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# INTERACTION OF THE LEGIONNAIRES' DISEASE BACTERIUM (*LEGIONELLA PNEUMOPHILA*) WITH HUMAN PHAGOCYTES

## II. Antibody Promotes Binding of *L. pneumophila* to Monocytes but Does Not Inhibit Intracellular Multiplication*

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The fate of an intracellular pathogen in mononuclear phagocytes appears to be an important determinant of the capacity of the pathogen to cause disease. Antibody against the pathogen can markedly alter the intracellular fate of the pathogen in mononuclear phagocytes; such an effect of antibody is reflected in vivo by enhanced resistance to infection of animals that have been passively immunized with hyperimmune serum. Under these conditions, the specific immunity expressed by these animals results from the presence of antibody and not from the presence of antigen-sensitive leukocytes. For example, antibody inhibited the intracellular multiplication of the obligate intracellular parasite *Rickettsia mooseri* in cultured human monocytes and rendered the organism susceptible to killing by these monocytes (1); paralleling this in vivo, the same antibody protected passively immunized mice against an otherwise lethal infection from *R. mooseri* ([1]; and C. L. Wisseman, Jr. Personal communication.). Similarly, human and mouse antitoxoplasma antibody rendered the obligate intracellular parasite *Toxoplasma gondii* incapable of multiplying in cultured human monocytes (2) and mouse peritoneal macrophages (3), respectively; mouse anti-toxoplasma antibody protected passively immunized mice from lethal infection with *T. gondii* (4).

With other intracellular pathogens, antibody does not significantly inhibit their multiplication in cultured leukocytes; this is reflected in vivo by a failure of antibody to protect passively immunized animals from infection. For example, immune mouse serum did not inhibit the multiplication of the facultative intracellular bacterium *Listeria monocytogenes* in cultured mouse peritoneal macrophages (5); and immune mouse serum did not protect mice from infection with *L. monocytogenes* (6). Similarly, antibody did not inhibit the multiplication of the facultative intracellular bacterium *Mycobacterium tuberculosis* in cultured mouse, rabbit, or guinea pig peritoneal macrophages (7–9); and immune mouse serum did not influence the survival of mice nor did immune guinea pig serum influence the course of infection in guinea pigs when these animals were challenged with *M. tuberculosis* (10, 11). In all these studies, there is a correlation between the capacity of antibody to inhibit intracellular infection of

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mononuclear phagocytes in vitro and to protect the passively immunized host against infection with the same pathogen, i.e., the in vitro studies appear to accurately reflect the role of antibody in host defense against diseases caused by intracellular pathogens. Therefore, to define the role of antibody in Legionnaires' disease, which is caused by the facultative intracellular bacterial pathogen *Legionella pneumophila* (12), we have studied the effect of anti-*L. pneumophila* antibody on host cell-*L. pneumophila* interaction.

In the accompanying paper (13), we described our studies on the influence of antibody on the interaction of *L. pneumophila* with human serum and human polymorphonuclear leukocytes (PMN).[1] We found that virulent egg yolk-grown *L. pneumophila* bacteria are completely resistant to the bactericidal effects of human serum whether or not the serum contains anti-*L. pneumophila* antibody. Moreover, we found that a significant proportion (30–40%) of an inoculum of *L. pneumophila* is resistant to killing by PMN even in the presence of anti-*L. pneumophila* antibody and complement.

In this paper, we describe our studies on the effects of anti-*L. pneumophila* antibody on the interaction of *L. pneumophila* (Philadelphia 1 strain) with human monocytes. We demonstrate (*a*) that anti-*L. pneumophila* antibody, in the presence of complement, promotes the binding of viable *L. pneumophila* to monocytes, as it does to PMN; (*b*) that monocytes, like PMN, require both specific antibody and complement to kill any *L. pneumophila* and that, under these conditions, monocytes kill only a limited proportion of a bacterial inoculum; and (*c*) that *L. pneumophila* surviving the confrontation with monocytes in the presence of specific antibody and complement multiply in monocytes as rapidly as when the bacteria enter monocytes in the absence of specific antibody.

## Materials and Methods

*Media, Agar, Sera, and Bacteria.* The media, agar, sera, and bacteria used in this study were obtained or prepared and stored as described in the accompanying paper (13). Normal human serum had an indirect fluorescent antibody (IFA) anti-*L. pneumophila* titer of <1:64 and immune human serum had an IFA anti-*L. pneumophila* titer of 1:4,096 (13).

*Human Blood Mononuclear Cells.* The blood mononuclear cell fraction was obtained by centrifugation over a Ficoll-sodium diatrizoate solution as previously described (12); the cells were >99% viable by trypan blue exclusion. Examination of a stained cytocentrifuged sample revealed that the mononuclear cell fraction contained ~40% monocytes, 58.5% lymphocytes, and 1.5% PMN. The adherent subpopulation (containing >90% monocytes) and the nonadherent subpopulation (containing >90% lymphocytes) were prepared as described (12). For use in the mononuclear cell-killing assay described below, adherent cells in Petri dishes were resuspended by incubating the cells for 10 min at 4°C (which causes most cells to round-up and many to detach) and gently scraping them off the bottom of the Petri dish with a rubber policeman. Recovered cells were 90% viable by trypan blue exclusion.

*Mononuclear Cell-killing Assay.* This was identical to the PMN-killing assay described in the accompanying paper (13) except that instead of PMN, $2 \times 10^6$ or $5 \times 10^6$ mononuclear cells, $2 \times 10^6$ resuspended adherent mononuclear cells, or $2 \times 10^6$ nonadherent mononuclear cells were incubated with *L. pneumophila*. At the end of the incubation, the contents of each tube were sonicated for 60 sec continuously with a micro-tip attached to a sonicator (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) with the output control of the sonicator set at the 4 position; this amount of sonic energy lysed the mononuclear cells completely but did not reduce bacterial colony-forming units (CFU) (12).

*Assay for the Binding of Viable L. pneumophila to Monocytes in the Presence or Absence of Anti-L. pneumophila Antibody.* *L. pneumophila* ($2.5 \times 10^5$ CFU/ml) were incubated in plastic test tubes

[1] *Abbreviations used in this paper:* CFU, colony-forming units; IFA, indirect fluorescent antibody assay; PMN, human polymorphonuclear leukocytes.

for 15 min at 37°C in 5.5 ml of medium that contained 30% fresh normal human serum or 30% fresh immune human serum. 1-ml quantities of these bacterial suspensions were then immediately transferred to 35-mm Petri dishes containing freshly prepared monocyte monolayers in 1 ml of RPMI-1640 medium (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.) (~$2 \times 10^6$ monocytes/Petri dish); the final vol in each Petri dish was 2 ml and the final serum concentration was 15%. Monocyte monolayers were incubated with bacteria for 1 h at 37°C in 5% $CO_2$-95% air on a gyratory shaker at 100 rpm. After the incubation, the monocyte monolayers were washed vigorously three times with RPMI-1640 (at 37°C) to remove non-cell-associated bacteria. Then 2 ml of RPMI-1640 was added to each Petri dish. The monocyte monolayers were then sonicated with a 2.54-cm-diam high-gain disrupter horn, attached to a sonicator (Heat Systems-Ultrasonics, Inc.) under sterile conditions with the output control of the sonicator set at the 4 position; this amount of sonic energy lysed the mononuclear cells completely but did not reduce bacterial CFU (12). CFU of *L. pneumophila* in each Petri dish were determined. Five replicate Petri dishes were used for each measurement.

*Infection of Mononuclear Cells in Suspension or Monocytes in a Monolayer in the Presence of Anti-L. pneumophila Antibody.* In the experiment depicted in Fig. 2, *L. pneumophila* ($5 \times 10^4$ CFU) were incubated at 37°C for 15 min in plastic test tubes in 750 μl of medium that contained 250 μl of fresh normal human serum or 250 μl of fresh immune human serum. Then $5 \times 10^6$ mononuclear cells (or RPMI-1640 as control) were added and the final vol in each tube was brought to 2.5 ml with RPMI-1640; the final serum concentration was 10%. A 100-μl sample from each tube was immediately removed and assayed for CFU of *L. pneumophila* on modified charcoal yeast extract agar as described (12). The tubes were then gassed to a pH of 7.4 with 5% $CO_2$-95% air, sealed, and incubated for 1 h at 37°C on a gyratory shaker at 250 rpm. Immediately after the incubation and at 24-h intervals for 4 d thereafter, the medium in each tube was assayed for CFU of *L. pneumophila.* Five replicate tubes were used for each measurement.

In the experiment depicted in Fig. 3, monocyte monolayers were infected in the presence of normal or immune serum exactly as in the assay for the binding of viable *L. pneumophila* to monocytes described above except that the concentration of bacteria preincubated with 30% normal or 30% immune serum was $10^6$ CFU/ml instead of $2.5 \times 10^5$ CFU/ml. After the monolayers were infected, Petri dishes were divided into two sets. Monocyte monolayers in the first set of Petri dishes (five that contained normal and five that contained immune serum) were washed vigorously three times with RPMI-1640 (at 37°C) to remove non-monocyte-associated bacteria and the cells were reincubated in 2 ml RPMI-1640 and 15% serum of the same type to which they had initially been exposed. The monolayers in two of these Petri dishes (one containing immune serum and in one containing normal serum) were then sonically disrupted, as in the binding assay, to determine the total number of CFU of *L. pneumophila* initially in these cultures. Monocyte monolayers in the second set of Petri dishes (four that contained immune and four that contained normal serum) were not washed free of non-monocyte associated bacteria. The remaining eight Petri dishes in the first set and the eight Petri dishes in the second set were incubated at 37°C in 5% $CO_2$-95% air under stationary conditions. The medium in each Petri dish was assayed for CFU of *L. pneumophila* at the start of this incubation period and daily for 4 d thereafter. Four replicate Petri dishes were used for each measurement in Fig. 3.

## Results

*Monocytes Require Both Specific Antibody and Complement to Kill Any L. pneumophila.* We incubated mononuclear cells with *L. pneumophila* in the presence of fresh normal human serum (a source of complement), heat-inactivated immune human serum ( a source of anti-*L. pneumophila* antibody), or fresh immune human serum (a source of both antibody and complement). Mononuclear cells killed *L. pneumophila* only when both a source of antibody and complement were present (Fig. 1 A). The mononuclear cells reduced CFU of *L. pneumophila* by only 0.25 log (45%).

In separate experiments, we incubated *L. pneumophila* with $2 \times 10^6$ lymphocytes (nonadherent mononuclear cells), $2 \times 10^6$ monocytes (adherent mononuclear cells), or

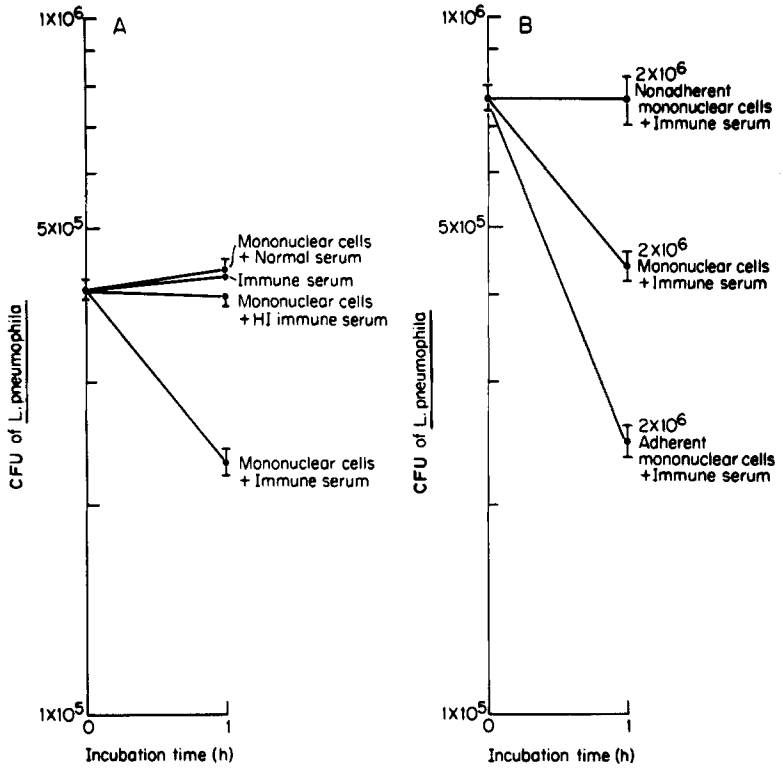


FIG. 1. Monocyte killing of *L. pneumophila*. (A) Mononuclear cells require both antibody and complement to kill any *L. pneumophila*. Mononuclear cells ($5 \times 10^6$) were incubated with $2.5 \times 10^5$ CFU of *L. pneumophila* at 37°C in 5% $CO_2$-95% air on a gyratory shaker for 1 h in medium that contained 10% fresh normal human serum (with an indirect fluorescent antibody assay (IFA) anti-*L. pneumophila* titer of <1:64), 10% fresh immune human serum (with an IFA anti-*L. pneumophila* titer of 1:4,096), or 10% heat-inactivated immune human serum (HI immune serum). CFU were determined initially and at the end of the incubation. Each point represents the average for three replicate tubes ± SE (B) Monocytes but not lymphocytes kill *L. pneumophila*. *L. pneumophila* ($7.5 \times 10^5$ CFU) were incubated with $2 \times 10^6$ lymphocytes (nonadherent mononuclear cells), $2 \times 10^6$ monocytes (adherent mononuclear cells), or $2 \times 10^6$ mononuclear cells at 37°C in 5% $CO_2$-95% air on a gyratory shaker for 1 h in medium that contained 10% fresh immune human serum. Adherent and nonadherent mononuclear cells were selected as described in Materials and Methods. CFU were determined initially and at the end of the incubation. Each point represents the average for three replicate tubes ± S.E.

$2 \times 10^6$ mononuclear cells in the presence of fresh immune human serum. Monocytes and mononuclear cells killed *L. pneumophila* (by 0.5 log and 0.25 log, respectively) but lymphocytes did not kill *L. pneumophila* (Fig. 1 B). This indicates that the monocytes in the mononuclear cell fraction are responsible for the killing.

Thus, monocytes, like PMN, require both specific antibody and complement to kill any *L. pneumophila*; even then they kill only a limited proportion of a bacterial inoculum.

*Specific Antibody Promotes the Binding of Viable L. pneumophila to Monocytes.* We preincubated *L. pneumophila* in medium that contained fresh normal or fresh immune human serum for 15 min at 37°C and then further incubated the bacteria with monocyte monolayers for 1 h at 37°C as described in Materials and Methods. We

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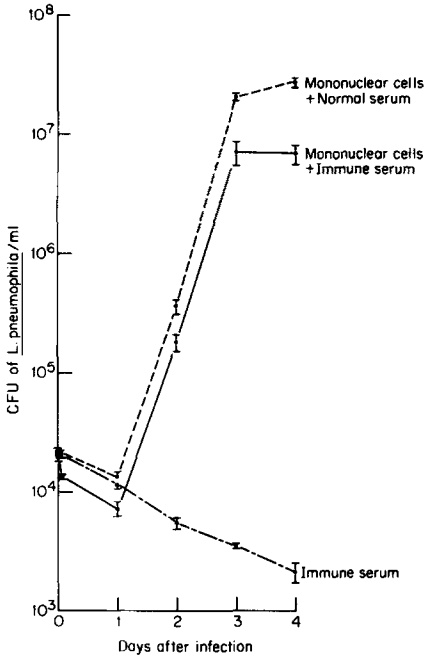


FIG. 2. Specific antibody fails to inhibit the multiplication of *L. pneumophila* in monocytes. *L. pneumophila* ($2.5 \times 10^4$ CFU) were incubated at 37°C for 10 min in 33% fresh normal human serum or 33% fresh immune human serum. The bacteria were then incubated at 37°C in 5% $CO_2$-95% air with $5 \times 10^6$ mononuclear cells (or RPMI-1640 as control) in medium that contained a final concentration of 10% of the same type of serum to which the bacteria were initially exposed. The cultures were shaken for 1 h and incubated under stationary conditions thereafter for 4 d. CFU were determined at 0, 1, 24, 48, 72, and 96 h after infecting the monocytes. Each point represents the average for five replicate tubes ± SE.

then sonically disrupted the monolayers and assayed for CFU of *L. pneumophila*. Monocyte monolayers incubated with *L. pneumophila* in the presence of normal serum contained an average of 2,490 ± 120 CFU of *L. pneumophila* (average for five replicate Petri dishes ± SE). Monocyte monolayers incubated with an equal number of *L. pneumophila* in the presence of immune serum contained an average of 8,290 ± 440 CFU of *L. pneumophila*, 3.3 times as many viable bacteria as when anti-*L. pneumophila* antibody was absent. Thus, specific antibody promotes the binding of viable *L. pneumophila* to monocytes as it does to PMN.

*Specific Antibody Does Not Inhibit the Multiplication of L. pneumophila in Monocytes.* We preincubated *L. pneumophila* in medium that contained fresh normal or fresh immune human serum for 15 min at 37°C and then further incubated the bacteria with mononuclear cells for 4 d. During the 1st h of incubation with the mononuclear cells, the cultures were shaken on a gyratory shaker; thereafter they were incubated under stationary conditions. Serum of the same type to which the *L. pneumophila* were initially exposed was present throughout.

During the 1st h of incubation with mononuclear cells, the number of CFU of *L. pneumophila* in cultures that lacked specific antibody remained constant. However, CFU decreased 0.25 log in cultures that contained specific antibody (Fig. 2). This amount of killing was comparable to that observed in the experiments depicted in

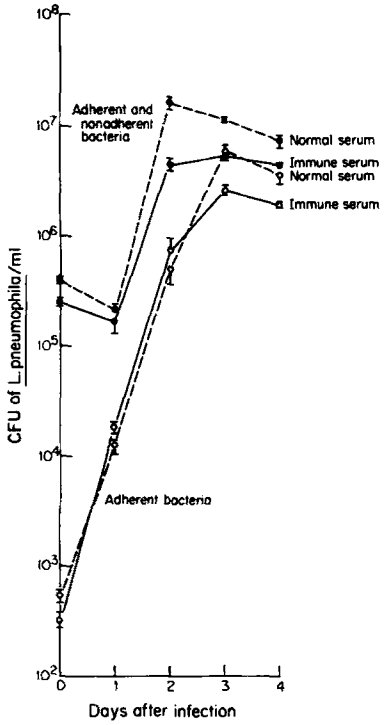


FIG. 3. Monocyte-adherent bacteria coated with antibody and complement multiply in monocytes. *L. pneumophila* ($10^6$ CFU) were preincubated with fresh immune or fresh normal human serum for 15 min at 37°C, and further incubated for 1 h at 37°C on a gyratory shaker with monocyte monolayers in Petri dishes (~2 × $10^6$ monocytes/Petri dish) containing 2 ml RPMI-1640 and 15% of the same type of serum to which they were initially exposed. Some of the monocyte monolayers were then washed to remove nonmonocyte associated bacteria and reincubated in 2 ml RPMI-1640 and 15% serum of the same type to which they were initially exposed. The medium in each Petri dish was assayed daily for CFU of *L. pneumophila*. Points on the lines labeled "Adherent bacteria" (○——○) represent CFU of *L. pneumophila* in the medium of monocyte monolayer cultures that were washed to remove non-monocyte-associated bacteria. Separate measurements showed that, at the start of the final incubation (0 d after infection on this graph), 98 and 86% of the bacteria in these cultures incubated in the presence of immune and normal serum, respectively, were monocyte associated. Points on the lines labeled "Adherent and nonadherent bacteria" (●) represent CFU of *L. pneumophila* in the medium of monocyte monolayer cultures that were not washed to remove non-monocyte-associated bacteria. Each point represents the average for four replicate Petri dishes ± SE.

Fig. 1. Subsequently, the number of CFU in cultures with and without specific antibody changed in parallel, first decreasing slightly and then increasing three logs.

To confirm that monocyte-associated bacteria coated with antibody and complement are capable of multiplying and initiating a cycle of infection in monocytes, and to study the growth kinetics of opsonized *L. pneumophila* in cultures that contained only monocytes, we did the following experiment. We preincubated *L. pneumophila* with fresh normal or fresh immune human serum for 15 min at 37°C, further incubated them with monocyte monolayers for 1 h, and washed some of the monolayers to remove non-monocyte-associated bacteria, as described in Materials and Methods. To determine the total number of CFU of *L. pneumophila* present in cultures after the non-monocyte-associated bacteria had been washed away, we sonically

disrupted the monolayers in two Petri dishes and assayed for CFU. We further incubated the remaining cultures and assayed the culture medium of these cultures for CFU of *L. pneumophila* at the start of this incubation and daily for 4 d thereafter.

Cultures that had been incubated for 1 h with *L. pneumophila* in the presence of fresh immune and fresh normal human serum contained 14,100 and 3,800 CFU/ml, respectively, after the cultures were sonically disrupted; a ratio of 3.7:1. This ratio is similar to the 3.3:1 ratio observed in the binding study discussed above. After washing away the bacteria, there were 300 CFU/ml in the medium of cultures that contained intact monolayers and immune serum (Fig. 3). Because there were 14,100 CFU/ml in a replicate culture containing a sonically disrupted monolayer, ~98% of the bacteria in the cultures containing intact monolayers and immune serum were monocyte-associated at the end of a 1-h incubation period, i.e., at the start (day 0) of the infection as shown in Fig. 3.

*L. pneumophila* multiplied to high levels in cultures initially containing only adherent bacteria and in cultures initially containing both adherent and nonadherent bacteria, whether or not antibody was present (Fig. 3). The rates of multiplication were similar in all cultures. We have obtained similar results in another experiment of this type in which we used dialyzed rabbit anti-*L. pneumophila* antiserum plus fresh normal human serum as the source of antibody and complement. In the accompanying paper, we showed that *L. pneumophila* incubated for 15 min at 37°C in immune serum are coated with antibody and complement (13). Therefore, opsonized *L. pneumophila* bacteria bound to monocytes in the presence of antibody and complement are capable of multiplying and initiating a cycle of infection in monocytes and of doing so as rapidly as unopsonized bacteria.

In all of these experiments, the peak level of *L. pneumophila* growth attained in cultures containing specific antibody was 0.25–0.5 log less than in cultures lacking specific antibody (Figs. 2 and 3). We do not know why this was so.

Thus, when monocytes are incubated with *L. pneumophila* in the presence of specific antibody and complement, the leukocytes kill a small proportion of the bacteria; however, the surviving bacteria multiply in monocytes as rapidly as when the bacteria are not exposed to antibody.

## Discussion

PMN and monocytes have similar requirements for killing *L. pneumophila* in that they require anti-*L. pneumophila* antibody and complement; antibody and complement increase the binding of viable bacteria to PMN and monocytes (5). In this respect, the interaction of PMN and monocytes with *L. pneumophila* resembles the interaction of these leukocytes with encapsulated bacteria (14). However, whereas encapsulated *Escherichia coli* are highly susceptible to the bactericidal effects of phagocytic leukocytes (14), *L. pneumophila* are relatively resistant to killing by PMN and monocytes. In their interaction with *L. pneumophila*, PMN and monocytes differ from each other in that only monocytes support the intracellular multiplication of *L. pneumophila* (12, 13).

The experiments reported here and in the accompanying paper (13) show that antibody and complement fail to bring about two major biological effects, at least in vitro. First, they fail to promote effective killing of *L. pneumophila* by PMN and monocytes. Second, they fail to inhibit the growth of *L. pneumophila* in monocytes. Although our findings do not exclude a role for antibody and complement in

conjunction with cell-mediated immunity, they suggest that humoral immunity by itself may not be an effective host defense against *L. pneumophila*; consequently, a vaccine that resulted only in antibody production against the Legionnaires' disease bacterium may not be efficacious.

These results are consistent with our previous suggestion that cell-mediated immunity plays an important role in host defense against *L. pneumophila* (4).

## Summary

In an accompanying paper (13), we reported that human polymorphonuclear leukocytes kill only a limited proportion (0.5 log) of an inoculum of *Legionella pneumophila* (Philadelphia 1 strain) in the presence of human anti-*L. pneumophila* antibody and complement. We now report on the effect of anti-*L. pneumophila* antibody on *L. pneumophila*-monocyte interaction. The studies were carried out under antibiotic-free conditions.

Monocytes bind more than three times as many viable *L. pneumophila* bacteria in the presence of both antibody and complement than in the presence of complement alone. Monocytes require both antibody and complement to kill any *L. pneumophila*: however, even then, monocytes kill only a limited proportion (0.25 log) of an inoculum. The surviving bacteria multiply several logs in the monocytes and multiply as rapidly as when the bacteria enter monocytes in the absence of antibody.

These findings suggest that humoral immunity may not be an effective host defense against *L. pneumophila.* Consequently, a vaccine that resulted only in antibody production against the Legionnaires' disease bacterium may not be efficacious.

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