVS growth in culture medium. The bacteria were also unable to replicate in culture medium supplemented with macrophage-conditioned medium (Fig. 3B). Thus, LVS growth in PC cultures was not simply the result of macrophage secretion of an essential growth factor into the extracellular milieu but the result of an intimate association with live cells.

LVS growth in resident and inflammatory macrophages. Resident peritoneal macrophages supported in vitro growth of LVS (Fig. 1 to 3), but macrophages differ greatly in function depending on the stage of maturation (12). Inflammatory peritoneal macrophages obtained from animals inoc-

ulated with Proteose Peptone or a suspension of colloidal starch also supported LVS growth (Fig. 4A). Growth curves for LVS in each of these inflammatory populations and resident cells were not significantly different (Fig. 4B). There was, however, a notably faster generation time in the inflammatory cell populations (doubling time of 3 to 3.5 h, estimated for two different populations on two different occasions) compared with that in resident cell populations (doubling time of 5 to 6.5 h from three different experiments).

Inhibition of growth by treatment of macrophages with lymphokines. Macrophages treated with lymphokines kill a variety of intracellular (bacteria and protozoa) and extracellular (tumor cells and helminths) targets; responsiveness to activation signals in lymphokines for killing of these different targets is dependent, in part, on the maturational status of the macrophage (38). The more-differentiated resident tissue macrophages were treated with lymphokines and assessed for capacity to inhibit replication of LVS (Fig. 5). These macrophages responded to a 1:10 dilution of lymphokines (which contained approximately 6 U of IFN- γ per ml) with complete inhibition of bacterial growth, and some (1-log) killing; infection at 2 h was 5×10^3 LVS CFU per ml, and at 72 h it was 3×10^2 LVS CFU per ml. The plateau for antimicrobial activity was reached at a 1:80 dilution, and half-maximal activity for lymphokines with this population of macrophages was a 1:20 dilution.

IFN- γ induction of anti-LVS activity in macrophages.

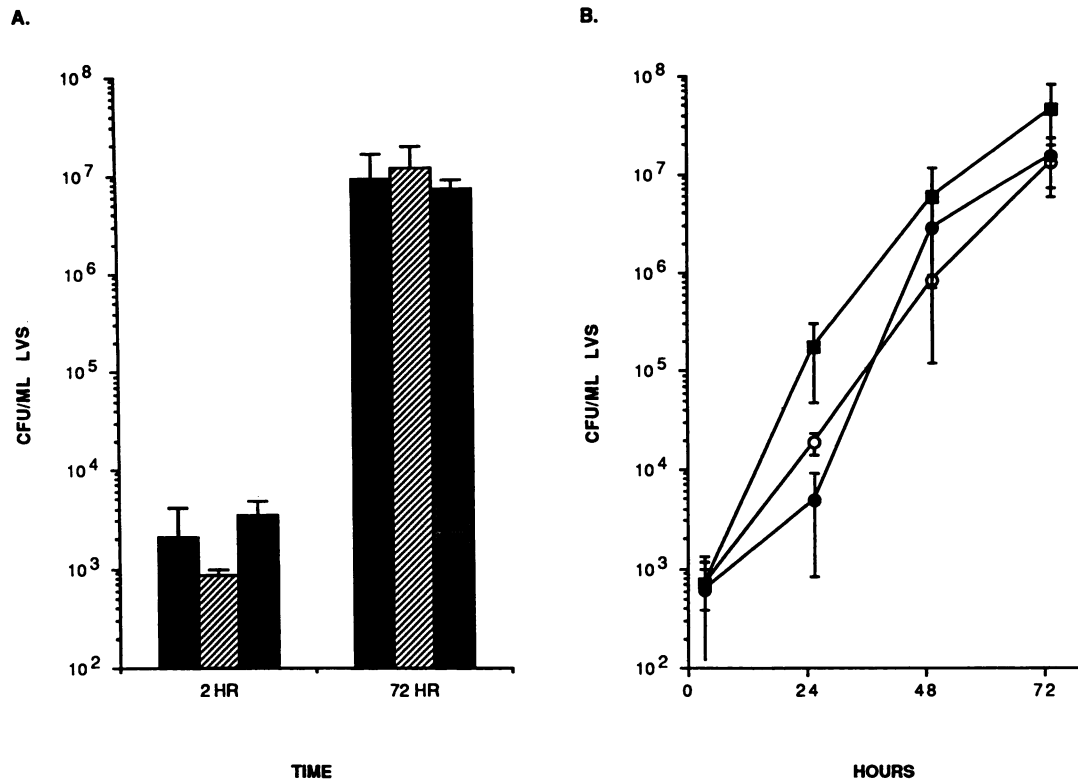


FIG. 4. Growth of LVS in resident or inflammatory macrophages. (A) CFU at 2 and 72 h after inoculation of LVS into macrophage cultures. Resident peritoneal (■), Proteose Peptone-elicited (□), or starch-elicited (▨) macrophages were seeded at 10^6 macrophages per ml in 1.0-ml volumes and exposed to 10^6 LVS for 2 h at 37°C in 5% CO_2 . Cell cultures were washed by centrifugation and resuspended in fresh medium. Replicate cultures were sonicated, and duplicate samples were serially diluted and plated on CHA for estimation of CFU per milliliter. The remainder of the cultures were incubated for an additional 72 h at 37°C in 5% CO_2 and then sonicated and assayed for CFU per milliliter as described above. (B) Estimation of CFU per milliliter in cultures of resident (○), Proteose Peptone-elicited (■), and starch-elicited (●) macrophages. Cells were infected and assayed as described above. Samples were removed every 24 h for estimation of CFU. Results are means \pm standard errors of the means from two separate experiments with starch-elicited cells and three separate experiments with resident and Proteose Peptone-elicited cells.

IFN- γ is a potent macrophage activation factor and was present in the lymphokines that induced anti-LVS activity in resident peritoneal macrophages. To determine if IFN- γ was responsible for the growth-inhibiting effects of lymphokine-treated macrophages, we removed IFN- γ by treating lymphokines with an MAb specific for IFN- γ and precipitating the immune complexes formed with protein A-Sepharose. Removal of IFN- γ from lymphokines effectively removed all macrophage antibacterial activity (Fig. 6).

Recombinant IFN- γ stimulated both resident and inflammatory macrophages to inhibit bacterial growth in a dose-dependent manner (Fig. 7). Interestingly, resident cells required higher concentrations of IFN- γ for bacterial growth inhibition than inflammatory cells: half-maximal activity of IFN- γ for induction of anti-LVS activity was 20 U/ml for resident cells and 5 U/ml for inflammatory macrophages. Recombinant IFN- γ alone (no cells) had no effect on the viability of LVS or its capacity to replicate in broth (data not shown). To further define the antibacterial activity induced with this macrophage-activating cytokine, we analyzed growth in IFN- γ -treated and untreated cells every 24 h for 5 days (Fig. 8). Untreated LVS-infected macrophages were less than 50% viable after 5 days in culture with LVS, and bacterial counts begin to decline. In contrast, IFN- γ -treated macrophages were over 70% viable after 5 days. While there was early growth of LVS (Fig. 8), bacterial killing was

apparent at 48 h and continued through day 5. The number of recoverable CFU in IFN- γ -treated cultures at 5 days was at least a log lower than the input inoculum ($\geq 90\%$ killing).

IFN- γ -induced anti-LVS activity and NO_2^- production. NO_2^- produced by activated macrophages correlates directly with killing of a variety of infectious targets in murine systems (1, 15, 25–27, 30); the presumed effector molecule for killing of these agents is NO, the short-lived reactive nitrogen intermediate that is rapidly oxidized to NO_2^- (23). NO_2^- , then, is a quantitative index of macrophage activation and killing capacity. Production of NO_2^- also correlated with anti-LVS activity in macrophages activated with IFN- γ (Table 1). The competitive inhibitor of L-arginine metabolism, N^G -MMLA, blocked IFN- γ -induced anti-LVS activity and production of NO_2^- in both resident and inflammatory macrophage populations (Table 1); TNF- α , the second signal for NO_2^- production and killing of intracellular targets (25, 28, 33), was also involved in destruction of *F. tularensis*, since an anti-TNF- α MAb also blocked growth inhibition and NO_2^- production in these cultures (Table 1).

DISCUSSION

To identify treatment modalities or prophylactic therapies against infectious threats, particularly intracellular pathogens, one must have a firm grasp of the biologic idiosyncra-

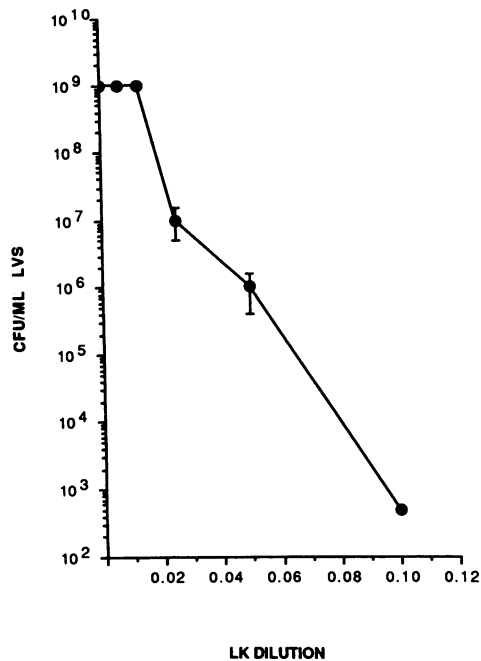


FIG. 5. Growth of LVS in macrophages exposed to lymphokines. Resident peritoneal macrophages were infected for 2 h as described in Materials and Methods, washed, and resuspended in medium with different concentrations of lymphokines. Results are mean CFU \pm standard error of the mean for triplicate cultures determined at 72 h after infection.

cies of the effector cell-target interaction. The macrophage is an important effector cell for both humoral and cellular immunity. Pathogens developed interesting strategies for intracellular survival in these cells, and the strategies are as diverse as the organisms themselves. Some pathogens evade the potent reactive oxygen intermediates released in the respiratory burst during phagocytosis by macrophages; others evade destruction in the phagolysosome, the digestive organelle of the macrophage. The most successful intracellular pathogens acquire multiple evasive strategies to ensure their survival. For example, *R. tsutsugamushi* buds from the surface of infected cells and passes from cell to cell wrapped in a protective coating of host cell membrane; once phagocytosed by an adjacent cell, it quickly dissolves its multilayered host cell coat to replicate freely in the cytoplasm (18). *L. monocytogenes* resident in the cytoplasm of cells actually propels itself into a pseudopod by polymerization of an actin filament tail, and the pseudopod is clipped off and internalized by the adjacent host cell by phagocytosis; the hemolysin of *L. monocytogenes* aids in its release from the phagosome and ensures its survival for replication in the cytoplasm (44). These two pathogens spend little time exposed to humoral defensive factors of the host since they remain sequestered within cells or coated by host membranes as they spread from cell to cell. *Rickettsia prowazekii*, on the other hand, does not bud from cells. It uses its phospholipase to break a hole in the host cell plasma membrane after it attaches; as the cell attempts to repair itself behind the bacterium, the rickettsia is actually pushed into the cytoplasm where it is then free to replicate (45). The rickettsiae and *L. monocytogenes*, then, escape the detrimental effects of phagosome-lysosome fusion by dissolving or avoiding the phagosome and replicating in the cytoplasm of the cell. Other pathogens that

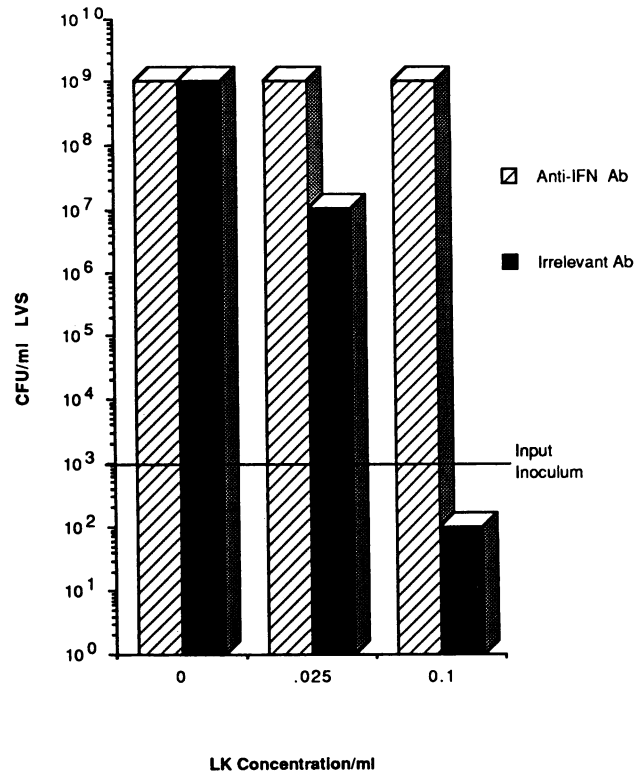


FIG. 6. Growth of LVS in macrophages exposed to lymphokines depleted of IFN- γ . Resident peritoneal macrophages were infected with LVS for 2 h as described in Materials and Methods. After being washed, cells were resuspended in medium containing dilutions of lymphokines treated with either a MAb prepared against IFN- γ , H22 (▨), or a MAb prepared against interleukin-4, 11B11 (■), and protein A-Sepharose. Untreated lymphokines contained 80 U of IFN- γ per ml; IFN- γ in lymphokines treated with H22 and protein A-Sepharose was undetectable by specific ELISA or bioassay; IFN- γ in lymphokines treated with 11B11 and protein A-Sepharose was 75 U/ml. Macrophage cultures were analyzed for CFU per milliliter at 72 h.

replicate in macrophages, such as toxoplasmas and mycobacteria, escape the innate antimicrobial armamentarium of this cell by actively preventing lysosome fusion: these pathogens replicate inside phagosomes (5, 31). Still others, like leishmaniae, replicate in the hostile environment of the phagolysosome, using the low pH of this organelle to their enzymatic advantage and neutralizing host-reactive oxygen effector molecules by specialized superoxide dismutases (10, 11). Clearly, host-target interactions are refined and diversified by pathogen-specific characteristics. That *F. tularensis* replicates in association with macrophages, and only viable macrophages under tissue culture conditions, suggests that it too has developed a number of evasive strategies for survival in the presence of these cells. The nature of these strategies is presently unknown, as is the actual site of replication, although the phagolysosome has been ruled out in one study (2). Once the intracellular compartment of *F. tularensis* replication is determined, identification of the intracellular survival strategies of this bacterium will follow quickly.

One of the most effective barrier defenses of vertebrate hosts is the tissue macrophage. The antimicrobial arsenal of tissue macrophages is extensive and includes both oxidative and nonoxidative killing mechanisms. Our studies suggest

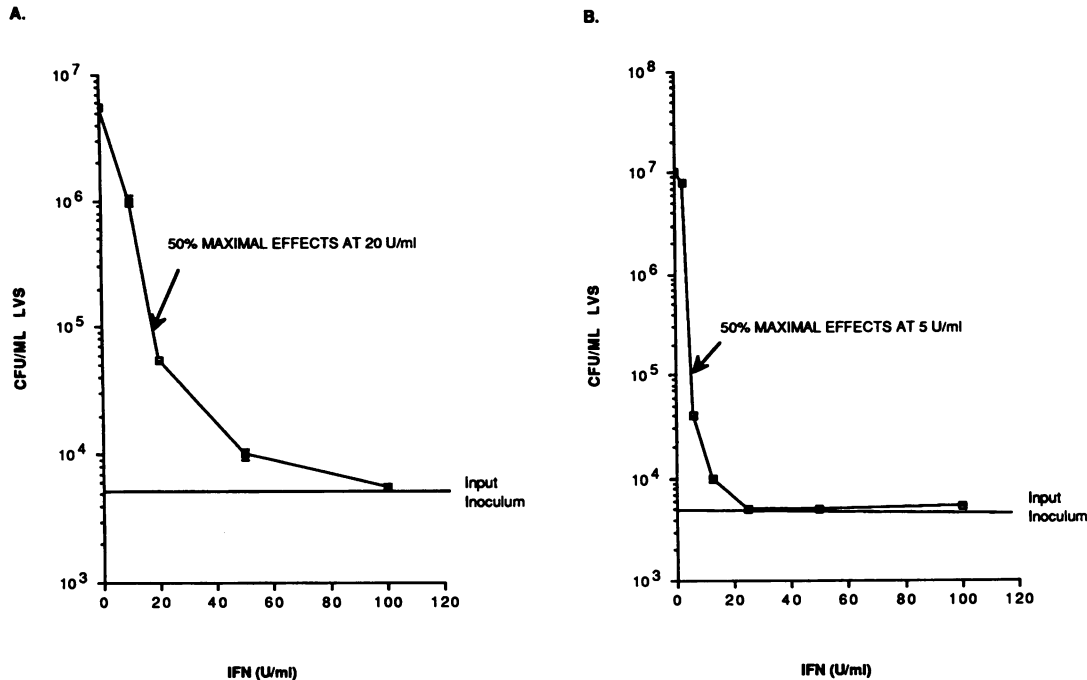


FIG. 7. Growth of LVS in macrophages exposed to recombinant IFN- γ . (A) Resident peritoneal macrophages were infected with LVS as described in Materials and Methods. Cells were washed and resuspended in medium with different concentrations of recombinant murine IFN- γ for 72 h. (B) Proteose Peptone-elicited macrophages were treated in a manner identical to treatment of resident cells for panel A. Data are reported in both panels as mean CFU per milliliter \pm standard error of the mean for duplicate samples plated at 72 h.

not only that LVS will survive innate antibacterial activities of macrophages in the tissues where it is inoculated but that these macrophages can also provide a safe haven for the replication of LVS (Fig. 1 to 3). A very early event (within minutes) in development of protective host responses is inflammation; the primary cellular participants in this early interaction with the pathogen are PMN. LVS is susceptible to oxidative killing mechanisms of PMN, particularly hypochlorous acid (34). Most extracellular bacteria will be phagocytosed and killed by these cells. LVS organisms that have already infected tissue macrophages, however, will not be susceptible to PMN killing and will replicate over time. Perhaps the capacity to survive within macrophages derived from the need of this pathogen to escape the lethal consequences of interactions with PMN. The PMN infiltrate is followed within hours by macrophages newly immigrated into tissue from circulation, inflammatory macrophages, which rapidly replace the PMN population as the predominant cell. Inflammatory macrophages, compared with resident cells, have increased enzyme activities and increased oxidative capacity and are better able to kill at least one intracellular pathogen (12, 13). LVS, however, is also not susceptible to the inherent killing capacity of inflammatory macrophages (Fig. 4). In fact, LVS replicates even more efficiently in the presence of these inflammatory cells, perhaps a reflection of their increased metabolic activity. Thus, LVS has clearly evolved mechanisms for evasion of both barrier and first-line (inflammatory) host defense mechanisms.

Neither resident nor inflammatory macrophages can eliminate *F. tularensis* without help from other cells. This help is provided by cytokines produced by antigen-specific T lymphocytes during initiation of an immune response and is characterized by development of qualitative changes in

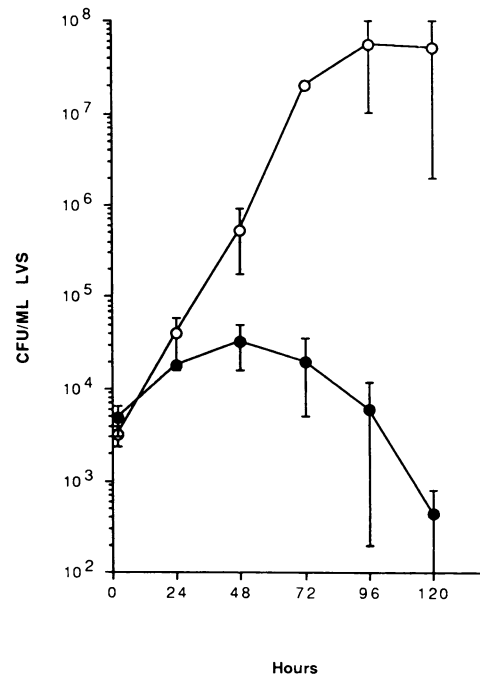


FIG. 8. Growth of LVS in macrophages exposed to IFN- γ . Resident peritoneal macrophages were infected with LVS as described in Materials and Methods. Cells were washed and resuspended in medium with (●) and without (○) 50 U of recombinant murine IFN- γ per ml. Duplicate samples were removed every 24 h for 5 days for an estimation of CFU. Results are means \pm standard errors of the means from two separate experiments.

TABLE 1. Correlation between NO₂⁻ production and anti-LVS activity of IFN-γ-activated macrophages

Expt and macrophage phenotype	Cell treatment	CFU of LVS/ml	NO ₂ ⁻ (μmol/10 ⁶ cells/72 h)
Expt 1			
Resident	Medium	2 × 10 ⁶	0
	IFN-γ ^a	5 × 10 ³	8
	IFN-γ + N ^G -MMLA ^b	8 × 10 ⁵	1
Inflammatory	Medium	7 × 10 ⁶	0
	IFN-γ	2 × 10 ³	8
	IFN-γ + N ^G -MMLA	9 × 10 ⁵	1
Expt 2			
Resident	Medium	2 × 10 ⁸	0
	IFN-γ	5 × 10 ⁵	21
	IFN-γ + N ^G -MMLA	3 × 10 ⁷	2
	IFN-γ + anti-TNF MAb ^c	5 × 10 ⁷	2
Inflammatory	Medium	8 × 10 ⁷	0
	IFN-γ	3 × 10 ³	16
	IFN-γ + N ^G -MMLA	8 × 10 ⁷	1
	IFN-γ + anti-TNF MAb	3 × 10 ⁷	3

^a 50 U of IFN-γ per ml.^b 500 μM N^G-MMLA.^c 40 μg of anti-TNF MAb TN3 per ml.

macrophage physiology and function. Cytokines such as IFN-γ activate macrophages to kill any number of intracellular and extracellular targets. This activity is modified, however, by the nature of the macrophage population and the nature of the target cell itself. For example, resident macrophages stimulated with IFN-γ kill intracellular protozoan parasites but inefficiently kill tumor cells; in contrast, inflammatory macrophages stimulated with IFN-γ kill extracellular tumor cells but are not effective against intracellular protozoan parasites (38). In the present study, we found that growth of LVS is inhibited in cultures of both resident and inflammatory cells stimulated with IFN-γ; inflammatory cells, however, required far less IFN-γ to achieve equivalent levels of killing than resident tissue macrophages (Fig. 7). Activated macrophages have many potential antimicrobial effector activities not present in resting macrophages, including the down-regulation of transferrin receptors for decreasing intracellular iron (8), degradation of tryptophan (9, 35), and production of effector molecules such as toxic oxygen and nitrogen intermediates (14, 15, 27, 39). NO derived from L-arginine is a potent effector molecule for intracellular destruction of *F. tularensis* (Table 1). For NO-mediated killing of other intracellular pathogens, the target itself triggers release of NO by the induction and autocrine action of TNF-α (25, 26, 32); in this, *F. tularensis* is similar to protozoan parasites. Destruction of LVS bacteria by IFN-γ-stimulated resident or inflammatory macrophages directly correlated with NO₂⁻ production and required TNF-α (Table 1).

In this study, then, we found that macrophages supported the replication of *F. tularensis* but can be activated to kill this bacterium in vitro by exposure to IFN-γ. The effector molecule for activated-macrophage killing of *F. tularensis* was a reactive nitrogen intermediate, and initiation of the nitrogen oxidation of L-arginine required LVS-induced production of TNF-α by macrophages. This TNF-α then served as the trigger for macrophage-mediated antibacterial activity. How these in vitro observations relate to pathogenesis and resolution of infections with *F. tularensis* in vivo is

presently unknown, but this study identified several critical concepts to explore in our experimental models of tularemia.

REFERENCES

- Adams, L. B., J. B. Hibbs, R. R. Taintor, and J. L. Krahenbuhl. 1990. Microbistatic effect of murine-activated macrophages for *Toxoplasma gondii*: role for synthesis of inorganic nitrogen oxides from L-arginine. *J. Immunol.* **144**:2725-2730.
- Anthony, L. S. D., R. D. Burke, and F. E. Nano. 1991. Growth of *Francisella* spp. in rodent macrophages. *Infect. Immun.* **59**:3291-3296.
- Anthony, L. S. D., E. Ghadirian, F. P. Nestel, and P. A. L. Kongshavn. 1989. The requirement for gamma interferon in resistance of mice to experimental tularemia. *Microb. Pathog.* **7**:421-428.
- Anthony, L. S. D., and P. A. L. Kongshavn. 1987. Experimental murine tularemia caused by *Francisella tularensis*, live vaccine strain: a model of acquired cellular resistance. *Microb. Pathog.* **2**:3-14.
- Armstrong, J. A., and P. D. Hart. 1971. Response of cultured macrophages to *Mycobacterium tuberculosis*, with observations on fusion of lysosomes with phagosomes. *J. Exp. Med.* **134**:713-740.
- Baker, C. N., D. G. Hollis, and C. Thornsberry. 1985. Antimicrobial susceptibility testing of *Francisella tularensis* with a modified Mueller-Hinton broth. *J. Clin. Microbiol.* **22**:212-215.
- Buchmeier, N. A., and R. D. Schreiber. 1985. Requirement of endogenous interferon-γ production for resolution of *Listeria monocytogenes* infection. *Proc. Natl. Acad. Sci. USA* **82**:7404-7409.
- Byrd, T., and M. Horwitz. 1989. Interferon gamma-activated human monocytes down-regulate transferrin receptors and inhibit the intracellular multiplication of *Legionella pneumophila* by limiting the availability of iron. *J. Clin. Invest.* **83**:1457-1465.
- Carlin, J. M., E. C. Borden, and G. I. Byrne. 1989. Interferon-induced indoleamine 2,3-dioxygenase activity inhibits *Chlamydia psittaci* replication in human macrophages. *J. Interferon Res.* **9**:329-337.
- Chang, K. P., and D. M. Dwyer. 1976. Multiplication of a human parasite (*Leishmania donovani*) in phagolysosomes of hamster macrophages in vitro. *Science* **193**:678-680.
- Channon, J. Y., and J. M. Blackwell. 1985. A study of the sensitivity of *Leishmania donovani* promastigotes and amastigotes to hydrogen peroxide. II. Possible mechanisms involved in protective H₂O₂ scavenging. *Parasitology* **91**:207-213.
- Cohn, Z. A. 1978. The maturation and activation of mononuclear phagocytes: fact, fancy, and future. *J. Immunol.* **121**:813-814.
- Czuprynski, C., P. M. Henson, and P. A. Campbell. 1984. Killing of *Listeria monocytogenes* by inflammatory neutrophils and mononuclear phagocytes from immune and nonimmune mice. *J. Leukocyte Biol.* **35**:193-208.
- Denis, M. 1991. Interferon-gamma-treated murine macrophages inhibit growth of tubercle bacilli via the generation of reactive nitrogen intermediates. *Cell. Immunol.* **132**:150-157.
- Drapier, J. C., and J. B. Hibbs. 1988. Differentiation of murine macrophages to express nonspecific cytotoxicity for tumor cells results in L-arginine-dependent inhibition of mitochondrial iron-sulfur enzymes in the macrophage effector cells. *J. Immunol.* **140**:2829-2834.
- Eigelsbach, H. T., and C. M. Downs. 1961. Prophylactic effectiveness of live and killed tularemia vaccines. I. Production of vaccine and evaluation in the white mouse and guinea pig. *J. Immunol.* **87**:415-425.
- Eigelsbach, H. T., D. H. Hunter, W. A. Janssen, H. G. Dangerfield, and S. G. Rabinowitz. 1975. Murine model for study of cell-mediated immunity: protection against death from fully virulent *Francisella tularensis* infection. *Infect. Immun.* **12**:999-1005.
- Ewing, E. P., A. Takeuchi, A. Shirai, and J. V. Osterman. 1978. Rickettsiae: an ultrastructural study. *Infect. Immun.* **19**:1068-1076.
- Florentin, I., J. Bird, Y. LeGarrec, V. Chung, and J. P. Giroud.

1985. Modifications of host defence mechanisms by an acute non-immunological inflammatory reaction. *Br. J. Exp. Pathol.* **66**:257-270.
20. Fortier, A. H., D. L. Hoover, and C. A. Nacy. 1982. Intracellular replication of *Leishmania tropica* in mouse peritoneal macrophages: amastigote infection of resident cells and inflammatory exudate macrophages. *Infect. Immun.* **38**:1304-1308.
 21. Fortier, A. H., B. A. Mock, M. S. Meltzer, and C. A. Nacy. 1987. *Mycobacterium bovis* BCG-induced protection against cutaneous and systemic *Leishmania major* infections of mice. *Infect. Immun.* **55**:1707-1714.
 22. Fortier, A. H., M. V. Slayter, R. Ziemba, M. S. Meltzer, and C. A. Nacy. 1991. Live vaccine strain of *Francisella tularensis*: infection and immunity in mice. *Infect. Immun.* **59**:2922-2928.
 23. Granger, D. L., J. B. Hibbs, J. R. Prefect, and D. T. Durack. 1990. Metabolic fate of L-arginine in relation to microbistatic capability of murine macrophages. *J. Clin. Invest.* **85**:264-268.
 24. Green, L. C., D. A. Wagner, J. Glogowski, P. L. Skipper, J. S. Wishnok, and S. R. Tannenbaum. 1982. Analysis of nitrate, nitrite, and ¹⁵nitrate in biological fluids. *Anal. Biochem.* **126**:131-134.
 25. Green, S. J., R. M. Crawford, J. T. Hockmeyer, M. S. Meltzer, and C. A. Nacy. 1990. *Leishmania major* amastigotes initiate the L-arginine-dependent killing mechanism in IFN- γ stimulated macrophages by induction of tumor necrosis factor- α . *J. Immunol.* **145**:4290-4297.
 26. Green, S. J., M. S. Meltzer, J. B. Hibbs, and C. A. Nacy. 1990. Activated macrophages destroy intracellular *Leishmania major* amastigotes by an L-arginine-dependent killing mechanism. *J. Immunol.* **144**:278-283.
 27. Green, S. J., C. A. Nacy, and M. S. Meltzer. 1991. Cytokine-induced synthesis of nitrogen oxides in macrophages: a protective host response to *Leishmania* and other intracellular pathogens. *J. Leukocyte Biol.* **50**:93-103.
 28. Havell, E. 1989. Evidence that tumor necrosis factor has an important role in antibacterial resistance. *J. Immunol.* **143**:2894-2899.
 29. Hoover, D. L., and C. A. Nacy. 1984. Macrophage activation to kill *Leishmania tropica*: defective intracellular destruction of amastigotes by macrophages elicited by sterile inflammatory agents. *J. Immunol.* **132**:1487-1493.
 30. James, S. L., and J. Glaven. 1989. Macrophage cytotoxicity against schistosomula of *Schistosoma mansoni* involves arginine-dependent production of reactive nitrogen intermediates. *J. Immunol.* **143**:4208-4214.
 31. Jones, T. C., and J. G. Hirsch. 1972. The interaction between *Toxoplasma gondii* and mammalian cells. II. The absence of lysosomal fusion with phagocytic vacuoles containing living parasites. *J. Exp. Med.* **136**:1173-1194.
 32. Keller, R., R. Gehri, R. Keist, E. Huf, and F. H. Kayser. 1991. The interaction of macrophages and bacteria: a comparative study of the induction of tumoricidal activity and of reactive nitrogen intermediates. *Cell. Immunol.* **134**:249-256.
 33. Kindler, V., A. P. Sappino, G. E. Grau, P. F. Piguët, and P. Vassalli. 1989. The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. *Cell* **56**:731-736.
 34. Lofgren, S., A. Tarnvik, M. Thore, and J. Carlsson. 1984. A wild and an attenuated strain of *Francisella tularensis* differ in susceptibility to hypochlorous acid: a possible explanation of their different handling by polymorphonuclear leukocytes. *Infect. Immun.* **43**:730-734.
 35. Murray, H., A. Szuro-Sudol, D. Wellner, J. Oca, A. Granger, D. Libby, C. Rothermel, and B. Rubin. 1989. Role of tryptophan degradation in respiratory burst-independent antimicrobial activity of gamma interferon-stimulated human macrophages. *Infect. Immun.* **57**:845-849.
 36. Nacy, C. A., A. H. Fortier, M. S. Meltzer, N. A. Buchmeier, and R. D. Schreiber. 1985. Macrophage activation to kill *Leishmania major*: activation of macrophages for intracellular destruction of amastigotes can be induced by both recombinant interferon- γ and non-interferon lymphokines. *J. Immunol.* **135**:3505-3511.
 37. Nacy, C. A., and M. S. Meltzer. 1984. Macrophages in resistance to rickettsial infections: protection against lethal *Rickettsia tsutsugamushi* infection by treatment of mice with macrophage-activating agents. *J. Leukocyte Biol.* **35**:385-396.
 38. Nacy, C. A., C. N. Oster, S. L. 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. *Contemp. Top. Immunobiol.* **13**:147-170.
 39. Sasada, M., and R. B. Johnston, Jr. 1980. Macrophage microbicidal activity: correlation between phagocytosis-associated oxidative metabolism and the killing of candida by macrophages. *J. Exp. Med.* **152**:85-91.
 40. Stevenson, M. M., P. A. Kongshavn, and E. Skamene. 1981. Genetic linkage of resistance to *Listeria monocytogenes* with macrophage inflammatory responses. *J. Immunol.* **127**:402-407.
 41. Tarnvik, A. 1989. Nature of protective immunity to *Francisella tularensis*. *Rev. Infect. Dis.* **11**:440-451.
 42. Thorpe, B. D., and S. Marcus. 1965. Phagocytosis and intracellular fate of *Pasteurella tularensis*. III. In vivo studies with passively transferred cells and sera. *J. Immunol.* **94**:578-585.
 43. Tigertt, W. D. 1962. Soviet viable *Pasteurella tularensis* vaccines. *Bacteriol. Rev.* **26**:354-373.
 44. Tilney, L. G., and D. A. Portnoy. 1989. Actin filaments and the growth, movement and spread of the intracellular bacterial parasite, *Listeria monocytogenes*. *J. Cell Biol.* **109**:1597-1608.
 45. Walker, T. S. 1984. Rickettsial interactions with human endothelial cells in vitro: adherence and entry. *Infect. Immun.* **44**:205-210.