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Antibody-Mediated Enhancement of *Legionella pneumophila*-Induced Interleukin 1 Activity

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The ability of antibody specific for *Legionella pneumophila* to enhance the induction of interleukin 1 (IL-1) production by murine peritoneal, splenic, and pulmonary macrophages in response to the bacterium was examined. Two preparations of *L. pneumophila* were utilized, a formalin-killed whole-cell preparation and viable bacteria. We measured both secreted (sIL-1) and cell membrane-associated (mIL-1) activities after incubation of the macrophages with the bacterial preparations in the presence or absence of the antibody. Both bacterial preparations induced sIL-1 and mIL-1 activities in each of the macrophage populations tested. These activities were generally enhanced by pretreating the bacteria with antibody, with the greatest enhancing activity observed for the formalin-killed preparations at lower doses of bacteria.

Interleukin 1 (IL-1) is produced primarily by mononuclear phagocytes in response to a variety of stimuli, including microorganisms and microbial products as well as various synthetic chemicals (16, 17, 39). IL-1 may be detected either as a soluble protein secreted into the culture supernatant (or plasma), referred to as sIL-1 activity, or as a membrane-associated form, referred to as mIL-1 activity (32, 33). Additionally, IL-1 activity is associated with two forms of peptide, referred to as IL-1 alpha and IL-1 beta, but the two forms appear to share the same receptor and to have overlapping biologic activities (18). Immune complexes, Fc sub-fragments, and aggregated immunoglobulin are reported to induce IL-1 activity (2, 10, 13, 19, 20, 24, 37, 38); however, we are not aware of any studies in which opsonized and unopsonized bacteria are compared for IL-1-inducing activity.

Legionella pneumophila is known to be a cause of lower-respiratory-tract infection (21, 45), although it also is apparent that subclinical infection occurs in a large number of individuals, as evinced by seropositivity surveys (22, 30, 43). The mechanisms of resistance to infection with *L. pneumophila* remain to be defined completely; however, it is apparent that cell-mediated immunity is of importance (28, 44). *L. pneumophila* grows within resting mononuclear phagocytes, whereas lymphokine-activated macrophages may effectively control bacterial growth (26, 27, 46). Further support for the role of cell-mediated immunity in resistance to *L. pneumophila* infection is derived from observations that patients who are receiving immunosuppressive therapy or who have malignancy are much more susceptible to Legionnaires' disease than are individuals with intact cell-mediated immunity responses (21, 25).

A major feature of *L. pneumophila* infection is the high fever seen in most patients (4, 45), and since one of the major systemic effects of IL-1 is fever induction, we hypothesized that *L. pneumophila* would be a potent inducer of IL-1. We previously demonstrated that *L. pneumophila* and its antigens induced IL-1 production by murine peritoneal, splenic and pulmonary macrophages and by human peripheral blood monocytes (31, 42). Here, we report on the effect that opsonizing *L. pneumophila* with a monoclonal antibody specific for the bacterium has on its ability to induce IL-1 activity.

MATERIALS AND METHODS

Experimental animals. Female BALB/c mice, 6 to 8 weeks old, obtained from Jackson Laboratory (Bar Harbor, Maine) were used as the source of macrophages in all of the studies. C3H/HeJ mice (6 to 8 weeks old), also purchased from Jackson Laboratory, were used as the source of thymocytes in the IL-1 assays. All mice were housed and maintained according to National Institutes of Health guidelines under the supervision of a licensed veterinarian.

Bacteria and antibody. A clinical isolate of *L. pneumophila* serogroup 1, originally derived from an autopsy specimen from Tampa General Hospital, was passed on buffered charcoal yeast extract agar and then maintained in frozen stock as described previously (23). The killed bacteria were prepared by placing the *L. pneumophila* organisms in 0.5% formalin for 24 h, washing the bacteria, and resuspending them in pyrogen-free saline. A monoclonal antibody (MAb) against whole-cell *L. pneumophila*, MAb 439-1, was generated by polyethylene glycol fusion of splenocytes from *L. pneumophila*-immunized BALB/c and NS1 myelomas. Following two limiting dilution procedures, a hybridoma, 439-1, was isolated. MAb 439-1, isotyped to be an immunoglobulin G, was tested by microagglutination with safranine-stained formalin-killed *L. pneumophila* and by enzyme-linked immunosorbent assay using *L. pneumophila* as the capture antigen. High-titer antibodies were obtained from ascites of pristane-primed BALB/c mice. MAb 439-1, at a microagglutination titer of 10,240, was mixed with 3×10^9 live or formalin-killed bacteria for 15 min at 37°C and then washed three times in pyrogen-free saline.

Macrophage cultures. Mice were sacrificed by cervical dislocation. Resident peritoneal macrophages were collected by peritoneal lavage with 5 ml of phosphate-buffered saline. Splenic leukocyte populations were prepared by processing

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freshly explanted spleens with a stomacher Lab-Blender (Tekmar, Cincinnati, Ohio). Pulmonary macrophages were prepared from washed, isolated lung tissue as described previously (9). Cell suspensions were washed twice with Hanks' balanced salt solution and resuspended in RPMI 1640 medium (GIBCO, Grand Island, N.Y.) containing 100 U of penicillin and 100 μg of streptomycin (GIBCO) per ml and 10% fetal bovine serum (HyClone, Sterile Systems, Logan, Utah). The macrophage content of the cell preparation from the different tissues was determined in preliminary studies by plastic adherence and the morphology of the adherent cells. These studies indicated that the pulmonary and peritoneal cell suspensions consisted of approximately 50% (40 to 55%) macrophages, whereas the spleen cells were about 10% (8 to 12%) macrophages (5, 6). By using these estimations, the viable cells, on the basis of on trypan blue dye exclusion, were adjusted to 10^6 macrophages per ml.

IL-1 production and assay. IL-1 was induced by incubating 10^5 macrophages with various numbers of either viable or formalin-killed *L. pneumophila* in wells of 96-well tissue culture plates for 24 h at 37°C in an atmosphere of 5% CO_2 in air. After 1 h of incubation with the *L. pneumophila*, erythromycin was added to the cultures to prevent further growth of the viable bacteria. Supernatants from these cultures were collected for assessment of sIL-1 activity.

Adherent macrophages were washed with RPMI 1640 and fixed with 1% paraformaldehyde in phosphate-buffered saline for 15 min, washed three times with RPMI 1640, and exposed to two changes of complete medium at 2-h and 1-h intervals at 37°C prior to testing for mIL-1 (42).

IL-1 bioassays were performed by using the thymocyte proliferation assay described previously (31). Briefly, 100 μl of a thymocyte suspension (15 × 10^6/ml) was added to 96-well plates along with 100 μl of supernatant fluids, to test for sIL-1. For mIL-1 activity, aliquots of the same thymocytes were added to the fixed macrophage cultures. Tritiated thymidine (0.5 μCi; specific activity, 2 Ci/mmol) (New England Nuclear, Boston, Mass.) was added after 48 h. After 18 h, the cultures were harvested onto glass fiber filters and radioactivity was counted with a liquid scintillation counter as described previously (31). Data are expressed as the change in counts per minute, which was obtained by subtracting the background counts per minute from the counts per minute in the test sample, and are means ± standard errors of three to six individual experiments.

Statistical analysis. Data were analyzed by utilizing Student's *t* test (47) to compare IL-1 activity induced by the formalin-killed bacteria with that induced by viable *L. pneumophila*.

RESULTS

Both the formalin-killed and the viable preparations of *L. pneumophila* induced sIL-1 production by macrophages from each of the tissue sources tested in a dose-dependent fashion (Fig. 1 through 3). As summarized below, pretreatment of the bacterial cell preparations with the MAb led in many cases to statistically significant increases in sIL-1 production in comparison with the levels of activity induced by equal concentrations of the bacteria alone.

Peritoneal macrophages (Fig. 1) produced significantly greater sIL-1 activity in response to opsonized formalin-killed legionellae used at concentrations of 10^6 and 10^7/ml (but not at 10^8/ml) than in response to equivalent concentrations of unopsonized bacterial preparations. In contrast, sIL-1 production by peritoneal macrophages was significantly greater with antibody-treated viable bacteria than with untreated viable bacteria at concentrations of 10^7 and 10^8/ml but not at 10^6/ml (Fig. 1).

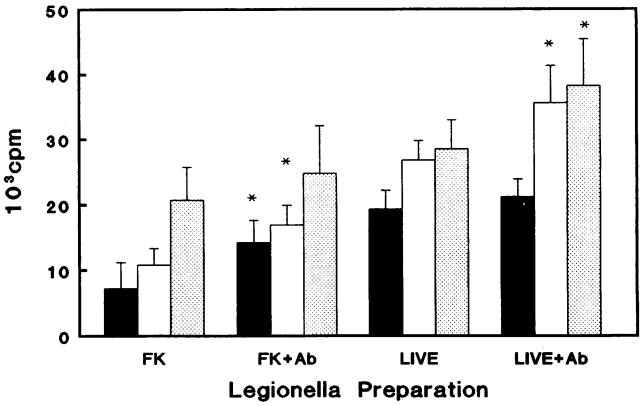

FIG. 1. Legionella-induced sIL-1 production by murine peritoneal macrophages. sIL-1 activities in supernatant fluids from murine peritoneal macrophages cultured for 24 h with various concentrations (10^6/ml [▩], 10^7/ml [□], and 10^8/ml [▥]) of either formalin-killed (FK) or live *L. pneumophila* preparations with and without antibody (Ab) treatment were assessed with the thymocyte proliferation assay described in Materials and Methods. The data are means + standard errors (bars) for three to six individual experiments. *, $P < 0.05$ for IL-1 activities in supernatants from cultures from macrophages stimulated with unopsonized organisms versus activities obtained with the antibody-opsonized preparation.

Pulmonary macrophage sIL-1 production in response to untreated and antibody-treated legionellae was significantly greater in response to the opsonized killed bacteria at 10^7 and 10^8/ml than to the unopsonized killed bacteria (Fig. 2). No differences were demonstrable between the opsonized and untreated viable bacteria in terms of induction of sIL-1 production by the pulmonary macrophages (Fig. 2).

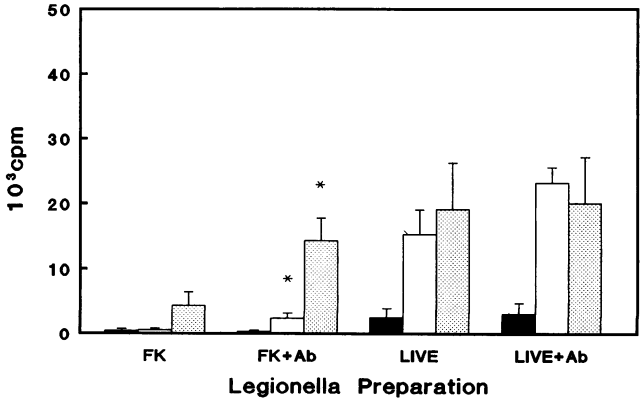

FIG. 2. Legionella-induced sIL-1 production by murine pulmonary macrophages. sIL-1 activities in supernatant fluids from murine pulmonary macrophages cultured for 24 h with various concentrations (10^6/ml [▩], 10^7/ml [□], and 10^8/ml [▥]) of either formalin-killed (FK) or live *L. pneumophila* preparations with and without antibody (Ab) treatment were assessed with the thymocyte proliferation assay described in Materials and Methods. The data are means + standard errors (bars) for three to six individual experiments. *, $P < 0.05$ for IL-1 activities in supernatants from cultures from macrophages stimulated with unopsonized organisms versus activities obtained with the antibody-opsonized preparation.

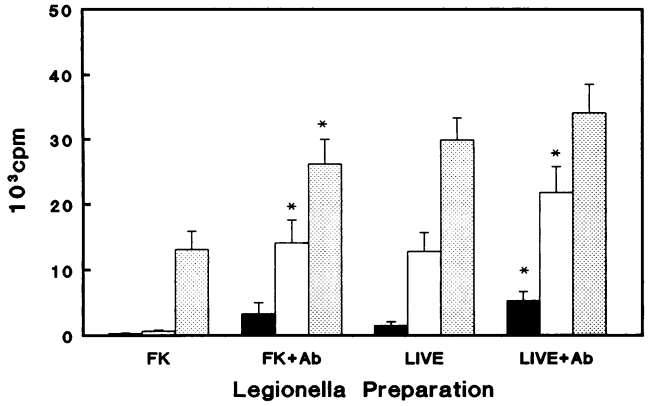

FIG. 3. Legionella-induced sIL-1 production by murine splenic macrophages. sIL-1 activities in supernatant fluids from murine splenic macrophages cultured for 24 h with various concentrations (10^6/ml [▩], 10^7/ml [□], and 10^8/ml [▥]) of either formalin-killed (FK) or live *L. pneumophila* preparations with and without antibody (Ab) treatment were assessed with the thymocyte proliferation assay described in Materials and Methods. The data are means + standard errors (bars) for three to six individual experiments. *, $P < 0.05$ for IL-1 activities in supernatants from cultures from macrophages stimulated with unopsonized organisms versus activities obtained with the antibody-opsonized preparation.

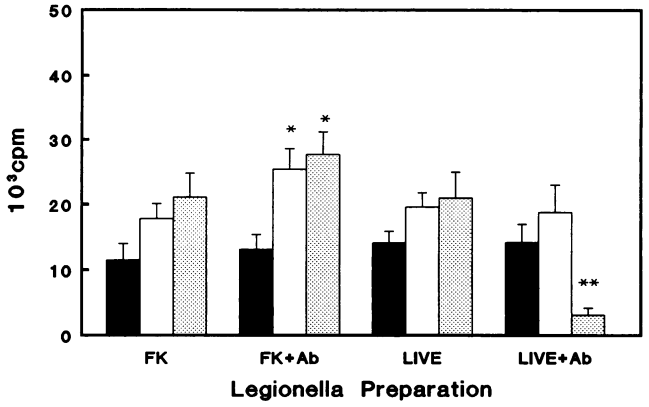

FIG. 4. Legionella-induced mIL-1 production by murine peritoneal macrophages. mIL-1 activities in supernatant fluids from murine peritoneal macrophages cultured for 24 h with various concentrations (10^6/ml [▩], 10^7/ml [□], and 10^8/ml [▥]) of either formalin-killed (FK) or live *L. pneumophila* preparations with and without antibody (Ab) treatment were assessed with the thymocyte proliferation assay described in Materials and Methods. The data are means + standard errors (bars) for three to six individual experiments. * and **, $P < 0.05$ and 0.01, respectively, for IL-1 activities in supernatants from cultures from macrophages stimulated with unopsonized organisms versus activities obtained with the antibody-opsonized preparation.

Opsonization of the bacteria prior to the addition of splenic macrophage cultures resulted in significantly greater sIL-1 activity produced in response to 10^7 and 10^8 formalin-killed bacteria as well as to 10^6 and 10^7 viable bacteria compared with untreated bacteria at the same concentrations (Fig. 3). There were no differences at the other concentrations tested with the splenic macrophages (Fig. 3).

The effect of opsonization on the ability of the bacterial preparations to induce mIL-1-like activity also was dependent on the concentrations utilized and on the responding cell type. With peritoneal macrophages, slight but statistically significantly increased levels of mIL-1 activity were produced in response to 10^7 and 10^8 opsonized formalin-killed bacteria compared with activity produced in response to unopsonized killed bacteria at the same concentrations (Fig. 4). No enhancement of mIL-1-inducing activity was observed when the viable bacteria were opsonized; indeed, at the highest concentration (10^8/ml) the mIL-1 response was significantly reduced compared with the response to the untreated viable organisms (Fig. 4).

Pulmonary macrophage mIL-1 production was significantly greater in response to opsonized formalin-killed bacteria at concentrations of 10^7 and 10^8/ml than were responses to the same concentrations of unopsonized killed bacteria (Fig. 5). Also (Fig. 5), opsonized viable bacteria had a significantly greater ability to induce pulmonary macrophage mIL-1 activity than did unopsonized viable organisms when used at a concentration of 10^7/ml.

Splenic macrophage mIL-1 production also was significantly greater in response to opsonized formalin-killed bacteria at concentrations of 10^7 and 10^8/ml than production in response to unopsonized bacteria at the same concentrations (Fig. 6). As in the case of peritoneal macrophages, opsonization of the viable bacteria failed to increase their ability to induce mIL-1 production and at 10^8/ml the opsonized viable bacteria induced significantly less mIL-1 than did the unopsonized organisms (Fig. 6).

DISCUSSION

IL-1 is an important mediator of fever (16, 17) and is associated with many of the physiologic effects of lipopolysaccharide (LPS). The effects are particularly evident when IL-1 is administered simultaneously with tumor necrosis factor to animals (16, 17, 41). IL-1 also is involved in amplification of immune responses to infectious agents, as demonstrated in both in vitro and in vivo models (15). IL-1 is detectable either as a secreted form or as a membrane-

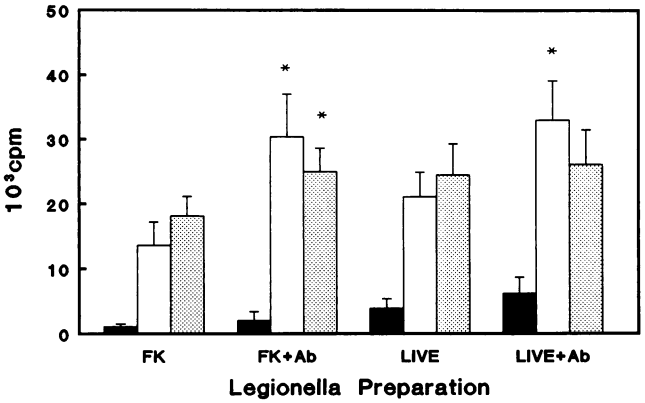

FIG. 5. Legionella-induced mIL-1 production by murine pulmonary macrophages. mIL-1 activities in supernatant fluids from murine pulmonary macrophages cultured for 24 h with various concentrations (10^6/ml [▩], 10^7/ml [□], and 10^8/ml [▥]) of either formalin-killed (FK) or live *L. pneumophila* preparations with and without antibody (Ab) treatment were assessed with the thymocyte proliferation assay described in Materials and Methods. The data are means + standard errors (bars) for three to six individual experiments. *, $P < 0.05$ for IL-1 activity in supernatants from cultures from macrophages stimulated with unopsonized organisms versus activities obtained with the antibody-opsonized preparation.

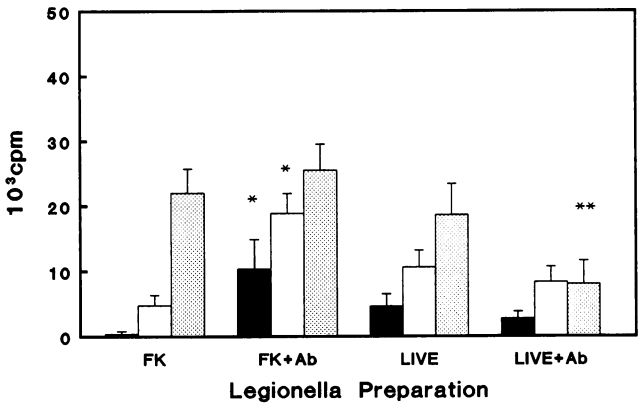

FIG. 6. Legionella-induced mIL-1 production by murine splenic macrophages. mIL-1 activities in supernatant fluids from murine splenic macrophages cultured for 24 h with various concentrations (10^6/ml [▩], 10^7/ml [□], and 10^8/ml [▦]) of either formalin-killed (FK) or live *L. pneumophila* preparations with and without antibody (Ab) treatment were assessed with the thymocyte proliferation assay described in Materials and Methods. The data are means + standard errors (bars) for three to six individual experiments. * and **, $P < 0.05$ and 0.01, respectively, for activities in supernatants from cultures from macrophages stimulated with unopsonized organisms versus activities obtained with the antibody-opsonized preparation.

associated cytokine (16, 17, 32, 33). The nature of the mIL-1 activity has been questioned in recent publications (36, 40). Minnich-Carruth et al., working with P388D_1 and thioglycolate-elicited murine macrophages (36), and Suttles, working with human monocytes (40), proposed that mIL-1 activity is an artifact of inadequate fixation in paraformaldehyde. They reported that extending the fixation period over 2 h eliminated detectable mIL-1 activity, and so they hypothesize that the mIL-1 activity is due to leakage of intracellular IL-1. We agree that there is a considerable amount of detectable intracellular IL-1 (generally in excess of 90,000 cpm); however, we believe that our data and the data of others support the concept that a portion of the IL-1 is membrane associated. In studies performed in our laboratory, *Legionella*-stimulated murine macrophages were fixed with 1% paraformaldehyde for 15 min and then cultured for 24 and 48 h prior to the thymocyte assay. Not only were the levels of IL-1 detected on the macrophages the same, but the supernatants collected from the fixed cells at 24 and 48 h contained no IL-1 activity and therefore indicated no leaking from the fixed cells (data not shown). Additionally, paraformaldehyde fixation for 2 h produced similar results. Furthermore, pretreatment of the fixed macrophages with anti-IL-1α antibody and washing of the macrophages prior to adding the thymocytes neutralized the detectable IL-1 activity (data not shown). These preliminary results indicated that our techniques were detecting a form of the IL-1 which associates with the membrane.

Brody and Durum (11) demonstrated that IL-1 binds to the macrophage surface via a lectin-like interaction that is dissociable with mannose but not other sugars. Additionally, Bakouche et al. (3) showed that a portion of the total IL-1 from LPS-activated monocytes is palmitylated. These findings may explain how IL-1 exists in a membrane-associated form even though its DNA lacks a predictable hydrophobic transmembrane sequence and an identifiable signal peptide (1, 34), both of which are generally associated with membrane proteins. Other data in the literature supporting the existence a membrane form of IL-1 include the release of IL-1 activity by trypsin proteolysis of monocytes (35) as well as the presence of IL-1 on the surface of cells as shown by radioiodination and immunoprecipitation (7) and by flow cytometry using fluorescent MAbs (14). Recent studies by Jirillo et al. (29) showed that lipid A-activated monocytes stimulated enhanced phagocytosis of *Candida albicans* by autologous polymorphonuclear leukocytes through a cell-to-cell contact which could be abrogated with anti-IL-1 treatment of the monocytes. Thus, these data indicated not only the presence of membrane-associated IL-1 but also a functional aspect of the IL-1.

The majority of the studies of sIL-1 and mIL-1 by other investigators utilized murine peritoneal macrophages or human peripheral blood monocytes. Few studies comparing the production of sIL-1 and mIL-1 activity by mononuclear phagocytes from various tissues from the same animal species in response to identical stimuli have been reported. We previously reported that formalin-killed and viable *L. pneumophila* induced both sIL-1 and mIL-1 activity in murine peritoneal, splenic, and pulmonary macrophages (42). We documented differences among the different cell populations in the magnitude of the responses, although macrophages from all three sources did respond to both stimuli. In the present study, we examined the effect of opsonization of the legionella preparations on their ability to induce sIL-1 and mIL-1 activity. We demonstrated that opsonization with a MAb preparation prior to addition to the macrophage cultures led to significantly greater levels of production of both forms of IL-1 in many of the experimental combinations of doses and cell types tested. Macrophages from all three tissue sources responded to opsonized formalin-killed bacteria at one or more concentrations with significant elevations of both mIL-1 and sIL-1 activity, although the responses did vary among cell sources. The responses of the macrophages to opsonized viable bacteria were more variable among the populations and differed with respect to the effects on sIL-1 and mIL-1. Peritoneal and splenic macrophages responded with significantly higher sIL-1 activity to opsonized live bacteria than to unopsonized organisms, although the effective concentrations differed with the cell types (Fig. 1 and 3). In contrast, opsonization of the live legionellae had no significant effect on sIL-1 responses of pulmonary macrophages (Fig. 2). The situation with mIL-1 activity was reversed, in that only the pulmonary macrophages demonstrated a significantly enhanced response to opsonized viable bacteria and they did so at only one concentration (Fig. 5). Opsonization of the live bacteria led to no increases in mIL-1-inducing activity for peritoneal or splenic macrophages (Fig. 4 and 6); indeed, the highest concentration of opsonized viable bacteria (10^8/ml) induced significantly less mIL-1 production by peritoneal and splenic macrophages than did the same concentration of unopsonized organisms.

These data indicate that opsonization of legionellae potentially enhances IL-1 production by mononuclear phagocytes under the appropriate conditions. The decrease seen with mIL-1 production in some cases when the opsonized live bacteria are utilized is probably related to an uptake of too many viable organisms, leading to cell death. Phagocytic assays in our laboratories have demonstrated that opsonization of legionellae leads to enhanced uptake and that with viable bacteria at high concentrations, the macrophages appear to be damaged because of toxicity or bacterial overgrowth (data not shown). Other investigators have dem-

onstrated that immune complexes induce IL-1 production (2, 10, 13, 19, 20, 24, 37, 38); however, we believe that this is the first demonstration of enhancement of IL-1 production by antibody treatment of a particulate bacterial stimulus, which is capable of inducing IL-1 production in the absence of the opsonin. The mechanism for the enhancement is not clear; however, it is possible that when the bacteria are opsonized, multiple signals are presented to the macrophage. One signal would be the presence of the factor(s) that induces IL-1 production by the cell even in the absence of the antibody. The opsonization may lead to increased concentrations of the stimulus at the cell surface or intracellularly because of increased binding and uptake. The second signal would be the binding of the antibody to cell surface Fc receptors, which seems to induce IL-1 synthesis, as revealed by the studies by other investigators using immune complexes or aggregated immunoglobulin (2, 10, 13, 19, 20, 24, 37, 38). Regardless of the mechanism, the results suggest that increased IL-1 synthesis, and possibly other cytokine synthesis, may occur in secondary exposures to antigens in the presence of antibody, which may in turn be in part responsible for the generally more rapid induction of secondary immune responses.

Several observations by ourselves and other investigators imply that IL-1 plays a role in some of the clinical features of *L. pneumophila* infection and possibly in resistance to infection with this bacterium. Firstly, one of the clinical features of legionella infection is high fever (4, 45), which is due to release of endogenous pyrogens such as IL-1 and tumor necrosis factor (16, 17). Investigations in our laboratories have demonstrated that *L. pneumophila* induces tumor necrosis factor activity from peritoneal macrophages (8). Therefore, both of these important endogenous pyrogens are produced by macrophages following their interaction with legionellae. Secondly, the macrophage is a host for the growth of *L. pneumophila*, and macrophages are the major producers of IL-1 in response to infections and other stimuli (16, 17). Cytokine-activated macrophages are important in limiting the growth of legionella (26, 27), again emphasizing the critical role of the macrophage in this infection. Studies by Chen and coworkers (12) indicate that IL-1 and tumor necrosis factor play a synergistic role with gamma interferon in achieving optimal activation of macrophages for tumoricidal activity. We are not aware of any published reports concerning the role of IL-1 in activation of macrophages for optimal resistance to intracellular bacteria, although the work of Chen et al. (12) indicates that IL-1 is an important factor in activation of macrophages measured in other assay systems. Finally, Czuprynski et al. (15) demonstrated that exogenously administered IL-1 increases the resistance of mice to challenge with *Listeria monocytogenes*, which like legionella is a facultative intracellular parasite controlled primarily by activated macrophages.

The demonstration that legionella induces both sIL-1 and mIL-1 activity is important in light of the recent suggestions that these two forms of the cytokine may have different roles in homeostasis, with the secreted form playing a greater role in systemic effects and the membrane-associated form playing a greater role in immune amplification (16, 32). Taken together with the observations listed above, these data suggest that IL-1 may play an important role in the outcome of infection with *L. pneumophila* and that opsonization of the bacteria by specific antibody enhances the IL-1-inducing potential of the organism.

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