Induction of Interleukin 1 by *Legionella pneumophila* in Murine Peritoneal Macrophage Cultures (42925)

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Abstract. ***Legionella pneumophila*-induced production of both membrane-associated and secreted interleukin 1 (mIL-1 and sIL-1, respectively) was examined utilizing peritoneal macrophages from BALB/c mice. The Legionella preparations for these studies included viable bacteria and formalin-killed whole cell preparations. Both of the preparations induced mIL-1 and sIL-1 in a dose-dependent fashion. However, the viable bacteria required about 1 log lower concentrations than the formalin-killed bacteria to induce the same level of IL-1 activity measured in the thymocyte proliferation assay. Kinetic studies showed that mIL-1 and sIL-1 were detectable within 4 hr after addition of either of the *L. pneumophila* preparations to the peritoneal macrophage cultures, with peak levels achieved within 24 hr. These results indicate that *L. pneumophila* is a potent inducer of both mIL-1 and sIL-1 in normal mouse peritoneal macrophage cultures.**

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Legionella pneumophila, first discovered in 1976 during the outbreak of Legionnaires' disease in Philadelphia, is the etiologic agent of diseases in man that range from relatively mild, febrile illness to life-threatening pneumonia (1). Seroepidemiologic studies indicate that a large percentage of individuals have been exposed to this bacterium based on antibody positivity. However, many of these individuals have no history of serious pulmonary disease (2–4). Such observations have led to the suggestion that the majority of individuals exposed to this bacterium either have subclinical infections or a mild illness (5).

During the years since the initial isolation of *L. pneumophila* numerous studies have been performed to determine the mechanisms of resistance to this bacterium, with the results suggesting a probable role of cell-mediated immunity (CMI) as being important. Evidence for the role of CMI includes the capacity of *L. pneumophila* to readily grow within resting mononuclear phagocytes while being inhibited or killed by "lymphokine-activated" macrophages (6-8) along with the lack of effect of antibody plus complement on survival of this bacterium (9). The role of CMI is further supported by observations that patients receiving immunosuppressive therapy or those with cellular immunosuppression due to a malignant state are significantly more susceptible to Legionnaires' disease than are individuals with intact CMI responses (10, 11).

One of the common characteristics of symptomatic infections with *L. pneumophila* is the presence of a relatively high fever (12). Since interleukin 1 (IL-1) is now recognized, along with tumor necrosis factor, as one of the major endogenous pyrogens (13), we performed a series of experiments to determine the ability of various preparations of this bacterium to induce IL-1 production by murine macrophages. Previous studies in this laboratory indicated that formalin-killed *L. pneumophila* can elicit IL-1 like activity in both murine peritoneal macrophage and human peripheral blood monocyte cultures as measured by the thymocyte comitogenic assay system (14). The M_r of this factor was 15.5 kDa as measured by gel filtration, which is similar to molecular weights reported for murine IL-1 induced by other stimuli (13). The studies presented in this report expand upon these observations to include assays for both secreted IL-1 (sIL-1) and macrophage membrane-associated IL-1 (mIL-1) using viable *L. pneumophila* as well as formalin-killed organisms as IL-1 inducers.

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Materials and Methods

Experimental Animals. Male BALB/c mice, 6–8 weeks old, obtained from The Jackson Laboratory (Bar Harbor, ME) were used as the source of macrophages

in all of the studies. C3H/HeJ mice (6–8 weeks old) also purchased from The Jackson Laboratory, were used as the source of thymocytes in the IL-1 assays. All mice were maintained in groups of five to six animals per cage and fed water and commercial mouse pellets *ad libitum*.

Bacteria. A clinical isolate of *L. pneumophila*, serogroup 1, was originally derived from an autopsy specimen from Tampa General Hospital and was maintained on buffered charcoal yeast extract agar as described previously (15). Preparations of formalin-killed bacteria and preparation of viable bacterial suspensions were performed exactly as described previously (15).

Macrophage Cultures. Mice were sacrificed by cervical dislocation and resident peritoneal macrophages were collected by intraperitoneal injection of 5 ml of phosphate-buffered saline solution and recovered after gentle peritoneal lavage. The cells were washed twice with Hanks' balanced salt solution and resuspended in RPMI 1640 medium (Gibco, Long Island, NY) containing 10% fetal bovine serum (Hyclone; Sterile Systems, Logan, UT). The cell concentration was adjusted to 1×10^6 viable cells/ml as determined by hemocytometer counts and exclusion of trypan blue dye.

Interleukin 1 Production and Assay. Interleukin 1 was induced by incubating 1×10^5 macrophages with varying numbers of either viable or formalin-killed *L. pneumophila* in wells of 96-well tissue culture plates for 24 hr at 37°C in an atmosphere containing 5% CO_2 in air. Supernatants from these cultures were collected for assessment of sIL-1 production. The adherent macrophages were washed with Hanks' balanced salt solution and fixed with 1% paraformaldehyde in phosphate-buffered saline for 1 hr, then washed three times with Hanks' balanced salt solution and two changes of complete medium for 30 min at 37°C prior to testing for mIL-1 as described below. IL-1 assays were performed using the thymocyte proliferation assay described previously (14). Briefly, 100 μl of a thymocyte suspension (15×10^6/ml) were added to wells of 96-well tissue culture plates along with supernatant fluids from the macrophage cultures to test for sIL-1 whereas mIL-1 activity was assessed by adding aliquots of the same thymocytes to the fixed macrophage cultures. Tritiated thymidine (0.5 μCi, specific activity 20Ci/mmol; New England Nuclear, Boston, MA) was added after 48 hr. The amount of label incorporated was determined 18 hr later by harvesting the cultures onto glass fiber filters and liquid scintillation counting as described previously (15). Data are expressed as the Δ cpm, which is obtained by subtracting the background cpm from the cpm in the test sample, and represent the mean ± SE of three experiments.

Statistical Analysis. The data presented represent the mean ± SE for four to nine separate experiments. Each experiment was performed on separate days using peritoneal macrophages from a pool of two to three mice per experiment. Differences between the level of IL-1 activity induced by the formalin-killed and viable Legionella were analyzed utilizing Student's *t* test (16).

Results

Previous results from this laboratory had indicated that *L. pneumophila* induces sIL-1 production by murine peritoneal macrophages during a 24-hr incubation with a formalin-killed preparation of the bacteria (14). Therefore, we designed a series of experiments to expand upon this observation to assess the effect of live bacteria and to examine mIL-1 as well. Additionally, we examined earlier time points for IL-1 induction. Figure 1 summarizes results of studies in which murine peritoneal macrophages were incubated for 4 hr with graded doses of viable or formalin-killed Legionellae and assayed for production of sIL-1. This form of IL-1 was readily detectable in the supernatants of macrophages incubated with either form of bacteria, with the viable bacteria inducing greater levels of sIL-1 than equal concentrations of the formalin-killed organisms, particularly at the lower concentrations (10^5/ml). Similar results were observed when the cultures were incubated 24 hr with the stimuli, i.e., the viable bacterial preparations led to substantially greater concentrations of sIL-1 than did equivalent concentrations of the formalin-killed preparations, with the exception of the highest concentration (10^7/ml) tested in which there was no demonstrable difference between the two inducers (Fig. 2).

The cells producing the sIL-1 also were examined for mIL-1 activity, using both the 4-hr and 24-hr time points. Results of the 4-hr incubation studies are summarized in Figure 3. Both the viable and formalin-killed *L. pneumophila* preparations induced production

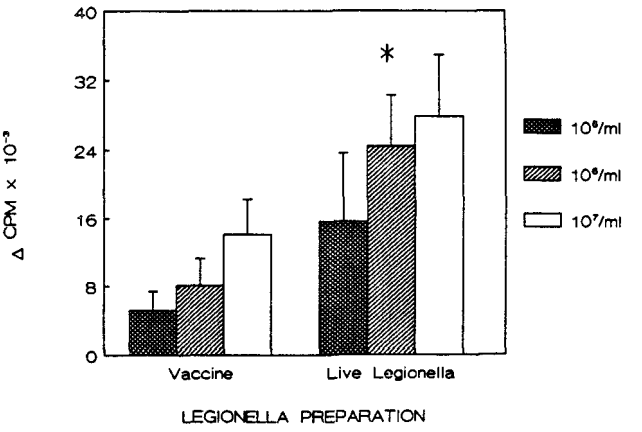

Figure 1. sIL-1 induction by *L. pneumophila*: 4-hr incubation. Supernatant fluids were collected from macrophage cultures incubated for 4 hr with the indicated concentration of either viable or formalin-killed Legionellae and tested for sIL-1 activity. Data are expressed as mean ± SE for four separate experiments. *$P < 0.05$ comparing induction of IL-1 by equivalent concentrations of vaccine and live Legionella preparations utilizing Student's *t* test.

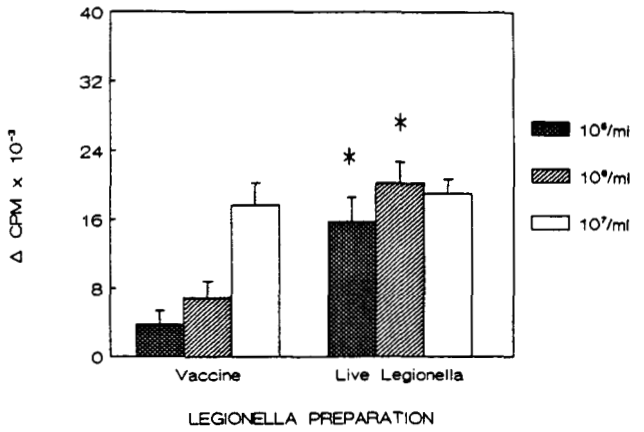

Figure 2. sIL-1 induction by *L. pneumophila*: 24-hr incubation. Supernatant fluids were collected from macrophage cultures incubated for 24 hr with the indicated concentration of either viable or formalin-killed Legionellae and tested for sIL-1 activity. Data are expressed as mean ± SE for nine separate experiments. $^*P < 0.05$ comparing induction of IL-1 by equivalent concentrations of vaccine or live Legionella preparations utilizing Student's *t* test.

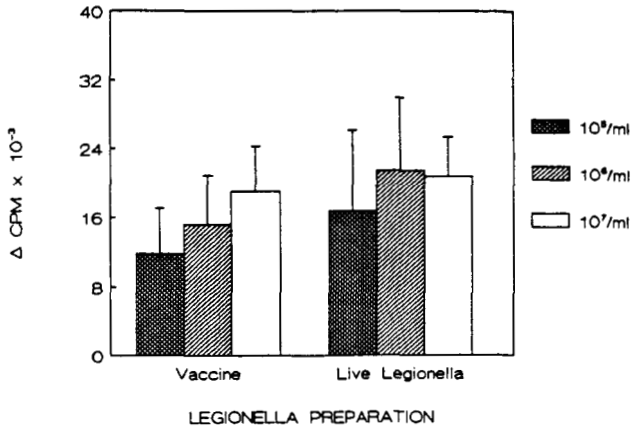

Figure 3. mIL-1 induction by *L. pneumophila*: 4-hr incubation. Macrophage cultures were incubated for 4 hr with the indicated concentration of either viable or formalin-killed Legionella, and then washed and fixed with paraformaldehyde as described in Materials and Methods. The fixed cells were then tested for mIL-1 activity. Data are expressed as mean ± SE for four separate experiments. There was no statistically significant difference between equivalent concentrations of the vaccine and live Legionella preparations when compared utilizing Student's *t* test.

of mIL-1 in a dose-dependent fashion during the 4-hr incubation, with the viable bacteria again inducing greater activity than the equivalent concentration of the formalin-killed preparation, except at the highest concentration (10^7) where the levels were approximately equal. Similar results were observed at 24-hr incubation (Fig. 4).

Additional studies were performed utilizing a lipopolysaccharide (LPS) preparation kindly supplied by Dr. H. K. Wong of the Centers for Disease Control and an outer membrane preparation of *L. pneumophila* supplied by Dr. William Johnson of the University of Iowa in induction of both mIL-1 and sIL-1 production by murine peritoneal macrophages. Both of these preparations were active in inducing the production of each

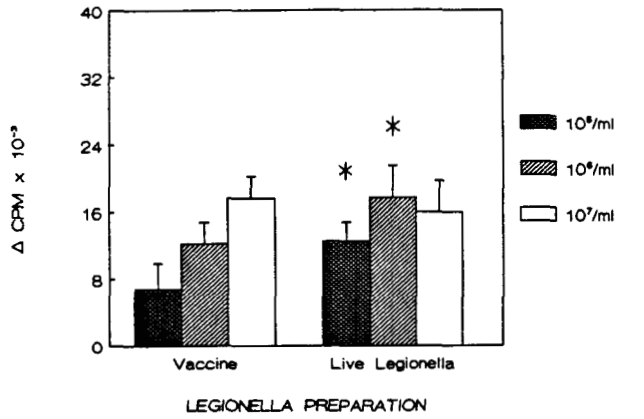

Figure 4. mIL-1 induction by *L. pneumophila*: 24-hr incubation. Macrophage cultures were incubated for 24 hr with the indicated concentration of either viable or formalin-killed Legionellae, and then washed and fixed with paraformaldehyde as described in Materials and Methods. The fixed cells were then tested for mIL-1 activity. Data are expressed as mean ± SE for nine separate experiments. $^*P < 0.05$ comparing IL-1 induction by equivalent concentrations of vaccine and live Legionella preparations utilizing Student's *t* test.

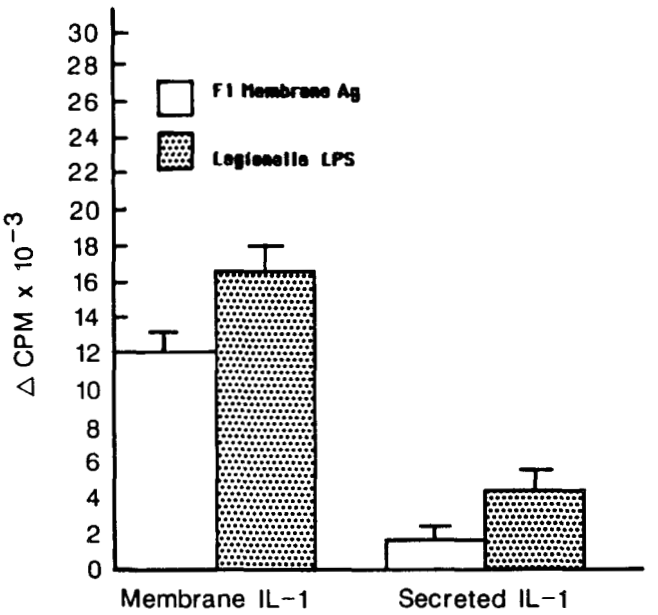

Figure 5. sIL-1 and mIL-1 induction by *L. pneumophila* LPS and outer membrane antigens. Macrophage cultures were incubated for 24 hr with 10 μg/ml of the indicated Legionella antigen preparation. The supernatants were collected for assay of sIL-1 activity and the macrophages were washed and fixed for assay of mIL-1 activity. Data are expressed as mean ± SE for three to four separate experiments.

of the forms of IL-1 tested (Fig. 5), indicating that Legionella LPS and possibly other outer membrane constituents may be important in the induction of IL-1 by this bacterium.

Discussion

Several observations by ourselves and other investigators suggest that IL-1 may be important in resistance to *L. pneumophila* and possibly in some of the clinical

features of infection with this bacterium. First, a high fever is one of the common features observed in patients infected with *L. pneumophila* (1, 12), which is now known to be due in part to the action of IL-1 on the central nervous system to increase the thermoregulatory set point (13). Second, the macrophage is a host for the growth of *L. pneumophila* (6-8) and the macrophage is a major producer of IL-1 in response to a number of stimuli, including gram-negative bacteria and their endotoxins (13). Third, lymphokine-activated macrophages appear to be important in the control of growth of this bacterium (7, 8). Recent studies by Chen *et al.* (17) indicate that IL-1, tumor necrosis factor, and γ-interferon are all required for optimal activation of macrophages for tumoricidal activity (17). We are not aware of any published reports concerning the requirement for IL-1 in activation of macrophages for enhanced killing of intracellular parasites; however, the observations of Chen *et al.* do indicate that Il-1 is an important component in the process of macrophage activation measured by other assays. Finally, Czuprynski *et al.* (18) have demonstrated that exogenously administered IL-1 increases the resistance of mice to challenge with *Listeria monocytogenes*, which is similar to Legionella in being a facultative intracellular parasite.

The data presented in this report clearly demonstrate that *L. pneumophila* is an inducer of both mIL-1 and sIL-1 production by murine peritoneal macrophages *in vitro*. Both forms of IL-1 were detectable within 4 hr after addition of the bacteria to the macrophage cultures, inducing IL-1 in direct correlation with the concentration of bacteria added. We compared the production of IL-1 in response to viable *L. pneumophila* and formalin-killed preparations of the organism. Interestingly, at lower concentrations (10^5 and 10^6/ml) the viable bacteria induced significantly greater levels of both membrane-associated and secreted forms of IL-1 activity than did the equivalent concentration of formalin-killed bacteria. The difference between the response to viable and formalin-killed forms of the bacteria was still present at the 10^7/ml concentration for mIL-1, although the magnitude of the difference was less while there was no difference seen with the two forms of the bacteria for the induction of sIL-1 at this higher concentration.

The reason for the enhanced production of IL-1 in response to viable vs formalin-killed *L. pneumophila* is not clear; however, it may be due to either continued production of a stimulatory factor(s) by the viable bacteria or possibly due to the inactivation of a stimulatory factor(s) during the formalin fixation procedure. It should be noted that erythromycin along with routine tissue culture antimicrobial agents are added to macrophage cultures 1 hr after addition of the bacteria to inhibit the growth of extracellular bacteria and to prevent the overgrowth of intracellular bacteria. This procedure is effective since we recover no viable Legionellae after 4–24 hr (data not shown). Therefore, the effects of bacterial growth would be limited to an early event in the macrophage-bacterial interaction. We have noted that as the concentration of viable *L. pneumophila* is increased to 10^7/ml (100 bacteria/macrophage) that we begin to see decreased viability in the macrophage cultures, even though we add erythromycin to the cultures and recover no viable bacteria after incubation. This suggests that the effect of the high concentration of viable bacteria on the subsequent production of IL-1 by the infected macrophages occurs early in the interaction of the macrophage with the bacterium or that the continued viability of the intracellular bacteria is not required for the decrease in IL-1 production observed in some experiments.

The demonstration that *L. pneumophila* induces both mIL-1 and sIL-1 activities is important in light of the recent suggestions that these two forms of IL-1 may have different *in vivo* significance, with the membrane-associated form possibly being more important in immune amplification whereas the secreted form may be important in the systemic reactions such as fever (19, 20).

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1. Winn WC. Legionnaires disease: Historical perspective. Clin Microbiol Rev **1**:60–18, 1988.
2. Klein GC, Jones WL, Feeley JC,. Upper limit for the detection of antibodies to *Legionella pneumophila* by the microagglutination test. J Clin Microbiol **10**:754–755, 1979.
3. Freedman AP, Katz SM. The prevelance of serum antibodies to *Legionella pneumophila* in patients with chronic pulmonary disease. Am Rev Respir Dis **123**:238-239, 1981.
4. Wilkinson HW, Cruce DC, Broome CL. Validation of *Legionella pneumophila* indirect fluorescence assay with epidemic sera. J Clin Microbiol **13**:139–146, 1981.
5. Winn WC. Cellular inflammatory defenses in *Legionella* pneumonia. Clin Immunol Newslett **4**:83–87, 1983.
6. Horwitz MA, Silverstein SC. The Legionnaires' disease bacterium *(Legionella pneumophila)* multiplies intracellularly in human monocytes. J Clin Invest **66**:441–450, 1980.
7. Horwitz MA, Silverstein SC. Activated human monocytes inhibit intracellular multiplication of Legionnaires' disease bacteria. J Exp Med **154**:1618–1635, 1981.
8. Yamamoto Y, Klein TW, Newton CA, Widen R, Friedman H. Differential growth of *Legionella pneumophila* in guinea pig versus mouse macrophage cultures. Infect Immun **55**:1369–1374, 1987.
9. Horwitz MA, Silverstein SC. Interaction of the Legionnaires' disease bacterium with human phagocytes. I. *L. pneumophila* resists killing by polymorphonuclear leukocytes, antibody and complement. J Exp Med **153**:386–397, 1981.
10. England AC III, Fraser DW, Plikaytis BD, Tsai TF, Storch G, Broome CV. Sporadic legionellosis in the United States: The first thousand cases. Ann Intern Med **94**:164–170, 1981.
11. Haley CE, Cohen ML, Halter J, Meyer RD. Nosocomial Legionnaires' disease: A continuing common source outbreak at Wadsworth Medical Center. Ann Intern Med **90**:583–586, 1979.
12. Beaty HN. Clinical features of Legionellosis. In: Thornsberry C, Balows A, Feeley JC, Jakubowski W, Eds. Legionella Proceedings

of the Second International Symposium. Washington DC, American Society for Microbiology, pp6–10, 1984.
13. Dinarello CA. Interleukin-1. Rev Infect Dis **6**:51–95, 1984.
14. Klein TW, Newton CA, Blanchard DK, Widen R, Friedman H. Induction of interleukin 1 by *Legionella pneumophila* antigens in mouse macrophage and human mononuclear leukocyte cultures. Zentralbl Bakteriol Hyg [A]**265**:462–471, 1987.
15. Friedman H, Widen R, Klein T, Searls L, Cabrian K. *Legionella pneumophila*-induced blastogenesis of murine lymphoid cells *in vitro*. Infect Immun **43**:314–319, 1984.
16. Zar JH. Biostatistical analysis. Englewood Cliffs, NJ: Prentice Hall, pp185–186, 1974.
17. Chen L, Yasuhiro S, Wheelock F. Interferon-gamma synergizes with tumor necrosis factor and with interleukin 1 and requires the presence of both cytokines to induce antitumor cytotoxic activity in macrophages. J Immunol **139**:4096–4101, 1987.
18. Czuprynski CJ, Brown JF. Young KM, Cooley J, Kurtz RS. Effects of murine recombinant interleukin 1α on the host response to bacterial infection J Immunol **140**:962–968, 1988.
19. Oppenheim JJ, Kovacs EJ, Matsushima J, Durum SK. There is more than one interleukin 1. Immunol Today **7**:45–49, 1986.
20. Kurt-Jones EA, Fiers W, Pober JS. Membrane interleukin 1 induction on human endothelial cells and dermal fibroblasts. J Immunol **139**:2317–2324, 1987.