INTRODUCTION

Background

During the year following the epidemic of pneumonia at a Legionnaires' convention in Philadelphia in 1976 (77), the causative bacterium was isolated (132) and named *Legionella pneumophila* (32). Within 2 years, a bacterium responsible for causing pneumonia in immunocompromised patients in Pittsburgh, Pa. (140), and Charlottesville, Va. (168), was isolated and proved to be a second *Legionella* species, *L. micdadei* (88). Since that time numerous additional species have been identified, and it has become apparent that some species contain more than one serogroup based on agglutinating surface antigens. Although proposals have been made to add additional genera, by common usage at present *Legionella* is the only designated genus in the family, *Legionellaceae*. As of mid-1991, a total of 32 *Legionella* species which contained 51 serogroups had been named (16), and this list is sure to grow further in the future. Sixteen of the *Legionella* spp., comprising 34 serogroups, have been reported as pathogenic for humans (16), whereas the others have as yet been obtained only from environmental reservoirs. However, it is possible that any species could cause human disease under the appropriate conditions.

The predominant clinical manifestation of *Legionella* infection is pneumonia. Approximately 85% of cases of *legionellosis* are due to *L. pneumophila*, with about 50% of all disease due to *L. pneumophila* serogroup 1 and 10% to serogroup 6 (164). The remainder of cases are due to other serogroups of *L. pneumophila*, *L. micdadei*, and a number of other species. It is not clear whether this distribution is due to greater inherent virulence of the more frequently isolated strains or, more likely, the prevalence of the various strains in the environment. The *legionellae* also cause an acute, febrile, nonpneumonic illness referred to as Pontiac fever. Although Pontiac fever follows the inhalation of environmental *legionellae*, as does pneumonia, there is no tissue
invasion and the disease is self-limited. The pathogenesis of Pontiac fever is obscure, and viable legionellae may not be required for its production. Alternatively, it has been proposed that Pontiac fever is produced by Legionella strains that are unable to multiply in human cells (73). Since the pathogenesis of Pontiac fever and Legionella pneumonia are so obviously different, the former will not be considered further in this review.

Although the source of the legionellae that infect humans is contaminated environmental water, the precise nutritional, pH, and temperature requirements that are necessary to cultivate Legionella strains in vitro suggest that it is not a free-living aquatic bacterium. It has been found that legionellae infect free-living amoebae and ciliated protozoa in vitro (1, 74, 170, 189) and, probably, in the natural environment (6, 31). Legionellae contained in amoebae, and especially in amoebal cysts, could survive environmental temperature extremes, chlorination, and other adverse conditions. Under certain conditions in vitro, ingested legionellae multiply within the vacuoles of free-living amoebae, so that amoebae may serve to amplify the numbers of legionellae in the environment. Furthermore, infected amoebae and amoebal vesicles containing legionellae would be present in the drift from contaminated aquatic environments and provide the vehicles whereby concentrated infectious particles could be delivered to humans.

**Purpose and Scope**

Whereas bacteria of the genus *Legionella* have emerged as relatively frequent causes of pneumonia, the mechanisms underlying their pathogenicity are poorly understood. The legionellae are facultative intracellular pathogens which multiply within the phagosome of monocytes (107, 199) and alveolar macrophages (120, 142). Furthermore, the legionellae are not killed efficiently after being phagocytized by polymorphonuclear leukocytes (106, 198). Since the legionellae have clearly been shown to be intracellular pathogens, their pathogenicity for humans may largely depend on the outcome of the interaction between the bacterium and the professional phagocytes it encounters in the host. Because of the intracellular location of *Legionella* spp., attention has naturally been focused on the cell-mediated immune responses to infection (103). The influence of specific cell-mediated immune mechanisms on the interaction of *Legionella* spp. with alveolar macrophages and mononuclear phagocytes is not doubt important in the clearance of the bacteria later in primary infection and in the immune host. However, the initial interactions with host phagocytic cells, especially neutrophils, may be particularly important in infection owing to facultative intracellular bacteria with relatively short generation times such as *Legionella* spp. Unless such organisms are controlled, they would reach overwhelming numbers before specific immunity has time to develop.

The aim of this review is to relate the basic biology, particularly the biochemistry, of *Legionella* spp. to its pathogenesis at the cellular level. Although much is known about virulence factors of extracellular pathogens, which generally serve to prevent phagocytosis mediated by host humoral factors, the mechanisms of pathogenicity used by intracellular parasites have not yet been fully elucidated (33). A variety of mechanisms have been implicated in the intracellular survival of different bacteria, including extraphagosomal location, resistance to oxidative and nonoxidative killing mechanisms, inhibition of phagosome-lysosome fusion, and interruption of phagocyte activation and the subsequent production of bactericidal oxygen metabolites (56). The functional defects that might permit the intracellular survival of the legionellae have remained an enigma until recently. The initial investigations of phagocytes that ingest *Legionella* spp. did not detect any of the variety of mechanisms used by other organisms to evade intracellular destruction. It has been demonstrated that both *L. pneumophila* and *L. micdadei* are phagocytized and remain within the phagosome. Furthermore, both species are susceptible to killing in vitro by H2O2 and other bactericidal oxygen metabolites produced by phagocytic cells (58, 122, 123). It has been thought that intracellular survival in phagocytes might be explained by the fact that phagosome-lysosome fusion is inhibited following the ingestion of *Legionella* spp. It now appears, however, that fusion may be inhibited by only a single strain (Philadelphia 1) of *L. pneumophila* serogroup 1 (99); no defect in phagosome-lysosome fusion has been found in monocytes or neutrophils which have phagocytized other species, other *L. pneumophila* serogroups, or even other strains of *L. pneumophila* serogroup 1 (159). Upon initial contact *L. micdadei* was a potent stimulus of the neutrophil and monocyte metabolic burst (60, 61). However, it was recently found that within 30 min following the ingestion of *L. micdadei*, the activation of neutrophils and monocytes in response to both soluble and particulate stimuli is profoundly impaired and the bactericidal activity of these cells for *Staphylococcus aureus* and *Escherichia coli* is attenuated (57, 59). These data suggest that one or more *Legionella* bacterial cell-associated factors have a striking inhibitory effect on phagocyte activation and, consequently, on subsequent antibacterial functions.

One strategy to elucidate the pathogenic potential of the legionellae at the cellular level is to attempt to discover the bacterial factors which are responsible for blocking the activation of phagocytes. Two factors that are elaborated by the legionellae and that inhibit phagocyte activation have been described. We found that *L. micdadei* bacterial cells contain a phosphatase which blocks superoxide anion (O2−) production by stimulated neutrophils (172), thus reproducing the refractoriness to stimulated activation which is seen following ingestion of *L. micdadei*. We have shown that the *Legionella* phosphatase disrupts the formation of critical intracellular second messengers in neutrophils (175), providing a logical mechanism by which the phosphatase blocks phagocyte activation. The second moiety known to block neutrophil oxidative metabolism in response to various agonists is the *Legionella* (cyto)toxin (79, 121). There is considerable evidence that the *Legionella* toxin is important in the pathogenesis of cellular infection. However, no group has yet succeeded in purifying the toxin to homogeneity, a prerequisite for defining its mechanism of action and establishing its role as a virulence factor.

In addition to the toxin and phosphatase, several other moieties elaborated by the legionellae may serve as virulence factors by promoting cell invasion or intracellular survival and multiplication. Genetic studies show that a cell surface protein named Mip is necessary for the efficient invasion of monocytes (45). The mechanism by which Mip promotes bacterial uptake by phagocytes is unknown. A possible role for a *Legionella* phospholipase C as a virulence factor is still largely theoretical. *L. micdadei* contains a protein kinase which catalyzes the phosphorylation of eukaryotic substrates, including phosphatidylinositol (PI) and tubulin (174). Although we have demonstrated that the enzyme phosphorylates PI in the plasma membrane of intact neutrophils, the function and significance of the *L. micdadei*
kinase are obscure. However, since the phosphorylation of either PI or tubulin might compromise phagocyte activation and bactericidal functions, the enzyme may well be a virulence factor.

When a purified *Legionella* exoprotease was aerosolized into the lungs of guinea pigs, it induced lesions resembling those of *Legionella* pneumonia and caused the death of many of the animals (49). Both the cytotoxic nature of the exoprotease and the detection of antibodies in human convalescent-phase serum reactive with the exoprotease (114, 157) suggested that this protein plays a role in the pathogenesis of legionellosis. However, recent work with a genetically engineered strain has convincingly shown that the protease is not necessary for intracellular survival or virulence (29, 184). Other *Legionella* moieties, including lipo polysaccharide (LPS), the major outer membrane protein (MOMP), the heat shock protein, oxygen-scavenging enzymes, and a hemolysin termed legolysin, which could conceivably be involved in virulence, will be mentioned only briefly since there is no evidence that any of these promote invasive or intracellular survival.

**Approach**

For the reader who is not as familiar with *Legionella* spp. as facultative intracellular pathogens, the vast amount of data concerning the interactions of the legionellae with host phagocytic cells will be briefly reviewed. Since it is only recently that functional lesions in phagocytes infected with *Legionella* spp. have been identified which explain the ability of the bacterium to survive in these cells, this is an opportune time to summarize these studies to provide the background for discussing the bacterial factors which might cause these impairments. Individual putative virulence factors will then be reviewed. The various factors have been characterized to a greater or lesser extent by quite different approaches. For example, the evidence suggesting that the phosphatase might be a virulence factor for intracellular bacterial survival is primarily biochemical, whereas for the Mip protein it is genetic and the mechanism of action is unknown. In contrast, the exoprotease, which was considered a possible virulence factor on pathological and biochemical grounds, does not appear to function in that capacity now that genetic analyses are complete. These data will be critically reviewed, for it is important to point out where the present evidence falls short of showing that a putative virulence factor contributes to cellular parasitism.

With the multiplicity of *Legionella* species and serogroups that have been described, it is possible that progress in identifying the determinants of a complex process such as pathogenicity will be made by comparing and contrasting the intracellular biology of different species and serogroups. When relevant biological differences are established, the biochemical basis for the difference can be sought. For example, in contrast to *L. pneumophila*, *L. micdadei* shows a predilection for causing disease almost exclusively in immunosuppressed patients (63, 140, 168). This suggests either that *L. micdadei* possesses virulence factors different from those of *L. pneumophila* or that the two species differ in their interaction with host defenses. Most of the research has utilized one of the serogroups of *L. pneumophila* or *L. micdadei*. One of the goals of this review is to integrate the work on the intracellular biology which has been accomplished with various species so that the similarities and differences may serve as the basis for an examination of the virulence factors of the genus. Unfortunately, however, each research group involved in studying putative virulence factors has tended to use a single *Legionella* strain. Therefore, in most instances it can be said only that a particular virulence determinant appears to be pertinent for that strain and that the relevance to other *Legionella* spp. and serogroups has not been addressed.

It is relatively easy to derive attenuated *Legionella* mutants by passing the wild-type bacteria on substandard media. The properties of these avirulent mutants have been compared with those of their virulent parental strains in a number of studies in an attempt to identify the attribute associated with virulence. A review of these investigations indicates that the mechanism leading to avirulence is different for the various mutants which arise during agar passage. These findings may support the possibility that virulence in the legionellae is multifactorial. However, it is not clear whether the various mechanisms identified by this approach are the ones which contribute to the virulent phenotype of the naturally occurring bacteria.

**INTRACELLULAR BIOLOGY OF LEGIONELLA SPP.**

**Pathology**

*Legionella* spp. produce an acute purulent pneumonia in which the alveoli are filled with polymorphonuclear neutrophils, macrophages, fibrin, and erythrocytes (203). The numbers of neutrophils and macrophages are usually approximately equal, but one or the other cell type may predominate in individual cases. Whether monocyte phagocytes or neutrophils predominate does not seem to be related to the duration of the pneumonia before tissue is obtained for examination (203). A dramatic microscopic feature of the exudate in many cases is the lytic destruction of the inflammatory cells, a process which has been termed leukocytolysis. There is a close association of the bacteria, or bacterial antigen, with the cellular component of the inflammatory infiltrate. The majority of bacteria are seen within inflammatory phagocytic cells; only a few are extracellular (84, 203). A careful immunofluorescence study by Hicklin et al. (91) documented the close association of *L. pneumophila* antigen with the cellular component of the inflammatory infiltrate and the rarity of bacilli in alveoli which were merely edematous or congested. Most of the intracellular bacteria are found within membrane-bound cytoplasmic vacuoles (phagosomes) or, later in infection following extensive intracellular bacterial proliferation, within the disrupted cytosol of the cells (40).

Similar histologic changes were observed in the lungs of guinea pigs inoculated with *Legionella* spp. intranasally (113), intratracheally (139, 150, 201), by aerosol (8, 9, 55), or even intraperitoneally (41). In the latter case the pneumonia presumably evентuates following bacteremia. Although occasional intracellular bacteria could be seen to be dividing within inflammatory cells when infected pulmonary tissue was examined by electron microscopy (41, 113), it could not be determined whether the bacteria multiplied intracellularly or were phagocytized after extracellular multiplication. Therefore, the initial approach of a number of investigators to understanding the pathogenesis of *Legionella* infection at the cellular level was to study in vitro the interactions of the bacterium with the three types of professional phagocytes it encounters in the host during natural infection (54), namely, polymorphonuclear neutrophils, alveolar macrophages, and peripheral blood monocyte-macrophages.
Polymorphonuclear Leukocytes

Antibody is required to fix the third component of complement (C3) to L. pneumophila, and only complement-coated bacteria are efficiently bound to or ingested by human neutrophils in vitro (106, 198). Even when the bacteria were preincubated with a source of specific antibody and complement, human neutrophils killed only about 0.5 log of an inoculum of virulent L. pneumophila (106). Under the same conditions neutrophils reduced the number of a serum-resistant, encapsulated strain of E. coli by 2.5 logs. Separation of the neutrophil-associated and unassociated components revealed that the majority of surviving legionellae were neutrophil associated, suggesting that the neutrophils fail to kill legionellae after they are bound and presumably internalized.

In contrast, human neutrophils phagocytized virulent L. micdadei with only normal human serum (complement) as the opsonin; specific antibody was not required (198). However, under these conditions significantly fewer L. micdadei cells (75%) were phagocytized than S. aureus cells (98%). Use of heat-inactivated serum abolished phagocytosis. There was essentially no killing of ingested L. micdadei cells by neutrophils, compared with the killing of 97% of S. aureus cells that had been phagocytized. In contrast, 89% of L. micdadei cells rendered avirulent by multiple passages on agar were killed by neutrophils.

FitzGeorge et al. (76) examined the effects of neutrophil depletion engendered by the administration of anti-polymorph serum on Legionella infection in guinea pigs. Elimination of polymorphonuclear leukocytes lowered the dose of aerosolized L. pneumophila necessary to cause pneumonia, increased the number of bacteria in the lungs, and produced much higher mortality. Neutrophil depletion did not change the extent or nature of the pulmonary lesions, except that neutrophils were absent from the infiltrate. These results indicate the importance of neutrophils in the defense of the lungs against L. pneumophila and also suggest that neutrophils and their enzymes are not responsible for the pulmonary lesions in legionellosis.

Working with L. micdadei, we have developed an in vitro system in which begins to elucidate how the legionelae escape intracellular destruction by neutrophils and monocytes. Initial experiments revealed that neutrophils phagocytosing L. micdadei appear to function normally. Neutrophil oxidative metabolism, as measured by reduction of Nitro Blue Tetrazolium dye, luminol-enhanced chemiluminescence, oxygen consumption, and hexose monophosphate shunt (HMPS) activity, was stimulated by L. micdadei ingestion to the same extent as when opsonized zymosan (OPZ). S. aureus, or E. coli served as the phagocytic stimulus (61). L. micdadei actually stimulated significantly more O$_2^-$ production than did either S. aureus or E. coli at bacterium-to-neutrophil ratios of 10:1, 100:1, and 1,000:1. Fusion of phagosomes with both primary (azurophilic) and secondary (specific) granules during phagocytosis of L. micdadei was equivalent to that obtained with S. aureus, which was used as the positive control. Furthermore, quantitation of extracellular myeloperoxidase and lysozyme indicated equivalent release of granule enzymes by neutrophils ingesting L. micdadei and S. aureus.

However, within 30 min after having phagocytosed L. micdadei, the activation of neutrophils in response to soluble and particulate stimuli is markedly depressed when compared with either nonphagocytizing control neutrophils or neutrophils which have ingested S. aureus or E. coli (59).

VIRULENCE FACTORS OF THE LEGIONELLACEAE

Once ingested, L. micdadei inhibited neutrophil chemotaxis in response to the chemotactic peptide, N-formyl-methionyl-leucyl-phenylalanine (fMLP). The quantity of O$_2^-$ produced in response to fMLP by neutrophils which had phagocytized L. micdadei 30 min or more previously was markedly depressed compared with either nonphagocytizing neutrophils or neutrophils ingesting S. aureus. The attenuation of stimulated O$_2^-$ production varied directly with the number of L. micdadei phagocytized and with the time elapsed following ingestion of the bacteria. The inhibitory effect of L. micdadei on neutrophil activation did not depend on the prior phagocytosis of viable bacteria, as neutrophils ingesting heat-killed L. micdadei had depressed fMLP-stimulated O$_2^-$ production compared with either nonphagocytizing neutrophils or neutrophils which had phagocytized heat-inactivated S. aureus. However, phagocytosis of either viable or dead bacteria was required for the inhibition of neutrophil activation. When neutrophils were exposed to L. micdadei at 4°C or in the presence of complement-depleted serum, conditions which preclude phagocytosis (180, 198), the subsequent production of fMLP-stimulated O$_2^-$ was normal.

Neutrophil phagocytic and bactericidal capacities were depressed concomitantly with the inhibition of activation following L. micdadei ingestion (59). The percentage of neutrophils which phagocytized viable S. aureus was significantly lower following ingestion of heat-inactivated L. micdadei when compared with either neutrophils which had previously ingested heat-killed S. aureus or nonphagocytizing neutrophils. Killing of staphylococci was likewise reduced, from virtually 100% killed by nonphagocytizing neutrophils and neutrophils which had previously ingested heat-killed S. aureus to 37% killed by neutrophils which had previously ingested killed L. micdadei. These results may be contrasted with the results of an experiment done by Horwitz and Silverstein (106) in which neutrophils simultaneously phagocytizing L. pneumophila and E. coli killed the latter as effectively as neutrophils ingesting only E. coli. This result again demonstrates that some time must elapse following the phagocytosis of Legionella cells before the inhibition of neutrophil function is evident.

Thus, we have demonstrated that L. micdadei, once ingested, significantly depressed several neutrophil functions including chemotaxis, phagocytosis, stimulated O$_2^-$ production, and bactericidal activity (59). Several characteristics of this inhibitory effect are noteworthy. First, ingestion of the organism is required; decreasing phagocytosis experimentally markedly reduced the degree of inhibition of neutrophil function. This suggests that the factor(s) responsible for the inhibition is released from the bacteria under the conditions existing in the phagolysosome. Second, viable bacteria are not required for inhibition to take place, since bacteria killed by heating are effective inhibitors of neutrophil function. Thus, a phagocyte may be able to kill the first Legionella cell ingested and yet could still become impaired and subsequently be incapable of handling additional phagocytized bacteria. On the other hand, the ingestion of viable Legionella cells impairs neutrophil function to a somewhat greater extent than does ingestion of heat-killed bacteria (59). This implies that the inhibitory effect is multifactorial, involving both a heat-stable and a heat-labile factor(s). Third, inhibition was not stimulus specific; significant inhibition of neutrophil activation occurred following stimulation with fMLP, OPZ, or phorbol myristate acetate (PMA). However, the inhibition of infected neutrophils stimulated with fMLP was greater than that produced in response to PMA. We conclude that the legionellae must possess the
capability to actively inhibit the phagocyte activation sequence leading to the respiratory burst, perhaps at multiple sites in the sequence.

A recent, preliminary report indicates that infection of neutrophils with \textit{L. pneumophila} inhibited the rise in intracellular Ca$^{2+}$ in response to fMLP (34). Both a virulent and an avirulent strain caused marked suppression of the Ca$^{2+}$ response at early time points, but the virulent bacterium produced significantly greater suppression at later times. \textit{E. coli} caused no inhibition of the fMLP-stimulated rise in intracellular Ca$^{2+}$. Thus, it appears that neutrophils which have phagocytized \textit{L. pneumophila} and \textit{L. micdadei} demonstrate similar blockade of the intracellular activation pathways.

**Mononuclear Phagocytes**

Alveolar macrophages. Kishimoto et al. (115) studied the interaction between \textit{L. pneumophila} and cell monolayers of avian alveolar macrophages that were obtained by bronchoalveolar lavage and cultured in vitro. Electron micrographs obtained 3 h after infection showed that about 5% of the alveolar macrophages contained intracellular bacteria. At 24 h later, many macrophages contained distended vacuoles filled with \textit{L. pneumophila}. Multiplication of the legionellae was so rapid and extensive that the cytoplasm of some macrophages became filled with vesicles containing bacteria and the cells were ultimately destroyed. Jacobs et al. (109) investigated the interaction between \textit{L. pneumophila} and pigtail monkey alveolar macrophages by using methods that distinguished between viable and dead bacteria. In the absence of antibody the alveolar macrophages phagocytized about 1% of the legionellae in the inoculum. The macrophages killed the majority (60 to 97%) of the ingested bacteria within 30 min. Phagocytosis of \textit{L. pneumophila} was associated with a respiratory burst, as visualized by Nitro Blue Tetrizolium reduction around ingested bacteria. Killing of the alveolar macrophage-associated \textit{Legionella} cells was inhibited by mannitol and by the combination of superoxide dismutase (SOD) and catalase, but not by either of these two enzymes alone. These results indicated that the killing of \textit{Legionella} cells by alveolar macrophages is mediated by the hydroxyl radical (OH$^-$), which is formed from O$_2^-$ and H$_2$O$_2$ in the presence of Fe$^{2+}$. The virulent legionellae which survived after the early killing multiplied more than 2 logs in the 96 h following infection. In contrast, avirulent \textit{L. pneumophila} replicated more slowly over the same period.

\textit{L. pneumophila} Philadelphia I also multiplied rapidly in human alveolar macrophages obtained by bronchoalveolar lavage and cultured as monolayers in vitro (142). Specific antibody combined with complement promoted the phagocytosis of \textit{L. pneumophila}, but alveolar macrophages were able to kill less than 10% of an inoculum even in the presence of both opsonins. \textit{L. pneumophila} multiplied 2.5 to 5 logs over 3 days, and at the peak of bacterial multiplication the macrophage monolayers were destroyed. Electron microscopy showed that the bacteria were located intracellularly within membrane-bound vacuoles which were studded with ribosomes. In the absence of serum, \textit{L. pneumophila} was also taken up by and multiplied rapidly in guinea pig and rat alveolar macrophages obtained by bronchoalveolar lavage (69). No extracellular multiplication of the bacteria occurred. Growth was inhibited when the alveolar macrophages were pretreated with cytochalasin D, which prevented phagocytosis of the legionellae.

In the absence of any opsonins, \textit{L. micdadei} cells were taken up by and multiplied within guinea pig alveolar macrophages, so that the cell-associated titer increased more than 100-fold over 20 h (120). \textit{L. micdadei} opsonized with either complement or specific antibody multiplied within alveolar macrophages to the same extent as did unopsonized bacteria. Treatment of the macrophages with cytochalasin B or incubation at 4°C reduced the percentage of alveolar macrophages containing intracellular bacteria, confirming that the uptake of \textit{Legionella} cells by alveolar macrophages is dependent on an intact macrophage microfilament system and occurs by a process compatible with phagocytosis. Guinea pigs infected with \textit{L. micdadei} by intratracheal inoculation and then treated with antimicrobial agents were immune to subsequent challenge with an otherwise lethal dose of \textit{L. micdadei}. However, the growth curves of \textit{L. micdadei} in alveolar macrophages obtained from these immune animals and infected in vitro were identical to those in alveolar macrophages from naive guinea pigs. On the other hand, cell-free supernatants from blood mononuclear cells stimulated with concanavalin A (ConA) inhibited the multiplication of \textit{L. pneumophila} in alveolar macrophages (142). This indicates that lymphokines may activate the alveolar macrophages in the lungs to restrict \textit{L. pneumophila} multiplication more effectively.

**Peripheral blood monocytes.** Horwitz and Silverstein (105) studied the interaction of virulent \textit{L. pneumophila Philadelphia 1} and human peripheral blood monocytes in vitro. \textit{L. pneumophila} cells multiplied several logs when incubated with the monocytes. Peak growth was associated with destruction of the monocyte monolayer. \textit{L. pneumophila} multiplied only in the adherent cell population, indicating multiplication in monocytes rather than lymphocytes. By electron microscopy, \textit{L. pneumophila} was found in membrane-bound cytoplasmic vacuoles studded with host cell ribosomes. Peripheral blood monocytes bound more than three times as many virulent \textit{L. pneumophila} in the presence of both specific antibody and complement than when opsonized with complement alone (107). Monocytes required both antibody and complement to kill any \textit{L. pneumophila} cells. However, even in the presence of both opsonins, monocytes killed only 0.25 log of an inoculum of virulent \textit{L. pneumophila}. The surviving bacteria multiplied several logs in the monocytes over 4 days following infection, regardless of whether they were opsonized with specific antibody in addition to complement.

Similarly, virulent \textit{L. micdadei} multiplied within human peripheral blood monocytes following phagocytosis (199). Intracellular bacterial growth was rapid, with a 100-fold increase to the peak titer occurring over 12 h. Under the same conditions, \textit{L. pneumophila} grew more slowly, reaching peak titer in 48 h, but \textit{L. pneumophila} multiplied to a higher final titer than \textit{L. micdadei} did. Electron microscopy after 18 h showed that the \textit{L. micdadei} cells were intracellular in normal-appearing phagosomes. At the same time, intracellular \textit{L. pneumophila} organisms were located in phagosomes studded with ribosomes. \textit{L. micdadei} activated the complement system and was opsonized by C3. However, the use of complement-depleted serum as the opsonic source had no effect on the ingestion or growth of \textit{L. micdadei} in monocytes.

As with neutrophils, oxidative metabolism, as measured by chemiluminescence, oxygen consumption, or HMPS activity, in monocytes actively phagocytizing \textit{L. micdadei} was equivalent to or greater than in monocytes ingesting \textit{S. aureus} or OPZ (60). Preliminary assays carried out on monocytes which had previously ingested heat-killed \textit{L. pneumophila}.
**VIRULENCE FACTORS OF THE LEGIONELLACEAE**

*micdadei* revealed a similar attenuation of function as was observed with neutrophils, although the degree of inhibition was more variable (57). In monocytes which had ingested *L. micdadei*, chemotaxis was inhibited by 50% compared with monocytes which had ingested *S. aureus* or nonphagocytizing monocytes (*P* < 0.01). Oxidative activity of monocytes which had phagocytized *L. micdadei* in response to OPZ, as measured by HMPSS activity, was reduced by 94% compared with controls (*P* = 0.01). However, chemiluminescence, another parameter of oxidative function, was reduced by only 50% (*P* < 0.02). Unlike neutrophils, monocytes which had ingested *L. micdadei* demonstrated no inhibition of phagocytosis of *S. aureus* and only minimal reduction in the killing of *S. aureus* (80% of ingested bacteria killed versus 100% killed; *P* < 0.05). Whether the inhibition of monocyte function would be greater following phagocytosis of viable rather than heat-killed *L. micdadei* cells remains to be determined.

Horwitz and Silverstein (108) showed that soluble products of stimulated lymphocytes were able to at least partially activate mononuclear phagocytes against *L. pneumophila*. Peripheral blood monocytes exposed to the products of ConA-treated mononuclear cells (primarily lymphocytes) exhibited decreased phagocytosis of *L. pneumophila*. Although these cells were still unable to kill ingested *L. pneumophila* cells, the intracellular multiplication of the bacilli was inhibited. Similarly, peripheral blood monocytes activated with human recombinant gamma interferon inhibited the multiplication of *L. pneumophila* (20). In the absence of complement and antibody, neither gamma interferon-activated monocytes nor unactivated monocytes killed *L. pneumophila*. Even in the presence of both opsonins, gamma interferon-activated monocytes killed only 0.5 log of an inoculum, which was not more than nonactivated monocytes did. These in vitro results were extended to show that, when incubated with formalin-killed *L. pneumophila*, the peripheral blood mononuclear cells from patients who had recovered from Legionnaires’ disease also produced cytokines which inhibited the growth of *L. pneumophila* in freshly explanted monocytes (100).

**Amoebae**

Rowbotham (170) was the first to demonstrate that legionellae are ingested by free-living amoebae of the genera *Acanthamoeba* and *Naegleria* in vitro. There are obvious parallels between *Legionella* uptake and infection of free-living amoebae and human phagocytic cells. After ingestion the legionellae within amoebae are confined to vacuoles and, under appropriate conditions, multiply to large numbers until the amoebal cell ruptures. Interestingly, the intracellular vacuoles containing a strain of *L. pneumophila* serogroup 1 (not further defined) demonstrated the same alignment of mitochondria and ribosomelike structures along the vacuole membrane (143) as was described for human phagocyte phagosomes containing the Philadelphia 1 strain (98, 105, 199). However, these morphologic findings and the intracellular multiplication of *Legionella* organisms were related to the medium in which the amoebae were maintained. The “abnormal” vacuoles were observed for amoebae maintained in saline, in which intracellular bacterial multiplication was absent, and apparently were not observed in amoeba culture medium, in which intracellular *Legionella* multiplication occurred. The interaction may also be influenced by the species of the amoeba and the legionellae (171) and, as with human phagocytes, the virulence of the *Legionella* strain employed (171, 189). For example, electron-microscopic studies have revealed two forms of *L. micdadei* bacteria: a smooth, thick-walled, banded form containing poly-(β-hydroxybutyrate) granules, and thinner, rumpled-wall, unbanded bacteria with little or no poly-(β-hydroxybutyrate) (85). The band appears to represent an unusually thick layer of peptidoglycan. Since the banded form was prevalent in infected human lungs and among *L. micdadei* cells propagated in cell cultures, whereas the unbanded form was predominant in agar-grown bacteria, the former may represent a more virulent form of the bacterium. It was found that the banded bacilli readily infect and multiply in *Acanthamoeba* cells while the unbanded *L. micdadei* organisms either do not infect or do not grow in these amoebae (171).

The similarity of the interaction of legionellae with amoebae and human phagocytic cells suggests that the former may be useful as a model to elucidate the latter. However, among amoeboid protozoa, phagocytosis is not only the means of defense against foreign cells and particles, but also the principal mode of ingesting food. Thus, there is a complex relationship between digestion of ingested bacteria and bacterial multiplication following ingestion. Low environmental temperatures favor digestion of ingested legionellae (1, 141), whereas higher temperatures favor infection and intracellular multiplication (1, 170). Moreover, virulence for amoebae does not necessarily coincide with virulence for human phagocytes. *L. anisa* and the amoeba *Hartmannella vermiformis* were both isolated from an indoor fountain which had been implicated as the source of an outbreak of Pontiac fever. The *L. anisa* strain multiplied in *H. vermiformis* in vitro, but failed to infect guinea pigs, cultures of the human mononuclear cell line U937, or human peripheral blood monocytes (73). These results suggested that the *L. anisa* strain could reach high concentrations in the environment as a result of growth in *H. vermiformis*, but produced Pontiac fever rather than pneumonia because of the inability to multiply in human phagocytic cells. When a large panel of *Legionella* strains of various species were cocultivated with *H. vermiformis* at 37°C in a system in which killed *Pseudomonas paucimobilis* cells served as food for the amoebae, the local environmental strains were more likely to multiply in the amoebae than were strains from other sources, including clinical isolates (194). However, it is possible that the difference in the ability of the legionellae to multiply in the cocultures was related to differences in passage histories of the strains. Almost all the environmental strains had been transferred on charcoal yeast extract agar fewer than five times, while the passage history of most of the other strains was unknown. More concerning the cell biology of the amoebae must be understood before the various factors involved in the *Legionella*-amoeba interaction which determine whether the amoebae will inhibit or support the growth of legionellae can be elucidated. From the data available at present, it does not appear likely that the virulence determinants for infection of, and multiplication in, amoebae and phagocytes are necessarily the same.

**Multiplication in Phagocytes Required for Infection and Disease**

The hypothesis that infection, and ultimately disease, depends on intracellular multiplication of the legionellae is based on three lines of evidence (43). First, in an experimental model of aerosol infection in guinea pigs, Davis et al. (54) found a rapid increase in the number of viable *Legionella* cells obtained by pulmonary lavage during the 24 h following
infection; by 16 h 86% of the viable bacteria were associated with the cell pellet. From 24 to 48 h after infection there was a rapid influx of neutrophils which resulted in a mixed inflammatory cell population that coincided with a leveling off of the Legionella titer. Most viable bacteria were found in the cell fractions containing the largest number of alveolar macrophages, whereas fewer viable bacteria were found in fractions containing predominantly neutrophils. Large numbers of morphologically intact bacteria were present only in alveolar macrophages. Morphologically intact bacilli were also found in neutrophils, but the majority of bacteria in these cells appeared structurally damaged. Similar results were obtained by Jepras et al. (112), who compared the effects of aerosol infection with virulent and avirulent Legionella strains in guinea pigs. The virulent strain multiplied rapidly in the lungs, reaching a peak titer of over 1011 viable bacteria per lung in 6 days, whereas the avirulent bacteria were unable to replicate and were cleared between 14 and 21 days following infection. Lung lavages performed on infected animals showed that the virulent legionellae were mainly intracellular, whereas the avirulent bacteria were predominantly extracellular. There were approximately 10 times the number of viable virulent Legionella cells in alveolar macrophages than in neutrophils. In contrast, there were about equal numbers of viable avirulent bacteria in macrophages and neutrophils. Therefore, during the early period after infection the primary site of Legionella multiplication appears to be the alveolar macrophage. Unlike the finding in short-term in vitro experiments, neutrophils appear capable of killing at least some Legionella cells in vivo over days, but this relatively inefficient killing is not sufficient to eliminate the bacteria.

Second, the susceptibility of a given animal species to infection is correlated with the ability of L. pneumophila to multiply within macrophages from that species (205, 206). However, there were exceptions, in that Legionella cells grew in the peritoneal macrophages of golden hamsters and rats, species which are resistant to infection (206). These results indicate that in at least some species other factors must be involved in susceptibility to infection. Third, L. pneumophila mutants that are impaired in intracellular growth possess reduced virulence for animals (45, 102, 112, 154).

**Phagocytosis and Phagosomal-Lysosome Fusion**

As indicated in the preceding sections, the most fundamental biological difference which has been found between various Legionella strains is their mode of entry into phagocytic cells and their intracellular fate. Both L. pneumophila and L. micdadei must fix complement in order to be ingested by neutrophils. However, in vitro opsonization of L. pneumophila Philadelphia 1 is dependent on specific antibody-mediated activation of the classical complement pathway; activation of the alternative pathway could not be detected (106, 190). On the other hand, L. micdadei fixes complement by the alternate pathway in the absence of specific antibody, and normal serum is a sufficient opsonin for neutrophil phagocytosis (180, 198). These findings lead to the conclusion that L. pneumophila should not be efficiently phagocytized by neutrophils early in primary infection before antibody is produced. Since neutrophils appear to be able to kill at least some legionellae in vivo, this could be an effective virulence mechanism. However, there is no evidence from pathological studies of human or animal material that L. pneumophila cells are not phagocytized by neutrophils to the same extent as L. micdadei. Perhaps the uptake of L. pneumophila by neutrophils is explained by later studies involving more sensitive methods which showed that the Philadelphia 1 strain does fix complement in nonimmune serum, albeit at 10-fold lower levels than E. coli does (14).

Horwitz (101, 103) observed that L. pneumophila Philadelphia 1 is ingested by human monocytes, alveolar macrophages, and polymorphonuclear leukocytes in an unique manner termed coiling phagocytosis. In this process, a long phagocyte pseudopod coils around the bacterium as the organism is internalized. The bacterium thus ends up in the center of a large coil and eventually comes to reside in an abnormal, ribosome-studded phagosome (98). The phagosome containing L. pneumophila Philadelphia 1 does not fuse with either primary or secondary monocyte lysosomes, as measured by acid phosphatase cytochemistry or by prelabeling lysosomes with thorium dioxide, respectively (99). The Philadelphia 1 strain also inhibits phagosome acidification in monocytes (104). Since live, glutaraldehyde-killed, and heat-killed L. pneumophila Philadelphia 1 cells are all internalized by coiling phagocytosis (101), the intracellular fate of the bacteria must be determined by factors other than the unusual mode of entry.

In contrast to the situation with Philadelphia 1, several workers observed that engulfment of other L. pneumophila serogroup 1 strains occurred by conventional phagocytosis in which the bacterium is surrounded by extensions of pseudopods until their tips meet and fuse on the distal side of the particle (43, 69, 148). Likewise, we found that L. micdadei is ingested by conventional phagocytosis and that the phagosome in which it is contained appears to be normal (198, 199). The apparent contradictions were clarified by Rechnitzer and Blom (159). They confirmed that the Philadelphia 1 strain of L. pneumophila serogroup 1 is internalized by coiling phagocytosis. However, the Knoxville 1 strain of L. pneumophila serogroup 1 and L. micdadei were phagocytized in the classical manner. More importantly, the formation of phagolysosomes was seen following phagocytosis of cells of the Knoxville 1 strain and L. micdadei, but not the Philadelphia 1 strain. Since pseudopod coil formation, formation of an abnormal phagosome, and inhibition of phagosome-lysosome fusion (and probably inhibition of phagosomal acidification) may be specific for the Philadelphia 1 strain of L. pneumophila, these phenomena are independent of bacterial virulence. It is improbable, therefore, that these processes are necessary for the intracellular survival of legionellae (159). On the other hand, these characteristics could promote or enhance the virulence of Philadelphia 1 vis-à-vis other strains of L. pneumophila and the other Legionella spp.

One can postulate that there may be one or more fundamental virulence factors possessed by all legionellae which explain their ability to survive and multiply within phagocytes. Both those fundamental mechanisms which are relevant to the entire genus and the additional or ancillary factors which are strain specific may be elucidated most rapidly by comparing and contrasting the virulence determinants of the various strains. The foregoing evidence suggests that the outcome of the interaction between Legionella spp. and phagocytes is multifactorial and could depend for each strain on at least the following factors: whether the strain inhibits phagosome-lysosome fusion and phagosomal acidification; whether the strain inhibits phagocyte activation and the subsequent generation of bactericidal oxygen metabolites by the particular phagocyte; and the susceptibility of the bacterial strain to the toxic oxygen metabolites produced by
phagocytes. It is also possible that the legionellae can defeat nonoxidative killing mechanisms of phagocytic cells, but this theoretical virulence mechanism has not yet been examined.

**BIOCHEMICAL OVERVIEW**

Since they multiply extracellularly on complex laboratory media, the members of the *Legionellaceae* are classified as facultative intracellular bacteria. They are obligate aerobes. In a comprehensive review of the biochemistry and physiology of *Legionella* spp., Miller and Hammel (133) concluded that although *L. pneumophila* can hydrolyze starch and can oxidize certain sugars, albeit slowly, its energy metabolism is based on oxidation-dependent rather than fermentative pathways. Furthermore, most of the glucose assimilated by the organism appears to be used to provide carbon skeletons for various biosynthetic pathways. Much of the glucose that is consumed is metabolized by the pentose phosphate pathway, presumably to provide pentoses for nucleic acid synthesis and NADPH to satisfy the need of the cell for reducing power in various biosynthetic pathways (e.g., fatty acid biosynthesis). *Legionella* cells seem to derive most of their energy from the oxidation of amino acids and related compounds by means of the tricarboxylic acid cycle, which, along with a complex electron transport chain, is completely expressed in these organisms. Serine, threonine, and glutamate are especially good substrates for *L. pneumophila*. In addition, the following organic acids stimulate oxygen consumption by these bacteria: lactate, pyruvate, acetate, malate, fumarate, and oxaloacetate.

*L. pneumophila* is capable of synthesizing its own fatty acids. A unique feature of the organism is that it produces mostly branched-chain fatty acids, the predominant one being isopalmmitate, a saturated, branched, 16-carbon fatty acid (136). This organism also contains small amounts of hydroxy fatty acids, (β-hydroxyisomyristic acid, β-hydroxyarachidic acid), most of which are confined to cell wall structures (130). The major phospholipids of *L. pneumophila* have been determined to be, in order of decreasing abundance, phosphatidylethanolamine, cardiolipin (diphasphatidylglycerol), phosphatidylethanolamine, phosphatidylglycerol, and phosphatidyl(dimethylethanolamine (75).

Thorpe and Miller (187) examined 10 strains of *L. pneumophila* for the production of extracellular enzymes. All strains produced detectable levels of extracellular protease, phosphatase, lipase, DNase RNase, and β-lactamase activity. Weak starch hydrolysis was also demonstrated for all strains. Elastase, collagenase, phospholipase C, hyaluronidase, chondroitinase, neuraminidase, and coagulase were not detected. However, all these strains had been passed on agar, so that the production of additional enzymes by wild-type legionellae could not be excluded. Unlike *L. pneumophila*, *L. bozemanii*, *L. dumoffii*, and *L. gormanii*, *L. micdadei* does not produce a β-lactamase (151).

**PROPERTIES OF POTENTIAL VIRULENCE FACTORS**

How the legionellae inhibit phagocyte functional activities is not clear. Since *L. micdadei* depress a variety of phagocytic functions (57, 59), it is likely that the bacteria disrupt a process which occurs early in the course of cell activation and is a common precedent to each of the functions we examined. Signal transduction linking cell surface stimulation with intracellular activation is such a process. During the last several years, a variety of signal transduction systems have been defined and shown to be important in neutrophil activation (179). These include (i) hydrolysis of the membrane phosphoinositide phosphatidylinositol-4,5-bisphosphate (PIP₂) by phospholipase C, (ii) arachidonic acid release via phospholipase A₂ activation, and (iii) intracellular fluxes of calcium (11, 19, 83, 128). Disruption of signal transduction by bacterial products such as pertussis toxin has clearly been shown to block a variety of cell functions similar to what we observed after ingestion of *L. micdadei* by neutrophils (12, 13, 30, 119, 191).

Two peptide factors elaborated by the legionellae which inhibit phagocyte activation have now been described. Material has been partially purified from supernatants and sonicates of *L. pneumophila* and *L. micdadei* that has demonstrated cytotoxic activity and lethality for animals and inhibits neutrophil oxidative metabolism (78, 79, 89, 90, 121). The second moiety elaborated by the legionellae which inhibits phagocyte activation is a specific phosphatase that we found blocked oxidative metabolism by stimulated neutrophils (172).

**Peptide Toxin**

The first *Legionella* factor known to inhibit neutrophil activation dates to the observation by Friedman et al. (78) that the culture filtrates in which *L. pneumophila* was grown contained cytotoxic activity for Chinese hamster ovary (CHO) cells. The cytotoxic activity was found to be heat stable, could pass through dialysis tubing with a molecular weight cutoff of 1,000, and was sensitive to pronase and papain but insensitive to trypsin. These properties suggested that the cytotoxin was a small polypeptide. One of the critical microbicidal actions of phagocytic cells is the generation of toxic oxidative metabolites including O₂⁻ and H₂O₂ from molecular oxygen. The reduced-oxygen species derived from O₂⁻ and H₂O₂ play an important role in the killing of bacteria and parasites. Quantities of toxin partially purified from *L. pneumophila* serogroup 1 (Knoxville 1) which had no effect on neutrophil viability or phagocytosis depressed HMPS activity and oxygen consumption during phagocytosis and inhibited both bacterial iodination and the killing of *E. coli* by neutrophils (79). Treatment of neutrophils with toxin partially purified by a different method depressed neutrophil HMPS activity stimulated by latex beads and the calcium ionophore A23187, but not shunt activity stimulated by ConA, PMA, or the potassium ionophore valinomycin (121). Treatment of neutrophils with crude toxin preparations also blocked membrane depolarization in response to A23187, but not in response to PMA. Independently, Hedlund and Larson (90) partially purified toxin from both *L. pneumophila* and *L. micdadei* bacterial cell sonicates and showed that the toxin preparations of the two species shared a common antigen unrelated to Legionella LPS. Their toxin preparations inhibited the chemiluminescent response of neutrophils (89).

Further characterization of the *Legionella* toxin has been hampered by difficulty in purifying this peptide factor to homogeneity. As was true for previous workers, we have been able only to partially purify the *L. micdadei* toxic factor from bacterial cell sonicates by conventional chromatographic methods. We noted that when neutrophils were treated with toxin-containing preparations, subsequent O₂⁻ production in response to FMLP was greatly inhibited, and we used this as an assay for biological activity. Gel filtration of toxin-containing preparations on a Bio-Gel P-2 column eluted with 10 mM phosphate buffer yielded numerous biologically active, low-molecular-weight peaks which had similar amino acids.
acid compositions (62). This suggested that spontaneous aggregation or oligomerization of the toxin molecules occurs at low salt concentration, which may be one of the impediments in attempts to purify the toxin. The predominant, biologically active $A_{250}$ peaks obtained from Bio-Gel P-2 gel filtration were examined for their effect on $O_2^\rightarrow$ production by neutrophils in response to various stimuli. The inhibition of the $O_2^\rightarrow$ response to fMLP was not abolished by heating the partially purified toxin to 100°C for 60 min. Preincubation of neutrophils with toxin for 10 min markedly inhibited subsequent $O_2^\rightarrow$ production in response to both ConA and OPZ to about the same extent as the inhibition of fMLP-stimulated production measured simultaneously; treatment with toxin had no effect on $O_2^\rightarrow$ produced in response to PMA. These results are not completely congruent with those of Lochner et al. (121), who found that partially purified $L$. pneumophila toxin did not inhibit neutrophil HMPS activity stimulated by either PMA or ConA. The difference found in the inhibition of ConA-stimulated neutrophils by toxin could represent true dissimilarities between the toxins derived from $L$. micdadei and $L$. pneumophila or could merely be a function of the specific neutrophil response (HMPS activity or $O_2^\rightarrow$ generation) being examined. However, this apparent inconsistency is more likely to be due to extraneous materials in the relatively crude toxin preparations which have been examined.

Since preincubation of neutrophils with toxin inhibited neutrophil $O_2^\rightarrow$ production in response to fMLP and OPZ, but had no effect on the $O_2^\rightarrow$ produced in response to PMA, treatment of neutrophils with partially purified toxin also does not exactly reproduce the refractoriness to stimulation which is seen following infection of neutrophils with $L$. micdadei (59). In the latter case, PMA-stimulated $O_2^\rightarrow$ production was attenuated, albeit to a lesser extent than fMLP- or OPZ-stimulated oxygen metabolism. However, since the toxin is the only heat-stable putative virulence factor presently known, it must be considered the prime candidate responsible for the inhibition of neutrophil activation which occurs following the ingestion of heat-killed $L$. micdadei.

The mechanism of toxin action is unknown. The pattern of stimulus specificity indicates that the Legionella toxin acts at an early step in the neutrophil activation sequence. The steps in signal transduction between stimulus binding and $O_2^\rightarrow$ production are best delineated for fMLP (179). As shown in Fig. 1, occupation of the neutrophil fMLP-receptor is coupled to the activation of a polyphosphoinositide-specific phosphodiesterase (phospholipase C) through a pertussis toxin-inhibitable guanine nucleotide-binding (G) protein (83). Phospholipase C catalyzes the hydrolysis of PIP$_2$ to yield the intracellular second messengers, myo-inositol-1,4,5-trisphosphate (IP$_3$) and sn-1,2-diacylglycerol. IP$_3$ is involved in the mobilization of Ca$^{2+}$ from intracellular stores in the endoplasmic reticulum and calciosomes. The increases in both intracellular Ca$^{2+}$ concentration and diacylglycerol lead to the activation of protein kinase C (110). The phorbol esters can substitute for cellular diacylglycerol by binding directly to and activating protein kinase C. It is not known whether the neutrophil NADPH oxidase, which catalyzes the reduction of molecular oxygen to $O_2^\rightarrow$, is activated directly by protein kinase C or through additional intermediaries; it is likely that at least one component of the NADPH oxidase complex is a substrate for protein kinase C (2). That $O_2^\rightarrow$ production by PMA-stimulated neutrophils pretreated with partially purified toxin is not inhibited would indicate that the toxin acts prior to the activation of protein kinase C. If ConA- as well as fMLP-stimulated $O_2^\rightarrow$ production is inhibited by the toxin, it implies that the toxin prevents the activation of phospholipase C without affecting fMLP binding or the pertussis toxin-inhibitable G protein. ConA binds to a neutrophil receptor which is distinct from the fMLP-receptor, since ConA binding, and the subsequent production of $O_2^\rightarrow$, can be reversibly inhibited by $\alpha$-methyl mannoside (47); ConA stimulation also bypasses the G protein, since $O_2^\rightarrow$ generation in response to ConA is not blocked by pertussis toxin (191). Furthermore, ConA trig-
gers the breakdown of PI, but not PIP2, and produces no significant increase in IP3 (117), indicating that ConA does not activate the polyphosphoinositide-specific phospholipase C. However, both ConA and FMLP stimulation produces a rapid increase in levels of diacylglycerol and phosphatidic acid. Although the source of the phosphatidic acid is not known, it appears that the *Legionella* toxin may affect other activation pathways in addition to blocking the polyphosphatidylinositol-specific phospholipase C.

The present evidence that the *Legionella* toxin is of importance in the pathogenesis of infection at the cellular level is compelling. First, toxin is produced by all five *Legionella* spp. which have been examined (89). Second, legionellae secrete the toxin into the medium in which they are grown, and exogenously applied, partially purified toxin blocks neutrophil activation, indicating that the toxin produced by both extracellular and intraphagosomal bacteria could adversely affect phagocytes. Third, the toxin is stable at pH 3.5, so that it would not be inactivated in the phagosome even if phagosomal acidification takes place. Fourth, with the possible exception of PMA stimulation, the treatment of neutrophils with toxin reproduces the refractoriness to stimulation and decrement in bactericidal activity which is seen following the phagocytosis of *L. micdadei*. Fifth, the toxin is heat stable and can therefore explain the inhibition of neutrophil functions which follow the ingestion of heat-killed *L. micdadei*. Finally, immunization of mice with toxin-containing material protects against a lethal challenge of *Legionella* cells (89), indicating that toxin production by legionellae is an important pathogenetic mechanism. This latter finding raises the possibility that a toxoid vaccine would be efficacious for protection against all species in the genus. However, it must be borne in mind that all of these studies were accomplished with crude preparations that must have contained considerable extraneous material. Further progress in elucidating the place of the toxin in *Legionella* pathogenicity at the cellular level and its mechanism of action awaits purification of this moiety to homogeneity.

**Enzymes**

Extracellular enzymes, such as proteases and hemolysins, have been implicated as factors that might be responsible for some of the pulmonary and extrapulmonary manifestations of legionellosis. Thus, the production of a cytolytic enzyme could explain the lysis of the pulmonary inflammatory infiltrate seen in many cases or the infarctlike necrosis observed in some cases (202). An exoprotein could also explain why prominent extrapulmonary manifestations, such as obtundation, diarrhea, abnormal liver function tests, hematuria, and azotemia, may occur in the absence of remarkable lesions outside the lungs (22, 139). Since Miller and Hammel (133) wrote their comprehensive review of the biochemistry and physiology of *Legionella* spp., a number of useful reports have been published on the enzymes and proteins that are localized to the surface of the various *Legionella* species.

**Phosphatases.** Few studies have been carried out on the *Legionella* lysosomal hydrolases. In 1981 Muller (138) showed that *L. pneumophila* produces acid phosphatase, and in 1982 Nolte et al. (146) documented the production of acid phosphatase by a total of nine *Legionella* strains including strains of *L. micdadei*. Using more traditional plate-substrate assays, as well as tube assays with culture supernatants, Thorpe and Miller (187) detected acid phosphatase in 10 strains of *L. pneumophila* representing six serogroups. During acid phosphatase cytochemistry studies of phago- some-lysosome fusion in peripheral blood monocytes infected with *L. pneumophila*, Horwitz (99) noted a thin layer of the lead phosphate reaction product between the inner and outer bacterial membranes of the majority of the intraplasomal bacteria.

The high-speed supernatant obtained after centrifuging a suspension of *L. micdadei* that had been freeze-thawed and sonicated contained two acid phosphatases (172). The two acid phosphatases can be separated by chromatography on hydroxylapatite. Both phosphatases are resistant to inhibition by l-(+) sodium tartrate. The predominant acid phosphatase, designated ACP2, was purified to homogeneity by chromatography on QAE-Sephadex, phenyl-Sepharose, and Sephadex G-200 (172). ACP2 runs as a single band and exhibits a molecular weight of 68,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Its isoelectric point is 4.5, and it has a pH optimum between 5.8 and 6.0 when assayed with 4-methylumbelliferyl phosphate as the substrate. Some of the preferred natural phosphomonoester substrates for ACP2 are fructose 1,6-diphosphate, AMP, and phosphotyrosine.

Preincubation of human neutrophils with a pure preparation of the *L. micdadei* acid phosphatase (ACP) inhibited the subsequent production of O2− in response to stimulation with FMLP (172). The second *Legionella* acid phosphatase that we purified (ACP) has no effect on the ability of neutrophils to produce O2− when tested at levels 100-fold above those at which ACP is inhibitory. Pretreatment of neutrophils with the *Legionella* phosphatase also inhibited O2− generation in response to ConA, but had no effect on O2− production stimulated by PMA (64).

The ability of the purified phosphatase to modulate the respiratory burst of phagocytes raised the question of the mechanism of action of the enzyme. The fact that the receptor-mediated hydrolysis of phosphoinositides to yield the intracellular second messengers diacylglycerol and inositol phosphates is involved in the regulation of phagocyte activation (19) suggested that dephosphorylation of one or more of these compounds might be responsible for the biological effects of the *Legionella* phosphatase on neutrophils. Furthermore, this approach had been successful in elucidating the mechanism of action of a cell surface acid phosphatase isolated from *Leishmania donovani* which similarly blocked O2− production by activated neutrophils (53). Indeed, we observed that PIP2 and IP3 are two excellent substrates for the *Legionella* phosphatase in vitro (175). In contrast to the nonphysiological substrate 4-methylumbelliferophosphate, which is hydrolyzed most rapidly at pH 6.0, PIP2 is hydrolyzed optimally by the *Legionella* phosphatase at pH 7.0. Because of this finding with the possible physiological substrate, we now refer to the enzyme as a phosphatase rather than an acid phosphatase.

Neutrophils which had been labeled with 32P, were treated with the *L. micdadei* phosphatase at pH 7.0, and the quantity of PIP2 in the cells was determined at various times (175). By 30 min, 20% of the radiolabeled [32P]PIP2 had been dephosphorylated to phosphatidylinositol-4-phosphate (PIP). None of the PIP which was formed was further dephosphorylated to PI. The production of inositol phosphates in neutrophils following FMLP stimulation was measured in [3H]inositol-labeled cells. When neutrophils were treated with the *Legionella* phosphatase prior to the addition of FMLP, the accumulation of IP3 30 s following stimulation was reduced 44% when compared with that in control cells that were not incubated with the enzyme (175). A 30-min preincubation of neutrophils with the phosphatase prior to addition to FMLP...
also decreased sn-1,2-diacylglycerol production by 45% at 2 min following stimulation (175).

Thus, the active phosphatase appears to reduce the amount of the second messengers IP3 and diacylglycerol produced following receptor-mediated stimulation by two mechanisms (175). First, the phosphatase catalyzes the dephosphorylation of PI3, both in vitro and in intact neutrophils. As shown in Fig. 1, this leaves less PI3 available as a substrate for phospholipase C following receptor-mediated stimulation, so that smaller amounts of both IP3 and diacylglycerol are generated. Second, the bacterial phosphatase may directly dephosphorylate some of the IP3 formed from the hydrolysis of PI3. Although the majority of the PI3 in eukaryotic cells is believed to be in the inner cytoplasmic membrane, the fact that PI3 in intact neutrophils is a substrate for exogenously added phosphatase implies that phosphatase from extracellular or intraphagosomal legionellae can reach this substrate. Although treatment of neutrophils with the phosphatase reproduces the refractoriness to FMLP-stimulated activation which is seen following ingestion of L. micdadei (59), the phosphatase cannot be solely responsible for the antiphagocyte effects of ingested legionellae. The phosphatase does not prevent O2− production in response to PMA, as occurs following phagocytosis of L. micdadei (59).

Also, the Legionella phosphatase is heat sensitive and loses both phosphatase activity and its inhibitory effect on stimulated neutrophil O2− production after heating (172), whereas ingestion of heated L. micdadei inhibits subsequent neutrophil (59) and monocyte (57) activation. Thus, it is doubtful that the enzymatic activities of the Legionella phosphatase fully account for the capacity of the bacterium to block the respiratory burst of phagocytic cells following stimulation. More probably, the phosphatase acts in concert with the toxin to produce blocks at multiple sites of the activation pathway following the phagocytosis of viable legionellae.

Phospholipase C. With the exception of L. micdadei, all of the various Legionella spp. that have been analyzed for their ability to produce phospholipase activity have been found to possess this capability. Baine (3) recognized that the histopathology of pneumonia caused by L. pneumophila was characterized by a cytotoxic picture with necrosis of mononuclear and polymorphonuclear leukocytes and acknowledged that the pathogenesis might involve an extracellular phospholipase. Baine et al. (5) had shown previously that cultures of L. pneumophila lyse erythrocytes from guinea pigs, horses, sheep, rabbits, and humans. Interestingly, it was the guinea pig and dog erythrocytes, which have the highest content of phosphatidylcholine, that were hemolyzed most rapidly by L. pneumophila (3). The same Legionella strains which lysed erythrocytes also caused clumping of egg yolk agar, suggesting that the bacteria were producing some sort of lecinthinas. In the same study, it was demonstrated that Washed cells of seven different Legionella spp., including L. micdadei, would all catalyze the hydrolysis of the nonphysiological sphingomyelinase-phospholipase C substrate p-nitrophenolphosphorylcholine. Five of six Legionella spp. that hydrolyzed p-nitrophenolphosphorylcholine would also cleave [3H]phosphorylcholine from [3H]choline-labeled phosphatidylcholine, confirming the hypothesis that the lecithin-cleaving factor is a C-type phospholipase. The only species which exhibited minimal hemolysis, did not digest egg yolk, and was incapable of splitting the tritiated lecithin substrate was L. micdadei. This observation illustrates the uncertainty inherent in drawing conclusions about the nature of enzyme reactions responsible for cleaving artificial substrates such as p-nitrophenolphosphorylcholine.

The hemolytic factor in the culture filtrates of a strain of L. bozemanii appeared to be a macromolecule, since it would not pass a filter with a 10,000-Da cutoff and was voided when chromatographed on a Sephadex G-100 column (80). The supernatant from sonicated L. bozemanii demonstrated far less hemolytic activity than the culture supernatant, indicating that the hemolysin was an extracellular product of the bacteria.

In 1988 Baine (4) reported on the purification and characterization of the L. pneumophila phospholipase C. High yield (85%) purification was achieved after only a 29-fold increase in the specific activity of the enzyme. Purification required only chromatography on DEAE-Sephadex, mannase chloride precipitation, and ammonium sulfate fractionation. Enzyme activity was assayed by the hydrolysis of p-nitrophenolphosphorylcholine and confirmed by the release of radioactivity from L-a-dipalmitylphosphatidylcholine labeled on choline. The molecular mass of the enzyme is 50 to 54 kDa, and maximum activity on p-nitrophenolphosphorylcholine substrate is observed at pH 8 to 9. Activity was stimulated three- to fourfold above the basal level in the presence of EDTA and by Ba2+, Ca2+, and Mg2+, but was inhibited by Cu2+ and Fe3+. Neutral detergents (e.g. Tween 20 and 80, Triton X-100) also stimulated activity two- to threefold. Interestingly, the purified phospholipase C was not hemolytic. Thus, although it was originally thought that hemolysis was a marker for phospholipase C activity, it eventually turned out that the hemolytic activity is due to another moiety, probably a metalloprotease (see below) and/or a recently described 29-kDa nonproteolytic hemolysin termed legionolysin (158).

Thus, it has been shown convincingly that, with the exception of L. micdadei, the Legionella spp. release considerable quantities of phospholipase C activity into the medium in which they are grown; the enzyme acts on phosphatidylcholine to produce diacylglycerol and phosphocholine. Baine (4) suggested three possible pathophysiological implications of the phospholipase C that is secreted by Legionella spp. First, hydrolysis of phosphatidylcholine, which is a major component of pulmonary surfactant, could impair pulmonary gas exchange. Support for this idea exists in that alveolar hyaline membranes have been described in Legionella pneumonia (203). With the availability of pure Legionella phospholipase C (4), it should now be possible to analyze the effects of the enzyme on isolated surfactant. Second, since phosphatidylcholine is an important constituent of eukaryotic membranes, the cytolysis of the phospholipase C might injure both inflammatory cells and lung tissue. It should be noted, however, that there is no direct evidence that the Legionella phospholipase C is cytolytic or affects eukaryotic cell membranes. Third, the perturbation of phagosome membranes by hydrolysis of phospholipid might disrupt pHaseosome-lysosome fusion. However, the evidence that Legionella strains which produce phospholipase C fail to inhibit fusion following ingestion (69, 148, 159) makes this possibility unlikely.

An additional mechanism by which bacterial phospholipase C might influence the interaction of the legionellae with host phagocytes is by mimicking the activation of cellular phospholipase C and catalyzing the hydrolysis of PI3, to the intracellular messengers IP3 and diacylglycerol. Exogenous bacterial phospholipase C has been shown to modify neutrophil activation. Phospholipase C from both Clostridium perfringens and Bacillus cereus elicited neutrophil O2− pro-
duction and enhanced the oxidative response to NaF when both stimuli were presented simultaneously (181). However, when the stimuli were added sequentially, bacterial phospholipase C abrogated the subsequent oxygen consumption response of neutrophils to latex beads. The authors suggested that bacterial phospholipase exoenzymes could activate neutrophils before they make contact with bacteria, leaving them unable to respond adequately to the microorganism. Whether the legionellae might avoid the bactericidal capacity of phagocytic cells by triggering a premature respiratory burst in this fashion remains to be examined. We (61) found that when *L. micdadei* cells are added to neutrophils, the amount of O$_2$⁻ generated (8.9 ± 2.0 nmol/10⁶ cells per 15 min) was not significantly greater than with *S. aureus* (5.0 ± 0.8 nmol/10⁶ cells per 15 min; *P* = 0.07). Since *L. micdadei* is the only species that does not produce phospholipase C, this would be the expected result, whereas the other phospholipase C-producing species should trigger an early, large release of O$_2$⁻. Indeed, a preliminary report suggests that purified *L. pneumophila* phospholipase may produce a dose-related inhibition of neutrophil function(s) (176).

**Proteases.** (i) General. The legionellae elaborate a number of proteolytic enzymes and aminopeptidases, some of which remain bound to the organism and some of which are secreted into the extracellular culture medium. One of the earliest studies demonstrating the proteolytic capability of the *Legionella* spp. was that of Muller (137); he showed that four strains of *L. pneumophila* could degrade a number of human serum proteins. Muller incubated suspensions of the bacteria with serum and then used immunoelectrophoresis to analyze for proteolytic degeneration of 23 different proteins. Five proteins were degraded: α₁-acid glycoprotein, α₁-antichymotrypsin, α₁-antitrypsin, β₂-globulin, and β₂-glycoprotein I. Muller speculated that inactivation of α₁-antichymotrypsin by *L. pneumophila* may contribute to the emphysemalike syndrome seen in legionellosis, which is comparable to that observed in some patients with α₁-antitrypsin deficiency. Thompson et al. (186) extended this work by demonstrating that cell-free culture filtrates of eight strains of *L. pneumophila* representing six serogroups contained proteolytic activity capable of digesting casein, gelatin, and hide powder azure, but not elastin.

In addition to showing that *L. pneumophila* produces proteases, Muller (138) demonstrated the presence of a variety of aminopeptidases. Again, washed, resuspended organisms were incubated with potential substrates, in this case synthetic, colorimetric naphthylamides, or nitroanilide derivatives of amino acids; collectively, these substrates constitute the API ZYM system. For example, L-alanylaminopeptidase activity is determined by using L-alanyl-2-naphthylamine or L-alanyl-4-nitroanilide as substrates. All four of the *L. pneumophila* strains that were tested were positive for 14 aminopeptidase activities; activities against D-hydroxyproline and L-proline were absent, as were activities against trypsin and chymotrypsin substrates (N-benzoyl-DL-arginine and N-benzoyl-L-phenylalanine-2-naphthylamine). In a parallel study, Nolte et al. (146) confirmed Muller’s observations with *L. pneumophila* and extended them to six additional *Legionella* spp. As will be described below, use of these single-amino-acid derivatives as protease substrates caused these investigators to fail to detect the prominent chymotrypsinlike activity produced by *Legionella* spp.

The first *Legionella* aminopeptidase to be isolated in pure form was phenylalanine-aminopeptidase (86). The enzyme was purified 400-fold from the culture supernatant of *L. pneumophila* by affinity chromatography on *O*-tert-butyl-Thr-Phe-Pro-Gly-aminosilochrom and by gel filtration and ion-exchange chromatography. It has the following properties: MW, 35,000; pl, 5.8; pH optimum, 8.0 to 9.5 with L-phenylalanine- p-nitroanilide as substrate. Although the enzyme could be inactivated by being incubated with EDTA, indicating that it is a metalloprotein, activity could not be reconstituted with Zn²⁺, Ca²⁺, Mg²⁺, Mn²⁺, or Cu²⁺. On the basis of a limited substrate specificity study, Gul’nik et al. (86) concluded that the role of this aminopeptidase appears to be the liberation of hydrophilic free amino acids from peptides present in the growth medium. These authors also speculated that the enzyme might hydrolyze intracellular host cell peptides or the products of host cell proteinolysis effected by *Legionella* proteases.

Whereas the studies of Muller (138) and Nolte et al. (146) analyzed *Legionella* strains for exo-aminopeptidases, Berdal et al. (17) used *para*-nitroanilide (*pNA*) peptides to test for the presence of endopeptidase activity in *Legionella* spp. Specifically, they used substrates in which the *pNA* moiety is attached to the carboxy-terminal end of tri- and tetrapeptides. When the amide bond of the *pNA* peptide is cleaved, the free *pNA* that is liberated can be estimated spectrophotometrically. The specificity of the endopeptidases is determined by the sequence of amino acids at the amino-terminal end of these exogenous substrates. Using *pNA* peptides of this type which possessed a variety of N-terminal amino acids attached to a prolyl-proline-para-nitroanilide chain (Pro-Pro- *pNA*), Berdal et al. (17) demonstrated the presence of a proline-specific endopeptidase in three *Legionella* species; the enzyme could cleave *pNA* from the following peptides: O-benzoyl-Ser-Thr-Pro-Pro- *pNA*, O-benzoyl-Ser-Pro-Pro- *pNA*, and benzoyl-Ser-Pro-Pro- *pNA*. The source of protease in these experiments was the concentrate obtained after subjecting the cell-free growth media to 100-fold concentration by pressure dialysis.

Using a similar approach, Berdal et al. (18) demonstrated that *L. pneumophila*, *L. bozemanii*, *L. dumoffii*, and *L. gormanii*, but not *L. micdadei*, produced soluble, extracellular chymotrypsinlike activity. Two compounds in particular were especially effective substrates: Suc-O-Met-Arg-Pro-Tyr- *pNA* and Suc-Ala-Pro-Tyr- *pNA*. Furthermore, the inhibitor pattern they obtained indicated that the chymotrypsinlike protease is a serine active-site enzyme. Interestingly, when sonicated bacterial cells were analyzed for chymotrypsinlike activity, little activity was found to be associated with the cells. Thus, these early studies with crude bacterial products demonstrated that the legionellae secrete proteases of the endopeptidase and exopeptidase varieties.

(ii) Zinc metalloprotease. By the mid-1980s, investigators began fractionating the proteases produced by *Legionella* spp. For example, in 1986 Conlan et al. (49) chromatographed the culture broth of *L. pneumophila* on a DEAE-cellulose ion-exchange column and a gel filtration column and demonstrated the presence of at least six different proteases, one of which digested collagen, casein, and gelatin and produced lung lesions in guinea pigs. This was the first evidence that a *Legionella* protease could produce lesions closely resembling those of *Legionella* pneumonia. The tissue-destructive protease eluted from a Sephadex G-100 column as though its native molecular weight was 38,000, and it cleaved collagen and the chromogenic peptides Suc-O-Met-Arg-Pro-Tyr- *pNA* and Suc-Ala-Pro-Tyr- *pNA*. This protein was devoid of elastase activity.

In the same year, Dreyfus and Iglewski (67) reported on the purification and properties of the major chymotrypsinlike
extracellular proteolytic enzyme of *L. pneumophila*. The enzyme appears to be identical to the tissue-destructive protease reported by Conlan et al. (49). In just four steps and with an overall enrichment of only 10-fold, the purification provided a 50% yield and 10 to 20 mg of pure enzyme protein from 6 liters of growth medium. It is a relatively acidic protein (pI 4.2 to 4.4), and its pH optimum is broad (pH 5.5 to 7.5); it has maximum activity against hide powder azure protein substrate. The protease is a zinc metalloprotein (1 mol of Zn²⁺ per mol of enzyme); however, it is not inhibited by phenylmethylsulfonyl fluoride, chymostatin, or trypsin inhibitor. Resistance to inhibition by these compounds is in conflict with the results obtained by Berdal et al. (18), who found that the *L. pneumophila* protease had the properties of chymotrypsin. This discrepancy probably occurs because whereas Dreyfus and Igleski (67) characterized a pure enzyme, Berdal et al. (18) were analyzing a relatively crude protease preparation. The protease is active as a 40-kDa monomer but is readily autolyzed to a 38-kDa species. Rechnitzer et al. (162) described a method that avoids enzyme autoproteolysis during purification and results in the isolation of a single 42-kDa protease which is cytotoxic to a variety of cell lines.

Several groups of investigators (15, 114, 162, 169, 185) have demonstrated that culture supernatants containing proteolytic activity or purified protease from *L. pneumophila* have cytoytic effects on tissue culture cells including cytotoxicity for CHO cells. Purified exoprotease from *L. pneumophila* has also been shown to elicit hemorrhagic and necrotic lesions when administered to guinea pigs intradermally (15, 49, 169). Conlan et al. (49) have used a guinea pig animal model to demonstrate the cytotoxic properties of the exoprotease. Intranasal or intratracheal administration of the partially purified *L. pneumophila* protease into the lungs of guinea pigs produced pathological changes which were very similar to those produced in experimental *Legionella* pneumonia (7, 49). Conlan et al. (50) also demonstrated that the tissue-destructive protease was present in the lung tissue of experimentally infected guinea pigs. On day 3 after infection, it was found in amounts equivalent to the lethal dose of the purified protease administered intranasally. In addition, Williams et al. (200) have shown by using double-labeling techniques that the protease is found at sites with large numbers of *L. pneumophila* cells, and both enzyme and bacteria are found only in association with pulmonary lesions. These studies suggested that production of this protease during natural infection may play an important role in causing cytology and the destruction of pulmonary tissue.

Work in several laboratories (15, 114, 157, 169) has demonstrated that the 38- to 42-kDa zinc metalloprotease from *L. pneumophila* produces the observed cytotoxic activities. Furthermore, the exoprotease was also shown to have hemolytic activity. Preliminary screening of selected *Legionella* species for proteolytic and hemolytic phenotypes suggested a correlation between these activities (114). Both virulent and avirulent strains of *L. pneumophila* and most other *Legionella* species expressed both extracellular protease and hemolytic activities. The exceptions were strains of *L. micdadei* and *L. feeleii*, which expressed neither protease nor hemolysin activity. When the *L. pneumophila* exoprotease was purified from culture supernatant, the hemolytic activity was found to copurify with the proteolytic activity, and analyses by SDS-PAGE and immunoblotting revealed a single protein species (114). Furthermore, an exoprotease-deficient mutant strain of *L. pneumophila* (PRT8) was isolated and was found to be nonhemolytic (114). Concentrated supernatants from strain PRT8 were no longer cytotoxic for CHO cells.

Quinn et al. (157) cloned the genetic sequence encoding the 38-kDa protease from *L. pneumophila* serogroup 1. They showed by transposon inactivation analysis that a single trinifunctional polypeptide encoded on a 1.2-kb cloned DNA fragment, designated *pro*, is responsible for proteolytic, hemolytic, and cytotoxic properties. These workers then compared the proteolytic, hemolytic, and cytotoxic activities of strains from several *Legionella* species (157). The concentrated supernatants from all strains of *L. pneumophila*, *L. dumoffii*, and *L. jordanis* and from one (of two) strain of *L. micdadei* demonstrated protease (caseinase) activity. Only the *Legionella* strains which demonstrated proteolytic activity showed hemolysis on canine blood agar. Cytotoxicity for CHO cells was apparent with culture supernatants or sonicates containing protease from strains of *L. pneumophila*, but the protease activities extracted from other *Legionella* species possessed only hemolytic activity. A probe consisting of the DNA sequence encoding the 38-kDa metalloprotease from *L. pneumophila* Philadelphia 1 hybridized to the chromosomal DNA of all serogroups of *L. pneumophila*, but not to any strains of *L. micdadei*, *L. dumoffii*, *L. feeleii*, or *L. jordanis* that were examined. Furthermore, Western immunoblotting analysis with antibodies raised against the *L. pneumophila* protease demonstrated cross-reactions among 38-kDa proteins from strains of *L. pneumophila*, but no reactions were observed with proteins from other *Legionella* species. The cloned protease from *L. pneumophila* reacted with convalescent-phase serum from patients infected with *L. pneumophila*, but not with antiserum from patients infected with other *Legionella* species (114, 157). Thus, despite some similarities among the proteolytic activities of *Legionella* spp., including proteolytic and hemolytic phenotypes, metal requirements for zinc or iron, sensitivity to EDTA, and temperature and pH optima, there are distinct genetic, immunological, and cytotoxicity differences among the proteolytic activities produced by *Legionella* spp. In particular, only in strains of *L. pneumophila* is the 38-kDa metalloprotease associated with cytotoxicity. The detection of antibodies to the protease during *L. pneumophila* infections indicated that the protease/cytotoxin is elaborated during natural infection and strengthened the possibility that the enzyme is involved in the pathogenesis of this species.

These same workers (21) then determined the sequence of the structural gene encoding the *L. pneumophila* zinc metalloprotease and found that it contains a large open reading frame which is preceded by consensus promoter and ribosome-binding sequences. The deduced polypeptide contained a putative signal sequence and a total of 543 amino acid residues with a computed molecular size of 60,775 Da, substantially larger than the 38,000-Da native and recombinant proteins. There was extensive amino acid identity with the *Pseudomonas aeruginosa* elastase, which is also encoded by an open reading frame larger than that predicted from the size of the mature protein. The structural identity of the *L. pneumophila* protease and the *P. aeruginosa* elastase was most pronounced in the regions forming the enzymatic active site of the elastase. Competitive inhibitors of *Pseudomonas* elastase were equally potent in inhibiting the *L. pneumophila* protease. These findings indicate that the *L. pneumophila* protease and the *P. aeruginosa* elastase share a similar molecular mechanism of proteolysis. An invasive-ness-defective mutant of the fish pathogen *Vibrio anguillarum* has been isolated and shown to produce lower levels of a zinc metalloprotease than the wild-type bacterium (134).
N-terminal amino acid analysis demonstrated considerable homology between the Vibrio enzyme and the *P. aeruginosa* elastase and small but significant homology with the *L. pneumophila* protease. The soluble hemagglutinin/protease produced by *V. cholerae* is also a zinc metalloprotease which is structurally, functionally and immunologically related to the *P. aeruginosa* elastase (87). The family of bacterial zinc metalloproteases also includes proteases from several *Bacillus* species and *Serratia marcescens*, which have been shown to be structurally related to *P. aeruginosa* elastase and to share amino acid homology in the regions forming the enzymatic active sites and zinc-binding domains (87). The ubiquity and conservation of these similar proteases among both pathogenic and nonpathogenic bacterial species (and in the case of *V. cholerae* including both pathogenic and nonpathogenic serotypes) suggest that they may provide some common survival advantage which is not necessarily essential for virulence but which, in the case of pathogenic species, may promote it (87).

Szeto and Shuman (184) succeeded in isolating the structural gene for the zinc metalloprotease from an *L. pneumophila* genomic library. They termed the protease the major secretory protein (Msp), and they termed the structural gene *mspA*. In *E. coli* which contained plasmids with the *mspA* gene, Msp protein and casemase activity were found in the periplasmic space and cytoplasm. Transposon mutagenesis with TsN9 insertions in the cloned structural gene for Msp in *E. coli* yielded mutants which no longer expressed protease activity. One of these TsN9 insertions was transferred to the *L. pneumophila* chromosome by allelic exchange. *L. pneumophila* strains that contained the transposon insertion within the *mspA* gene failed to synthesize the exoprotease and failed to produce any hemolysis. However, the mutant strain was able to grow within and kill human macrophages (PMA-differentiated HL-60 cells) as well as the wild-type, isogenic strain was. The culture supernatants from HL-60 cells infected with the *mspA* mutant were examined for the presence of revertants which had regained increased levels of protease production, and none were detected.

Blander et al. (29) found that when guinea pigs were challenged with various doses of aerosolized Msp" and Msp" *L. pneumophila*, the protease-negative and -positive strains demonstrated equivalent 50% and 100% lethal doses. The Msp" mutant and Msp" isogenic parental strains multiplied in the lungs of challenged guinea pigs at comparable rates; both strains multiplied 4 logs over 72 h. They also produced comparable pathological lesions in infected guinea pigs. By SDS-PAGE and Western blot analysis, no reversion of the Msp" strain occurred following passage in the animals. On the basis of these observations, the cytotoxic exoprotease is not required for intracellular infection or multiplication nor for lethality in vivo. Furthermore, since there was no difference in the pathological findings of the lungs of guinea pigs infected with the protease-negative mutant strain, the protease does not appear to be necessary for the production of acute pneumonitis. The cytotoxic exoprotease might still be considered a potential factor that enhances virulence through the destruction of macrophages (114). It has been suggested that self-limited *Legionella* infections, such as Pontiac fever, may result from a proinflammatory process colonizing lung macrophages but failing to elicit the cytotoxic damage necessary for the development of the more acute disease (114). However, the work of Szeto and Shuman (184) clearly showed that a 1,000-fold decrease in caseinolytic activity does not decrease the cytotoxic effect of *L. pneumophila* in differentiated HL-60 cells. Surprisingly, these results make it likely that there must be some other moiety which is responsible for any in vivo cytopathic effect; the *Legionella* toxin would be the leading candidate for this phenomenon.

Even if the zinc metalloprotease is not a virulence factor for intracellular infection, it could have effects on specific host proteins of potential relevance to the pathogenesis of legionellosis. Conlan et al. (51) demonstrated that the tissue-destructive protease produced by *L. pneumophila* and released into the growth medium was capable of thoroughly inactivating the important blood proteinase inhibitor α₁-antitrypsin. Unlike most other proteases such as trypsin and chymotrypsin, which consume and irreversibly inactivate α₁-antitrypsin by forming 1:1 molar complexes with the protease inhibitor, as little as one molecule of the *L. pneumophila* protease is capable of completely inactivating 500 molecules of α₁-antitrypsin. The inactivation of α₁-antitrypsin by tissue-destructive protease appears to proceed by cleavage of a 5,000-Da polypeptide from the native, 57,500-Da parent α₁-antitrypsin molecule. The ability of the *L. pneumophila* protease to inactivate many times its own mass of α₁-antitrypsin, one of the major defenses against proteolytic attack, raises the possibility that this particular enzyme, as well as other proteases released by necrosis from both the host and phagocytized bacteria, has the potential to cause more pulmonary damage than if it was inhibited by α₁-antitrypsin. However, as already noted, there does not appear to be any difference in the lung abnormalities of guinea pigs infected with Msp" and Msp" strains (29).

The *Legionella* protease may also directly impair phagocytic and natural killer cell function. There is a preliminary report indicating that the purified *L. pneumophila* protease at concentrations that were not cytotoxic inhibited human neutrophil and monocyte chemotaxis toward various chemoattractants in a dose-dependent manner (160); any effects on phagocyte bactericidal functions were not addressed in this study. This suggests that production of the bacterial enzyme could result in impaired recruitment of phagocytic cells to the site of infection. Sahney et al. (177) prepared 10-fold-concentrated supernatants by ultrafiltration in a cell with a 10,000-Da cutoff from overnight cultures of a virulent *L. pneumophila* serogroup 1 strain and a protease-deficient mutant derived from an avirulent *L. pneumophila* serogroup 1 strain by ethyl methanesulfonate mutagenesis. The concentrated supernatant from the virulent strain inhibited spontaneous neutrophil chemotaxis and chemotaxis toward FMLP and O₂⁻ generation in response to zymosan-activated particles, FMA, A23187, and FMLP at concentrations that had no effect on cell viability. Heat-treated supernatant from the virulent strain had no inhibitory effect on O₂⁻ generation in response to any of the four stimuli. In contrast, the supernatant from the protease-negative mutant failed to inhibit neutrophil O₂⁻ response to zymosan-activated particles and PMA and only partially inhibited neutrophil response to A23187 and FMLP. Neutrophil spontaneous migration was unaffected by the culture supernatant from the mutant, whereas directed chemotaxis was partially inhibited. Since the inhibitory effects on neutrophil function were absent or smaller when the concentrated supernatant from the protease-deficient mutant strain was used, the conclusion that the protease was responsible for the effects of the supernatant from the virulent strain seems inescapable. However, the protease-deficient strain was derived from an avirulent parent strain, but the supernatant from it was tested in parallel with the supernatant from an unrelated virulent *L. pneumophila* serogroup 1 strain. This leaves open
the question of whether the avirulent parent, and the protease-deficient mutant derived therewith, lacked another exoprotectant(s) which is inhibitory for neutrophils. Culture supernatants might contain phospholipase, phosphatase, and a number of other heat-labile exoproteins in addition to protease; the low-molecular-weight toxin should have been eliminated in these experiments by the ultrafiltration step. As noted above, a Legionella phosphatase blocks neutrophil activation. Also, preliminary studies by this same group indicated that purified Legionella phospholipase inhibited neutrophil function (176). It therefore remains to be determined whether the protease adversely affects the bactericidal mechanisms of phagocytic cells.

Rechnitzer et al. (161) found that purified protease inhibited natural killer cell cytolytic activity in a concentration- and time-dependent fashion. The inhibitory effect was not reversed by either gamma interferon or interleukin-2. The protease appeared to act by inhibiting the binding of effector cells to target cells. In vivo, this action might reverse the previously described lysis of L. pneumophila-infected mononuclear cells by a natural killer subset population (25).

Before there was evidence indicating that the tissue damage caused by the legionellae cannot be attributed to the effects of the zinc metalloprotease, the possibility that antibodies to this enzyme would reduce the severity of the disease and thus offer a means of prophylaxis and therapy was considered (77). This approach has been explored for the acute bacterial pneumonias due to the extracellular bacteria S. marcescens and P. aeruginosa. Guinea pigs and mice vaccinated with purified S. marcescens protease developed anti-protease antibodies and were partially protected against a subsequent lethal challenge of the bacterium (124). Similarly, immunization of mink with a protease toxoid vaccine of P. aeruginosa resulted in the production of anti-protease antibodies and protection against pneumonia due to a lethal inoculum of bacteria (97). Blander and Horwitz (27) observed that guinea pigs infected with L. pneumophila develop cell-mediated immunity to the zinc metalloprotease. Guinea pigs immunized with a subcutaneous dose of purified Legionella protease developed strong humoral and cell-mediated immune responses to the enzyme (27). When these animals were challenged with a lethal aerosol dose L. pneumophila, they exhibited significant protective immunity, which was associated with the ability to limit Legionella multiplication in their lungs. When combined with the previous studies indicating that the protease is not a necessary virulence factor for intracellular infection or tissue destruction, these results suggest that immunization with the protease fosters immunity through the induction of specific cell-mediated immunity, rather than protection mediated by anti-protease antibody.

When guinea pigs were immunized with L. pneumophila serogroup 6 protease, protective immunity developed against challenge with L. pneumophila serogroup 1 organisms (28). However, as would be expected from the data indicating that the proteases of various species are not genetically identical, immunization with the protease from L. bozemanii provided significant protection against challenge with L. pneumophila serogroup 1, but less than that provided by immunization with L. pneumophila serogroup 1 protease. Surprisingly, when challenged with L. bozemanii, rather than L. pneumophila, guinea pigs immunized with purified L. pneumophila protease demonstrated protective immunity. Thus, the drawback of this approach to prophylaxis is that it only partial or no protection may be produced against species, other than L. pneumophila, which possess proteases genetically different from that of L. pneumophila. Nevertheless, if these results can be extended to humans, the purified protease appears to be a promising vaccine candidate for protection against the L. pneumophila serogroups, which cause the majority of cases.

**Enzymes that scavenge reduced-oxygen metabolites.** Host phagocytes produce microbicidal reduced-oxygen metabolites such as O$_2^-$ and H$_2$O$_2$ when they phagocytize bacteria. Enzymes which eliminate these toxic reduced forms of oxygen, or which block their production in the first place, might allow intracellular pathogens to escape killing. Pine et al. (155) analyzed 40 Legionella strains for the following reduced-oxygen scavenging enzymes: SOD, catalase, and peroxidase. SOD inactivates O$_2^-$ by converting it into O$_2$:

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Catalase converts H$_2$O$_2$ into H$_2$O and O$_2$ in the absence of any other substrate:

$$H_2O_2 \rightarrow O_2 + 2H_2O$$

A peroxidase uses H$_2$O$_2$ to oxidize some organic compound [R(OH)$_2$]$_2$:

$$HO--R--OH + H_2O_2 \rightarrow O=H--R=O + 2H_2O$$

One commonly used assay for peroxidase activity employs o-dianisidine as the reductant; the peroxidase-catalyzed oxidation of o-dianisidine by H$_2$O$_2$ to O$_2$ and H$_2$O yields a yellow product in the absence of an organic compound just as catalase does. In this case the peroxidase is said to express catalatic activity.

Pine et al. (155) found basically that whereas SOD was ubiquitous among the Legionella spp., catalase and peroxidase activities were not distributed uniformly among the 40 strains they analyzed. Two of the Legionella spp., L. pneumophila and L. gormanii, had no catalase and contained only a peroxidase which, in L. pneumophila, exhibited low catalytic activity. L. dumoffii appeared to have this same peroxidase plus a separate catalase. All the remaining species had a catalase only. Because none of these aforementioned enzymes have been isolated in pure form from any of the legionellae, final conclusions about the enzymatic activities responsible for the metabolism of H$_2$O$_2$ by live Legionella spp. or cell extracts derived must therefore be regarded as provisional. When Pine et al. (155) examined these enzymological data from the standpoint of potential relationships of these enzymes to the frequency with which the various species cause disease, they concluded that (i) there does not appear to be a direct association of the peroxidase with pathogenicity because the peroxidase appears equally distributed among all of the serogroups of L. pneumophila, and (ii) the distribution of SOD and catalase among the Legionella spp. does not support the view that these enzymes play a role in pathogenesis.

L. pneumophila appears to be highly sensitive in vitro to externally added H$_2$O$_2$ (96, 122, 123), to in vitro products of the myeloperoxidase (MPO)-H$_2$O$_2$-halide system (122, 123), and to products of the xanthine oxidase system (122, 123). The latter system generates O$_2^-$ and H$_2$O$_2$, and, in the presence of Fe$^{2+}$, OH$^-$ and possibly singlet oxygen. Since killing of L. pneumophila by products of the xanthine oxidase system in the presence of Fe$^{2+}$ was reversed by the addition of catalase or SOD and by the OH$^-$ scavenger mannitol, it appears that H$_2$O$_2$, O$_2^-$ and OH$^-$, or products derived from them are the bactericidal species (122, 123). Likewise, we (58) found that L. micdadei is as susceptible to killing by H$_2$O$_2$ in vitro as E. coli or S. aureus is. However,
both *L. pneumophilia* and *L. micdadei* were significantly more resistant to killing by hypochlorite, the major bactericidal product of the MPO-H₂O₂-halide system of neutrophils, than *E. coli*, *Klebsiella pneumonias*, or *S. aureus* were (58, 118). Whether the latter property is of biologic significance remains to be assessed.

There is evidence that catalase produced by *S. aureus* protects intraphagocytic bacteria by destroying H₂O₂ produced by neutrophils (127). It has also been determined that the facultative intracellular bacterial pathogen *Nocardia asteroides*, which is susceptible to killing by oxygen metabolites, is protected from oxidative killing mechanisms in vivo by the production of SOD (10). The nocardial SOD is secreted into the growth medium and is neutralized by treatment of the whole bacteria with anti-SOD antibody (10).

The reason why the legionellae are not protected by either SOD or catalase is presumably that both of these enzymes are located in the cytoplasm (96), suggesting that the targets for damage by peroxide may be located in or external to the cytoplasmic membrane.

Conversely, there is some evidence that legionellae may possess quantitatively less oxygen metabolite-scavenging enzymes than other gram-negative bacilli. Phagocytosis of *L. pneumophilia* by primate alveolar macrophages was associated with a respiratory burst indicated by localized reduction of Nitro Blue Tetrazolium around the ingested organism (109). However, when assayed quantitatively, ingestion of either virulent or avirulent *L. pneumophilia* by alveolar macrophages stimulated three- to fourfold less Nitro Blue Tetrazolium reduction than did the phagocytosis of *E. coli*, yet fewer *E. coli* cells were killed than *L. pneumophilia* cells. *L. pneumophilia* was more susceptible than the *E. coli* strain to the toxic effects of H₂O₂ and the oxygen metabolites generated by the xanthine oxidase system (123). A combination of SOD and catalase, but not either scavenger of oxygen metabolites alone, significantly reduced the killing of *L. pneumophilia* by alveolar macrophages. Mannitol also inhibited the killing of *Legionella* cells by alveolar macrophages, and its effect was significantly greater than that of SOD and catalase combined. These findings suggested that OH⁻ plays a role in the early killing of *Legionella* cells by alveolar macrophages as well as in vitro (109). The difference in susceptibility of *Legionella* spp. and *E. coli* to early killing by alveolar macrophages and by oxygen metabolites correlated with the significantly lower content of the oxygen metabolite scavengers catalase, glutathione peroxidase, glutathione reductase, and glutathione in *Legionella* spp. than in *E. coli* (125).

**Protein Kinases.** Protein kinases catalyze the phosphorylation of specific serine, threonine, or tyrosine residues of protein substrates. Evidence demonstrating the presence of multiple species of endogenous phosphorylated proteins and protein kinases in prokaryotes has been reported for *Salmonella typhimurium* (197) and *E. coli* (70, 126). For *E. coli*, it has been shown convincingly that NAD⁺:isocitrate dehydrogenase undergoes phosphorylation and dephosphorylation (144, 163). Interestingly, both reactions are catalyzed by a single bifunctional enzyme. Protein kinases have been described in protozoa (125), and we have extensively purified and partially characterized protein kinases that are present on the outer surface of *L. donovani* promastigotes (52).

The demonstration of this ectokinase activity, together with the finding of acid phosphatase activity on the outer surface of *L. donovani* (165, 166), led us to speculate that this obligate intracellular parasite may be capable of regulating the properties and function of host phagocytic cells by the phosphorylation and dephosphorylation of critical proteins. Therefore, we (173) examined the possibility that *Legionella* spp. have protein kinase activity in addition to phosphatase activity.

The high-speed supernatant from a sonicate of *L. micdadei* bacterial cells was applied to a QAE-Sephadex column (173). Approximately 70% of the protein kinase activity appeared in the QAE-Sephadex breakthrough fractions and was designated PK I. When the column was developed with an NaCl gradient, a single peak of protein kinase activity was eluted and designated PK II. Both PK I and PK II were further purified on a histone affinity column followed by high-performance gel filtration. The *Kₐ₅₀* values with mixed histones as the substrate were nearly identical for PK I (0.27 mM) and PK II (0.29 mM). Both protein kinases exhibited maximum activity at pH 6.8 to 7.0. PK II is cyclic nucleotide dependent, and PK II activity is stimulated by calmodulin, a Ca²⁺-calmodulin mixture, mixtures of Ca²⁺ and phosphatidylserine, or Ca²⁺ and PI, and a Ca²⁺-PI-diolein mixture. None of these agents markedly influenced the activity of PK I. All the experiments which follow concern the cyclic nucleotide-independent PK I, which hereafter will be referred to simply as the *L. micdadei* protein kinase. This bacterial kinase was purified to homogeneity by isoelectric focusing and chromatography on Sephadex G-150 (174). The purified protein kinase electrophoresed as a single band with a molecular weight of approximately 55.000 in SDS-containing gels.

To approach the question whether the bacterial protein kinase might affect host phagocytes, we determined whether neutrophils possess substrates for the enzyme (174). Neutrophil homogenates were centrifuged to produce cytosol and membrane fractions. Either the cytosol or solubilized membrane fraction was incubated with the purified protein kinase in the presence of [γ-³²P]ATP, and the [³²P]-labeled proteins were resolved by SDS-PAGE and visualized by autoradiography. The bacterial enzyme catalyzed the phosphorylation of 11.5-, 14-, 19-, 23-, 25-, and 38-kDa proteins present in the extract of neutrophil membranes and 11.5-, 13.5-, 25-, and 38-kDa proteins in the cytosol. Furthermore, when purified *Legionella* protein kinase was incubated with calf brain tubulin and [γ-³²P]ATP in the presence of Mn²⁺, extensive phosphorylation of tubulin occurred.

Surprisingly, when the purified *L. micdadei* kinase was incubated in the presence of [γ-³²P]ATP and PI, a linear, time-dependent incorporation of [³²P] into PIP was observed; the PIP was not further phosphorylated to PIP₂ (174). The *Kₐ₅₀* of the kinase for ATP was 1.5 mM. The PI kinase activity of the *L. micdadei* enzyme was optimal at pH 7.0, close to that observed when histone is used as the substrate (pH 7.2 [173]). Neutrophils which had been labeled with mio-[2-³²H]inositol were treated with the purified PI-tubulin kinase, and the labeled inositol lipids were extracted and separated. After a 4-h incubation with the bacterial kinase, 19 to 24% of the PI in the neutrophil plasma membrane had been phosphorylated to PIP, resulting in a 73 to 87% increase in the quantity of labeled PIP, while the level of PIP₂ did not change by more than 10% (174).

In summary, *L. micdadei* contains at least two protein kinases. One of these kinases has the capability of catalyzing, at the expense of ATP, the phosphorylation of not only tubulin (and histone) but also PI. The function and significance of the *Legionella* protein kinase are obscure. Although we have demonstrated that the enzyme is capable of phosphorylating PI and tubulin in vitro and PI in intact neutrophils, its physiologically relevant substrates remain to be established.
Although it seems unlikely, the possibility that there are endogenous substrates for the bacterial enzyme remains to be excluded. The presence of PI is rare in prokaryotic cells; it has been found in only the mycobacteria (193) and myxobacteria (36) and was not detected in Legionella spp. (75). Since prokaryotes do not contain tubulin, it would seem most likely that host phagocyte PI or tubulin or both are potential physiological substrates. The phosphorylation of either of these eukaryotic substrates might block phagocyte activation or function. For example, since phosphorylation of tubulin monomers inhibits assembly into microtubules (195, 196), the L. micdadei kinase might affect various antimicrobial activities mediated by tubulin, including motility, phagocytosis, and granule-membrane fusion. Also, all of the eukaryotic PI kinases that are closely associated with protein kinases have been found to phosphorylate PI at the D-3 position of inositol, rather than the usual D-4 position (38). If this is the case for the Legionella enzyme, the PI-3-P formed would not be in the pathway for the formation of PI-4,5-P₂, which is the progenitor for IP₃ production upon cell activation.

Legionella Cell Envelope

Hindahl and Iglewski (92) fractionated a whole-cell lysate of L. pneumophila Knoxville 1 into five membrane fractions by sucrose gradient centrifugation. Membranes were characterized by enzymatic, chemical, and SDS-PAGE analyses. Two forms of cytoplasmic membrane (CM-1, CM-2), a band of intermediate density (IM), and two forms of outer membrane (OM-1, OM-2) were detected. The CM-1 fraction was the purest form of cytoplasmic membrane, whereas fraction CM-2 was primarily cytoplasmic membrane associated with small amounts of peptidoglycan. The IM, CM-1, and OM-2 fractions were all enriched in peptidoglycan. The IM, OM-1, and OM-2 fractions were expected to be enriched in total hexose relative to CM-1 and CM-2 as a result of increased amounts of LPS associated with these fractions. However, the amount of carbohydrate and 2-keto-3-deoxyoctonic acid (KDO) was not appreciably greater in outer membrane than in cytoplasmic membrane. The high molar ratio of hexosamine to KDO in the membrane fractions suggested that the great majority of hexosamine detected was derived from peptidoglycan rather than LPS. Phosphatidylethanolamine and phosphatidylcholine were found to be the major phospholipids in the membrane fractions, confirming the unusual phospholipid composition of whole Legionella cells found by Finnerty et al. (75). Phosphatidylcholine is seldom observed in bacterial cells. The major outer membrane proteins had molecular sizes of 29,000 and 33,000 Da and were both modified by heating. The 29,000-Da protein was tightly associated with the peptidoglycan and was equally distributed in the IM, OM-1, and OM-2 fractions.

Gabay and Horwitz (82) isolated cell envelopes by treating whole L. pneumophila Philadelphia 1 cells with lysozyme and EDTA to convert them to spheroplasts and then lysing the spheroplasts osmotically or sonically. They resolved the cell envelopes into two membrane fractions by isopycnic centrifugation. NADH oxidase was localized to the fraction of buoyant density 1.145, which was designated cytoplasmic membrane, and LPS was found in the fraction of density 1.222, which was designated outer membrane. In contrast to the results of Hindahl and Iglewski (92), KDO was localized to the outer membrane fraction. SDS-PAGE revealed that the L. pneumophila outer membrane contains a single major protein species migrating at 28,000 Da; this is the MOMP of the bacterium. The cytoplasmic membrane also contains a single major protein species migrating at 65,000 Da. Surface iodination of the bacteria and agglutination and immunofluorescence studies with rabbit antibody produced against the purified MOMP revealed that this protein is exposed at the cell surface. These workers isolated LPS from L. pneumophila membranes by SDS-EDTA treatment. The pattern obtained by subjecting the LPS to SDS-PAGE and staining the gel with silver nitrate indicated that L. pneumophila LPS might be atypical. Two other lines of evidence suggest that Legionella LPS is not typical of the LPS of most gram-negative bacilli. First, lipid analysis has shown that Legionella LPS has an unusual fatty acid composition consisting predominantly of branched-chain fatty acids; hydroxy-fatty acids, which are generally associated with classical endotoxins as structural components of lipid A, are absent (75, 136, 204). Second, Legionella LPS lacks the classical endotoxicity associated with LPS in that it induces a weak pyrogenicity response in rabbits and has relatively low toxicity for mice (204).

Gabay and Horwitz (82) also studied serologic responses of patients to cell envelope components of L. pneumophila Philadelphia 1. Serum samples from patients with evidence of infection with L. pneumophila serogroup 1 contained large amounts of antibody to this strain. Few of these antibodies recognized the MOMP of L. pneumophila. In contrast, more than 98% of the antibodies were directed against the LPS. These results and the work of others (46, 48, 145, 149) have established that LPS is the dominant serogroup-specific antigen and the major antigen responsible for the reactivity of patient serum in the indirect fluorescent-antibody assay. Given the similarity of the patterns of LPS from virulent and avirulent strains of the same serogroup on SDS-PAGE, it seems unlikely that LPS is a virulence determinant for L. pneumophila (48).

Cell Surface Legionella Proteins

About 10% of the total protein mass of Legionella spp. is localized to the outer membrane of the organism. Although outer membrane proteins are of potential use for purposes of identification, they are also of interest because they may play a role in pathogenesis and because the outer membrane antigens come into primary contact with the inflammatory cells and immune system of the host.

MOMP. In the preliminary study of the outer membrane proteins of L. pneumophila by Ehret and Ruckdeschel (68), it was reported that all 10 serogroups examined possessed a 29-kDa MOMP. The MOMP, which is associated with peptidoglycan, has been isolated by several groups of investigators (35, 81, 93). Gabay et al. (81) purified MOMP from L. pneumophila by a three-step procedure that involved extraction of bacterial cells with calcium and detergent followed by ion-exchange and molecular-sieve chromatography. Preparation of MOMP monomers required the reducing agent 2-mercaptoethanol, indicating that the protein aggregates through the formation of interchain disulfide bridges (35, 81). Several researchers have demonstrated that the MOMP of L. pneumophila has a molecular mass between 24 and 29 kDa (35, 68, 81, 93). It is generally agreed that all Legionella species, with the possible exception of serogroup 1 strains of L. bozemanii (35), possess a disulfide-cross-linked MOMP of similar molecular weight, although Hindahl and Iglewski (93) did not observe MOMP in species other than L. pneumophila. Gabay et al. (81) demonstrated that the MOMP of L. pneumophila is a cation-selective porin. Porins are a class of
bacterial proteins that are capable of inserting themselves into membranes, including those of host cells, and opening up channels through which ions can pass. Horwitz (103) has speculated that one mechanism by which *L. pneumophila* Philadelphia 1 might inhibit acidification of the monocyte phagosome is by the insertion of a proton ionophore, namely, the cation-selective MOMP porin, into the monocyte membrane. Although as yet unproven, a 95-kDa complex may represent the porin consisting of four disulfide-cross-linked subunits of the MOMP, and this complex is bound to peptidoglycan in situ (35).

The finding that human monocyte complement receptors (CR), CR1 and CR3, and complement component C3 in serum mediate the phagocytosis of *L. pneumophila* (152) prompted Horwitz to investigate the identity of the C3 acceptor molecule on the bacterial surface. Bellinger-Kawahara and Horwitz (14) showed that C3 was fixed to *L. pneumophila* which were opsonized in fresh, nonimmune serum, and C3 fixation took place via the alternate pathway of complement activation. Immunoblot analysis of opsonized *L. pneumophila* indicated that C3 was fixed exclusively to the MOMP; C3 did not fix to LPS on these blots. Furthermore, when purified MOMP was incorporated into liposomes, the MOMP-liposomes avidly fixed C3 and were efficiently phagocytized by monocytes. Since the MOMP is present in virulent and avirulent *Legionella* strains (35), its role in pathogenesis is unclear. However, it is a substance deserving of further study. C3 is the major opsonin for the phagocytosis of the legionellae (152, 180), so that if the MOMP were modified it might lead to decreased internalization of the bacterium and subsequent failure to multiply. A preliminary report indicates that a strain of *L. pneumophila* rendered avirulent by prolonged agar passage showed a greatly diminished expression of the MOMP (167). When a 750-bp DNA fragment from the virulent *L. pneumophila* strain was transformed into *E. coli*, the resulting clones expressed the *Legionella* MOMP and had increased virulence in chicken embryos compared with the parent *E. coli* strain. Thus, reduced expression of MOMP could be the mechanism behind the attenuation of some strains which do not appear to be phagocytized as effectively as their virulent counterparts (see below).

**Mip protein.** Instead of using biochemical analyses to identify components of *Legionella* spp. that may be virulence factors, Cianciotto, Eisenstein, Engleberg, and their coworkers initiated a genetic analysis of intracellular parasitism. To identify targets for mutagenesis, they cloned genes encoding surface protein antigens and chose the gene encoding a 24-kDa *L. pneumophila*-specific antigen that induced strong antigenic reactivity in rabbits immunized with killed bacterial cells (153) for further investigation. This protein is distinct from the MOMP of *L. pneumophila*. The gene encoding the 24-kDa protein was deleted by site-specific mutagenesis involving allelic exchange with specific loss of 24-kDa antigen expression (45). Compared with the isogenic parent, the mutant was significantly impaired in its ability to infect transformed human U937 cells and human alveolar macrophages. U937 cells are monoblastic in continuous culture, but lose their proliferative capacity and develop features of tissue macrophages after treatment with inducers such as PMA. An 80-fold-greater inoculum of the mutant strain than of the parent strain was required to infect U937 cells. The mutant strain regained full infectivity following reintroduction of the cloned 24-kDa protein gene. Although the titer of the mutant strain was lower immediately following infection, the growth rates of the mutant and parent strains in U937 cells were similar over the next 40 h, indicating that the mutant strain was less able to initiate infection, but remained capable of intracellular multiplication. When opsonized with specific antibody, the mutant strain still demonstrated reduced infectivity despite equivalent cell association, indicating that the mutant did not lack a ligand required for macrophage attachment. Thus, the 24-kDa protein appears to be required either for optimal internalization of *L. pneumophila* by macrophages or for resistance to the bactericidal mechanisms that are operative immediately following phagocytosis. These workers have designated the gene encoding the 24-kDa protein as *mip* (for macrophage infectivity potentiator) and the gene product as Mip.

To substantiate the importance of Mip in the pathogenesis of *L. pneumophila* infection, the lethality of the parent and mutant strains for guinea pigs inoculated intratracheally was examined (44). The *mip* mutant killed fewer animals and produced illness later after inoculation than did the isogenic parent strain. Although the mutant strain was capable of causing illness and death at a high dose, it was significantly less virulent than the parent strain. Furthermore, bacteria derived from the mutant strain into which the *mip* gene had been reintroduced were as virulent for guinea pigs as the parent strain was. The titer of the *mip* mutant in the lungs of animals 48 h after infection was lower than that of the parent strain, but the difference was not statistically significant; the titers of lung homogenates from animals infected with the reconstituted strain were intermediate between those of the other two. Interestingly, the spleen titers of animals infected with the *mip* mutant were 23- and 20-fold lower, respectively, than those of animals receiving either the parent or reconstituted *mip*+ strain; these are highly significant differences. There was no discernible difference between the *mip* mutant and parent strains in proteolytic and phosphatase activities, complement fixation, serum resistance, or LPS structure. A derivative of the *mip*+ strain which had been rendered avirulent by multiple passages on agar still expressed a 24-kDa Mip protein. Therefore, a different mechanism appears to be operative in legionellae rendered avirulent by agar passage, suggesting that multiple virulence factors may be operative in wild-type bacteria.

The DNA sequence encoding the Mip protein has been established (71). The inferred polypeptide is a potent polycation with an estimated pi of 9.8. Although the mechanism by which the Mip protein promotes the invasion of host phagocytes is unknown, the polycationic character of this surface molecule raises some logical possibilities (71). Polycations are known to induce the phagocytosis of inert particles, and increased phagocytosis of legionellae might promote their multiplication. Alternatively, following bacterial uptake, this protein could act as a cationic lysosomotopic agent to inhibit phagosome-lysosome fusion and acidification. However, if inhibition of phagosome-lysosome fusion were the mechanism of Mip action, it is unclear why only the Philadelphia 1 strain of *L. pneumophila* has been observed to inhibit fusion, since the *mip* gene is conserved throughout the species (44). It is intriguing that the carboxyterminal 114 residues of the Mip protein share 39% identity with a protein isolated from *Neurospora crassa* which binds the new immunosuppressive drug, FK 506 (188). The *Neurospora* protein exhibits peptidyl-prolyl cis-trans isomerase activity. Prolyl isomerases are thought to catalyze a step in protein folding. It is not known whether Mip acts as a prolyl isomerase or binds FK 506.

A large panel of *Legionella* strains were examined by
Southern hybridization and immunoblot analyses for the presence and expression of the mip sequence (42). Strains representing all 14 serogroups of L. pneumophila contained a mip gene and expressed a 24-kDa Mip protein. Although the DNAs for 29 other Legionella species did not hybridize with the mip DNA probe under high-stringency conditions, they did so at reduced stringency. Furthermore, these species each expressed a 24- to 31-kDa protein that reacted with specific Mip antiserum. It was also shown that the cloned mip-like gene from L. micdadei encoded the cross-reactive protein. Thus, although the mip gene is conserved in, and is specific to, L. pneumophila (44), mip-like genes are present throughout the genus. Whether the various Mip proteins are functionally similar remains to be determined. If the Mip-related proteins of the other Legionella species are functionally dissimilar to the Mip protein of L. pneumophila itself, it may help to explain the apparent greater virulence of the latter species. The mip gene family appears to be limited to the genus Legionella. DNAs from a number of gram-negative bacteria, including Yersinia pestis, Neisseria gonorrhoeae, and Mycoplasma pneumoniae, did not hybridize with probes known to contain mip (42). Likewise, Mip-related proteins were not detected in other gram-negative bacilli or Mycoplasma spp. (72).

Heat shock protein. All Legionella species and serogroups that have been examined express a 58- to 60-kDa protein which contains a genus-specific epitope recognized by monoclonal antibodies, as well as epitopes which are cross-reactive with many species of gram-negative bacteria (94, 178). The gene coding for the L. pneumophila 58- to 60-kDa common antigen has been cloned in E. coli, and its complete nucleotide sequence has been determined (94, 95, 178). The protein is preferentially synthesized upon heat shock and is serologically cross-reactive and demonstrates considerable homology with other heat shock proteins including the GroEL protein of E. coli, the Mycobacterium tuberculosis 65-kDa antigen, and Coxella burnetii HtbB. The protein is highly immunogenic and is the predominant Legionella protein reactive with human convalescent-phase serum from patients with confirmed cases of legionellosis. It has been shown that the purified L. pneumophila 60-kDa protein is antigenic for human T lymphocytes (95). Furthermore, indirect fluorescent-antibody studies indicated that this protein may be located in the periplasmic space or expressed on the surface of intracellular bacteria. Thus, although no evidence has accrued to establish the Legionella heat shock protein as a virulence factor, it seems likely that this protein would be induced by the unfavorable conditions in the phagosome. What effects the release of this protein might have on phagocyte function which could lead to the abrogation of cellular bactericidal mechanisms remain to be determined.

AVIRULENT LEGIONELLA MUTANTS

Mutants, particularly avirulent mutants, can be powerful tools for analyzing virulence determinants. One strategy for elucidating the virulence determinants of the legionellae is to isolate and characterize mutants that are unable to multiply in monocytes or monocytic cell lines (71) or to survive in neutrophils infected in vitro. In this manner it might be possible to identify mutants blocked at each of the hypothetical steps required for successful intracellular parasitism: uptake by or entry into the cell, resistance to cellular bactericidal mechanisms, and ability to multiply within the phagosome. Two strategies have been used for the production of avirulent Legionella mutants. Some investigators have examined spontaneously occurring avirulent mutants, whereas others have genetically engineered the bacteria and then screened for possible decreased virulence. As just described for definition of the Mip protein, the latter approach has the advantage that a fully virulent, isogenic parent strain is available for comparative purposes.

When passaged on suboptimal artificial media, L. pneumophila and L. micdadei spontaneously convert to mutant forms which are avirulent for guinea pigs. The medium commonly used for this purpose is supplemented Mueller-Hinton agar. The casein hydrolysate component of supplemented Mueller-Hington medium has been shown to be inhibitory to the growth of virulent, but not avirulent, bacterial cells, and the inhibitory component of the hydrolysate was identified as NaCl (39). The importance of intracellular growth to virulence expression by Legionella cells is underscored by the inability of avirulent strains produced by agar passage to replicate intracellularly. Kishimoto et al. (116) were the first to find that virulent strains of L. pneumophila serogroup 1 survived and proliferated in guinea pig peritoneal macrophages, whereas an avirulent strain derived from Philadelphia 1 (passage history unknown) was killed by normal macrophages. The phagocytosis of the virulent and avirulent strains appeared to be equivalent. Jacobs et al. (109) undertook extensive studies of the interaction of virulent and avirulent L. pneumophila serogroup 1 strains with pigtail monkey alveolar macrophages; the avirulent strain was obtained by repeated passage of originally virulent bacteria on modified Muller-Hinton agar. Primate alveolar macrophages phagocytized comparable numbers of virulent and avirulent bacteria in the absence of specific antibody. The majority of the bacteria which were associated with alveolar macrophages were killed, and virulent and avirulent Legionella cells were equally susceptible to this early killing. The virulent L. pneumophila cells that survived intracellularly increased in number by over 2 logs during the 96 h after infection. In contrast, the avirulent bacteria multiplied much more slowly over the same period. Neither the mechanism of the survival of a fraction of both virulent and avirulent Legionella cells following phagocytosis nor the mechanism for the subsequent unrestrained multiplication of the virulent bacteria is known. Similarly, virulent L. pneumophila serogroup 1 cells multiplied in the human macrophage-like U937 cell line and produced cytotoxic effect during intracellular growth, whereas the avirulent mutant obtained after more than 200 passages on suboptimal medium did not (45).

Horwitz (102) obtained 44 mutant clones of L. pneumophila Philadelphia 1 by batch passaging wild-type bacteria nine times on supplemented Mueller-Hinton agar. Then 44 colonies were selected, and each was passaged individually an additional three times. None of the 44 mutant clones multiplied in human monocytes in experiments in which wild-type L. pneumophila multiplied 2.5 to 4.5 logs. Like the wild type, the avirulent mutants were resistant to the bactericidal effects of complement in the presence or absence of high-titer antibody. Both mutant and wild-type bacteria bound to and were ingested by monocytes, and both entered by coiling phagocytosis. The wild type formed the distinctive ribosome-lined phagosome, inhibited phagosome-lysosome fusion, and multiplied intracellularly. The avirulent mutant did not form the distinctive phagosome or inhibit phagosome-lysosome fusion; it survived intracellularly but did not replicate in the phagosome. Essentially the same results were obtained by using the promyelocytic cell line, HL-60, after its differentiation into macrophage-like cells (129).

All of the preceding studies indicate that there is no quantitative difference in the uptake of virulent and avirulent
L. pneumophila strains by phagocytic cells. In contrast, Dreyfus et al. (66), using an assay in which the extracellular bacteria are killed with an antibiotic, found that L. pneumophila serogroup 1 strains rendered avirulent by passage on agar entered HeLa cells approximately 1,000-fold less efficiently than did virulent strains. When radiolabeled bacteria were used, no difference was found in the numbers of virulent and avirulent bacteria which were associated with the HeLa cell monolayers after 1 h of incubation. These findings may apply only to nonprofessional phagocytes, or the divergent results may be due to the different methods used to enumerate intracellular bacteria. Alternatively, the various strains derived by prolonged agar passage may differ in the particular virulence mechanism which has been altered.

One possible differentiation between virulent and avirulent legionellae was suggested by prior work indicating that avirulent strains of Salmonella typhi and S. aureus stimulated oxygen consumption by neutrophils whereas a virulent strain of Salmonella typhi did not (134). Indeed, there was a preliminary report indicating that a virulent strain of L. pneumophila stimulated significantly less O$_2^-$ consumption and chemiluminescence by neutrophils than an avirulent strain did (192). Immune human serum was used as the opsonin in these studies, and no qualitative difference in bacterial uptake by the neutrophils was observed. Unfortunately, the method by which the avirulent strain was derived was not reported. On the other hand, we (60, 61) found that virulent L. micdadei stimulated the metabolic burst of neutrophils and monocytes during phagocytosis to the same extent as S. aureus and E. coli.

Summersgill et al. (182) examined four strains of L. pneumophila serogroup 1 and their avirulent variants (created by multiple passages on agar) for their effect on neutrophil function, as determined by measurement of peak chemiluminescence and O$_2^-$ production during phagocytosis. When neutrophils were exposed to either virulent or avirulent strains of L. pneumophila in the presence of normal serum, their O$_2^-$ production and chemiluminescence were markedly lower than those observed with E. coli. This observation may be explained by the fact that both specific antibody and complement are required for efficient phagocytosis of L. pneumophila by neutrophils (106); a reduced uptake in the absence of specific antibody would be expected to result in less stimulation of oxidative metabolism. However, the reduction in neutrophil oxidative function was more pronounced with the virulent member of each pair of strains in both assays. To determine whether the difference in reduced neutrophil function was due to C3 binding, the four pairs of bacteria were incubated in normal human serum and examined by quantitative immunofluorescence. Both virulent and avirulent legionellae bound less C3 than E. coli did, but the relative quantities of C3 bound did not match the relative reduction in oxidative function produced by each strain. Three of the four virulent bacteria bound less C3 than the avirulent member of the pair did. These workers concluded that some of the depressed neutrophil function following exposure to virulent L. pneumophila may be related to reduced C3 binding, although other virulence-associated factors may also be involved. The relationship of virulence and opsonization was supported by Plouffe et al. (156), who studied two subtypes of L. pneumophila serogroup 1 isolated at one medical center. The attack rate for one strain was 10-fold higher than for the other. The clinically less virulent strain was killed by fresh serum and fixed qualitatively more complement, whereas little killing of the more virulent strain occurred in fresh serum and it fixed less complement.

Summersgill et al. (183) then examined the interactions of these same four virulent-avirulent pairs of L. pneumophila with human peripheral blood monocytes. All of the L. pneumophila strains elicited less oxidative response by monocytes in the presence of normal serum, as measured by both O$_2^-$ and H$_2$O$_2$ production, than did E. coli. Also, the avirulent member of each pair of bacteria evoked more oxidative response than did the virulent strain. All of the virulent strains were capable of multiplying in monocytes to a high titer over a period of 3 days. The avirulent strains failed to multiply, and the titers fell to undetectable levels after 24 h of incubation. To assess the effects of complement fixation by each strain, phagocytic indices were determined under various conditions. In the presence of autologous donor serum, all L. pneumophila strains had phagocytic indices markedly lower than that of E. coli. There were no differences between the phagocytic index of the virulent and avirulent member of each pair. When heat-inactivated serum was used, all L. pneumophila phagocytic indices fell markedly, as did that for E. coli, and the indices were restored by the addition of human complement to heat-inactivated serum. Therefore, although the importance of complement in the adherence and uptake of L. pneumophila was confirmed, the binding of complement did not provide an explanation of the difference between virulent and avirulent bacteria in eliciting an oxidative response or multiplying in monocytes.

There is conflicting evidence regarding whether avirulent Legionella strains are more susceptible to killing by oxygen metabolites than are virulent strains (111, 122, 123). Locksley et al. (123) and Lochner et al. (122) reported that virulent and avirulent strains of L. pneumophila were equally susceptible to the antimicrobial species generated by the xanthine oxidase reaction and the MPO-H$_2$O$_2$-halide system. In contrast, Jepras and Fitzgeorge (111) found that two virulent L. pneumophila serogroup 1 strains resisted the bactericidal activity generated by the xanthine oxidase system while two avirulent strains did not. In addition, the catalase activity of the strains was directly correlated with their virulence and resistance to H$_2$O$_2$ and the toxic oxygen metabolites generated by the xanthine oxidase system. All four strains of L. pneumophila were equally susceptible to MPO-mediated killing. These results would help explain why the legionellae can survive and multiply in mononuclear phagocytes but are killed, albeit inefficiently, by neutrophils. It is not clear why different results were obtained in the three studies. The earlier workers (122, 123) used strains which had their virulence defined by intraperitoneal infection of guinea pigs and lethality for embryonated eggs, whereas Jepras and Fitzgeorge (111) defined virulence by the dose required to kill guinea pigs inoculated by aerosol. Although the latter is a more relevant definition of virulence, it may still be that avirulent strains derived in the laboratory by agar passage differ in the particular virulence mechanism(s) which is altered.

Mintz et al. (135) isolated several independent thymidine-requiring auxotrophs of L. pneumophila serogroup 1. The thymidine auxotrophs exhibited a marked decrease in viability when they were deprived of thymidine, and they were incapable of intracellular survival or multiplication in peripheral blood monocytes. In contrast, both the wild-type strain and prototrophic revertants were capable of multiplication in monocytes. It was not clear whether the thymidine auxotrophs died intracellularly directly as a result of thymidine deprivation or because they were unable to produce a...
passage in different laboratories necessarily have a defect in the same virulence mechanism. The phagocytosis of these agar-passed mutants is decreased in some cases, whereas for other attenuated strains uptake is normal. The mechanism of reduced uptake may be decreased complement fixation, perhaps owing to decreased expression of the MOMP (167), in some, but not necessarily all, instances. Avirulent mutants which are ingested normally presumably have a defect in their ability to withstand or nullify cellular bactericidal mechanisms. However, the particular mechanism has not been defined for any avirulent mutant, so it is not possible to link the work done on these mutants with the effects of any defined antiphagocyte moiety. Interpretation of these results is further complicated by the fact that mutants derived by passing a virulent strain on artificial medium may contain multiple mutations (45).

CONCLUSIONS AND FUTURE RESEARCH

The systematic search for Legionella virulence factors which promote survival within phagocytes can proceed along one of two routes. In the first approach individual virulence factors can be isolated and purified, utilizing some model system to demonstrate virulence. Once purified, the biochemical or molecular mechanism of the action of the factor can be pursued: if this is consistent with the functional activity of the factor, it adds credence to the importance of the moiety in pathogenesis. Virulence factors identified by this route can be classified as sufficient to promote virulence in the particular model system used. The second systematic route to the identification of virulence factors is to use molecular biological methods to create mutants which are isogenic with the parent strain in all respects except the production of a single gene product and to show that the mutant is avirulent in some model system in which the parent is virulent. It would appear that this plan would have to be used to identify moieties which are structural components of the bacterium rather than exproducts. This scheme would seem the only one whereby necessary, as opposed to sufficient, virulence factors may be identified. However, the data obtained may not always be clear-cut. The second approach was the one used in identifying the Mip protein as a virulence factor, but the Mip mutant, although attenuated, was not totally avirulent in the model systems studied. The limitation of both schemes is that the acceptance of the moiety as a virulence factor depends entirely on the relevance of the model system for virulence which was used and how faithfully the system represents the events which take place in nature.

Although all investigations of Legionella virulence have not been as systematic as the schema just described, the evidence that a particular moiety is important in the ability of the legionellae to invade and persist in host phagocytic cells takes one of three forms: functional, biochemical, or genetic. The evidence that the Legionella toxin is a virulence factor is entirely functional in that partially purified toxin adversely affects neutrophil activation. The biochemical basis for toxin action cannot be addressed until this moiety is purified by homogeneity and identified. In addition to functional effects on neutrophils, a biochemical mechanism of action has been established for the phosphatase. However, as yet, other legionellae have not been examined to determine whether they contain the same active phosphatase which is present in L. micdadei. It has not been rigorously determined whether the phosphatase is secreted by Legionella spp. or is found in the periplasmic space. nor is it known how the phosphatase
reaches its substrates in the phagocytic cell. Also, the basic work required to engineer a phosphatase-negative mutant and determine whether it is avirulent has not been done.

At the other end of this spectrum is the Mip protein. The gene encoding this Legionella cell surface 24-kDa protein has been cloned, and an isogenic mutant lacking a functional gene has been constructed. Compared with the parent strain, the mutant was impaired in its ability to infect macrophage cell lines and produce disease in guinea pigs. The function of the Mip protein is unknown, but it appears to be involved with the initiation of intracellular infection: multiplication of the mutant lacking Mip after being internalized was apparently normal. The possibility remains that these properties are not functions of Mip itself. Mip is probably a prevalent protein in the outer cell membrane. The absence of Mip could so alter the structure of the outer membrane that some other component required for invasion or intracellular survival is altered or the overall surface is so distorted that ingestion is impaired. It remains to be determined whether the Mip protein is a virulence factor only for L. pneumophila or is operative throughout the genus. Either the function of Mip must be elucidated and the Mip-like proteins of the other species shown to have similar actions, or genetically engineered legionellae of other species lacking their Mip-like proteins would have to be shown to be attenuated. It should also be noted that, although Mip appears to be necessary for optimal cellular invasion, the mip mutant did multiply in macrophages and produced illness in guinea pigs. That L. pneumophila mip mutants are not totally avirulent indicates that additional factors are involved in virulence. In addition, all putative virulence factors other than the toxin are heat labile. Since it has been shown that following the phagocytosis of heat-killed L. micdadei the activation of neutrophils and monocytes is severely impaired, heat-labile factors may play only secondary or ancillary roles to that of the toxin in regard to defeating phagocyte bactericidal functions.

In addition to the toxin, phagatase, and Mip protein, there are other putative virulence factors which may involved in the intracellular survival and multiplication of legionellae. The demonstration of protein kinase activity in L. micdadei together with phosphatase activity indicates that the bacterium may possess the capability of regulating the properties and function of host cell proteins and lipids through phosphorylation and dephosphorylation reactions. Although it was shown that the L. micdadei enzyme phosphatases PI in the plasma membrane of intact neutrophils, there is no evidence that the purified protein kinase adversely affects phagocyte function. It also has not been determined whether other Legionella species possess a PI-protein kinase, where the kinase is located in the bacterial cell or if the bacterial kinase phosphorylates PI or other cellular substrates during infection. The possibility that the Legionella phospholipase C is a virulence factor is entirely theoretical. Although a number of mechanisms whereby a phospholipase C might contribute to intracellular parasitism can be put forward, there are no functional or biochemical data in this regard for the purified protein.

The zinc metalloprotease secreted by Legionella spp. is cytotoxic and hemolytic and induces pulmonary lesions which resemble those found in infection. However, despite these indications that the extracellular protease might be a virulence factor, there is now strong evidence that the protease is not necessary for cellular invasion and growth of the legionellae. Not only is the protease produced by avirulent legionellae, but also a genetically engineered, protease-negative strain is fully virulent. On the other hand, guinea pigs immunized with the purified L. pneumophila protease are resistant to challenge with an otherwise lethal quantity of L. pneumophila cells. Although the protease turns out not to be a virulence factor, the research effort expended on this moiety has yielded a very promising immunogenic agent. In fact, one generalization that can be made is that there may be little relationship between the role of a particular moiety as a virulence factor, defined as promoting invasiveness and intracellular survival or multiplication, and the utility of the moiety in producing protective immunity. Not only does the purified protease engender protection, but also guinea pigs immunized by aerosol exposure to L. pneumophila rendered avirulent by multiple agar passage are protected against an otherwise lethal aerosol challenge of wild-type bacteria (26). Apparently, the common denominator for the protective efficacy is that immunization with either the protease or avirulent bacteria invokes strong cell-mediated immune reactions (26, 27). Cell-mediated immune responses which are effective in eliminating extracellular bacteria or killing Legionella-infected cells are then redirected to challenge with virulent bacteria. This scheme does not exclude the possibility that a necessary virulence factor will be found, immunization with which would protect by preventing the infection of host phagocytes in the first place. For example, Hedlund (89) demonstrated that inoculation of AKR/J mice with crude toxin preparations from either L. pneumophila or L. micdadei protected against a lethal dose of either crude toxin or viable bacteria from the same or the other species. These promising experiments have not been followed up because it has not yet been possible to purify and characterize the Legionella toxin.

The foregoing considerations indicate that the capacity of the legionellae to survive and multiply intracellularly is multifactorial. This is not surprising since there are at least four attributes which Legionella spp. must possess to be a successful intracellular pathogen. First, the bacteria must adhere or bind to the cell and, second, they must be phagocytized in order to gain access to the favored site for multiplication. In very few studies has any attempt been made to distinguish attachment from ingestion. However, it appears that some avirulent strains obtained by prolonged agar passage fix complement like wild-type Legionella strains and are ingested normally by phagocytes, whereas other similarly derived avirulent mutants are not taken up by phagocytic cells. Third, the legionellae must either inhibit phagosome-lysosome fusion and/or oxidative metabolism or resist killing by bactericidal oxygen species and phagosomal contents, in order to survive immediately after ingestion. Finally, a virulent bacterium must be capable of multiplication within the cell, including, for example, having the capacity to obtain the appropriate nutrients within the phagosome. The inability of thymidine-requiring mutants to grow intracellularly may be an example of avirulence based on inability to acquire a necessary nutrient. Loss of the ability to carry out any one of these steps may cause the bacterium to be attenuated to some extent, but perhaps not totally. Therefore, it is probable that more than one factor is necessary for the virulent phenotype and unlikely that a single entity will be found which is, of itself, sufficient to explain the intracellular multiplication of Legionella cells. This conceptualization may explain why multiple putative virulence factors are being discovered, some of which appear to promote entry into the cell and others which block various antibacterial properties of phagocytes. If there is redundancy on the part of the legionellae, there may be an even greater number of virulence factors. This does appear
to be the case since at least two *Legionella* moieties (toxin and phosphatase) have similar inhibiting effects on neutrophil activation.

As is obvious from the data on the cytotoxicity and protein synthesis inhibition engendered by the *legionellae*, as well as the similar effects of various moieties on phagocyte functions, a primary future task is to determine which bacterial factor(s) is responsible for each of the defects in phagocyte activation and bactericidal activity produced by *Legionella* spp. As the number of possible virulence factors grows, determining the role of each in pathogenesis at the cellular level will depend on the development of mechanistic data. At first, this data may be descriptive; e.g., a particular factor inhibits phagocyte oxygen metabolism or phagosome-lysosome fusion. Eventually, the molecular or biochemical mechanism of action should be determined; to date this has been accomplished only for the *Legionella* phosphatase. As the ultimate test, the techniques of molecular biology should be applied to each putative virulence factor. It should be demonstrated that a genetically engineered bacterium which lacks the virulence factor is less pathogenic in a relevant cellular or animal model than the isogenic parent strain.

The elucidation of the pathogenetic mechanisms by which the *legionellae* evade host phagocyte bactericidal properties during the early stages of *Legionella* infection may be expected to yield two more immediate practical benefits in the therapy and prevention of legionellosis. First, the limits of conventional antimicrobial therapy may have been reached now that it is understood that inhibitory or bactericidal antimicrobial agents which penetrate phagocytes must be used (65, 150). Further therapeutic success may be possible only by using immunotherapeutic modalities based on a knowledge of pathogenesis at the molecular level. For example, if the basis of the failure of neutrophils to kill *legionellae* is due to a *Legionella*-induced block in cell activation, therapy with a specific cytokine, such as tumor necrosis factor, might reverse the inhibition (23, 24). Second, it may be possible to develop an efficacious vaccine that functions by counteracting one or more of the intracellular virulence mechanisms rather than by stimulating cell-medi-ated immunity. The more that is known about pathogenesis at the cellular and molecular level, the more likely the ability to specifically attack the weakest link in the chain of virulence determinants.

ACKNOWLEDGMENTS

Work in our laboratory was supported in part by Public Health Service grant R01 AI17047 from the National Institutes of Health. We thank our coinvestigator, Gerald R. Donowitz, for reviewing the manuscript and for his generous permission to include unpublished data from his laboratory.

ADDENDUM

After it was demonstrated that other *Legionella* species possess analogs of the *L. pneumophila* Mip protein (42), the *L. micdadei* mip gene was cloned and expressed in *E. coli* (5a). DNA sequence analysis of the *L. micdadei* mip gene disclosed 71% homology with the *L. pneumophila* gene. The predicted secondary structures of the two Mip proteins are quite similar. An extended alpha helix from residues 55 to 120 of *L. micdadei* Mip protein could represent an elongated structure projecting from the surface of the bacterium, as previously suggested (71). A similar, extended alpha-helical structure was identified in the corresponding proteins of *L. pneumophila* and *Chlamydia trachomatis* (125a). The most obvious difference between the *L. pneumophila* and *L. micdadei* Mip proteins is in the initial 22 carboxy-terminal amino acids. These residues are hydrophobic and have the predicted structure of an alpha helix. This probably represents a secretory signal, but in *L. micdadei* no typical signal cleavage site can be identified. In contrast, the *L. pneumo-

phil* *a* mip gene has an easily distinguishable leader sequence with a typical cleavage site. Southern hybridization experiments indicated that the mip gene of *L. micdadei* has extensive homology with the mip-like genes of several *Legionella* species. It was suggested that the mip gene family of various *Legionella* strains can be divided into three homology groups: (i) the mip gene of *L. pneumophila*, which has moderate homology to its analogs in all other *Legionella* species (42); (ii) the mip-like genes of *L. micdadei*, *L. feelei*, *L. jamestowniensis*, *L. oakridgensis*, *L. quinlivanii*, *L. saintheleni*, *L. spiritensis*, and *L. wadsworthii*, which have moderate homology to the *L. pneumophila* gene but extensive (≥90%) homology with *L. micdadei* mip; and (iii) the mip-like genes of the remaining *Legionella* species, which have moderate homology with both the *L. pneumophila* and *L. micdadei* genes. Whether this genetic heterogeneity has implications regarding the virulence of the various species remains to be determined. Also awaiting elucidation is the striking sequence similarity between the carboxy-terminal end of the two *Legionella* mip genes and the *N. crassa* (188) and human FK 506 binding proteins, as well as a cryptic *N. meningitidis* sequence (179a). The extended alpha helix in the N-terminal part of the Mip proteins may form a rodlike spacer arm extending from the bacterial cell which may bring the enzymatically active carboxy-terminal end close to a target membrane structure. Whether the Mip proteins act enzymatically as prolyl-isomerases, as do the FK 506-binding proteins, remains to be determined. Preliminary experiments showed that a recombinant *E. coli* expressing the *L. micdadei* Mip protein was taken up better by human monocytes than was *E. coli* carrying only the vector (5b), indicating that the *L. micdadei* Mip protein, like its *L. pneumophila* counterpart, is necessary for optimum uptake by phagocytic cells.

A specific DNA probe for the legiolysin gene (ily) cloned from *L. pneumophila* Philadelphia 1 has been used in Southern hybridizations to detect ily-specific DNA in the genomes of *legionellae* and other gram-negative pathogenic bacteria (15a). Under conditions of high stringency, the ily reacted only with DNA from *L. pneumophila* isolates. However, under low-stringency conditions, hybridization was observed for all the *Legionella* strains tested. No hybridization occurred with DNAs from bacteria of other genera. All but one *L. pneumophila* strain also produced ily proteins that were detected in Western blots by using anti-ily antibodies. The protein was not detected in non-*L. pneumophila* strains or in other gram-negative bacteria, so that legiolysin appears to be specific to *L. pneumophila*. Since the zinc metalloprotease is also specific to *L. pneumophila* (157), the hemolysis observed for numerous *Legionella* species appears to be due to a moiety other than legiolysin or the protease. As yet, there is no evidence the legiolysin is a virulence factor that plays a role in pathogenesis at the cellular level. In fact, ily was detected in an avirulent strain of *L. pneumophila* Philadelphia 1.

REFERENCES

1. Anand, C. M., A. R. Skinner, A. Malle, and J. B. Kurtz. 1983. Interaction of *L. pneumophila* and a free living amoeba (*Acantu-

moeba palestiensis*). J. Hyg. Camb. 91:167–178.
VIRULENCE FACTORS OF THE LEGIONELLACEA

2. Badvey, J. A., and M. L. Kurnovsky. 1986. Production of superoxide by phagocytic leukocytes: a paradigm for stimulus-response phenomena. Curr. Top. Cell. Regul. 28:183–208.

3. Baine, W. B. 1985. Cytolytic and phospholipase C activity in Legionella species. J. Gen. Microbiol. 131:1381–1391.

4. Baine, W. B. 1988. A phospholipase C from the Dallas 1E strain of Legionella pneumophila serogroup 5: purification and characterization of conditions for optimal activity with an artificial substrate. J. Gen. Microbiol. 134:489–498.

5. Bangsberg, J. M., N. P. Cianciotto, and P. Hindersson. 1991. Nucleotide sequence analysis of the Legionella micdadei mip gene, encoding a 30-kDa pyelonephritis associated protein. Mol. Microbiol. 5:979–986.

6. Barbee, J. M., B. S. Fields, J. C. Feeley, G. W. Gorman, and W. T. Martin. 1986. Isolation of protozoa from water associated with Legionellosis outbreak and demonstration of intracellular multiplication of Legionella pneumophila. Appl. Environ. Microbiol. 51:422–424.

7. Baskerville, A., G. C. Conlan, L. A. E. Ashworth, and A. B. Dowsett. 1986. Pulmonary damage caused by a protease from Legionella pneumophila. Br. J. Exp. Pathol. 67:527–536.

8. Baskerville, A., A. B. Dowsett, R. B. Fitzgeorge, P. Habib, and M. Broster. 1983. Ultrastructure of pulmonary alveoli and macrophages in experimental legionnaires’ disease. J. Pathol. 140:77–90.

9. Baskerville, A., R. B. Fitzgeorge, M. Broster, and P. Habib. 1983. Histopathology of experimental legionnaires’ disease in guinea pigs. Resusc rhinos monkeys and marmosets. J. Pathol. 139:349–362.

10. Beaman, L. E. 1988. Monoclonal antibodies demonstrating that superoxide dismutase contributes to protection of Nocardia asteroides within the intact host. Infect. Immun. 56:443–450.

11. Becker, E. L. 1986. Leukocyte stimulation: receptor, membrane and metabolic events. Fed. Proc. 45:2188–2190.

12. Bier, E., J. C. Kermode, P. H. Naccache, R. Yassin, M. L. Marsh, J. J. Munoz, and R. I. Shafai. 1985. The inhibition of neutrophil granule enzyme secretion and chemotaxis by pertussis toxin. J. Cell Biol. 106:1641–1646.

13. Becker, E., J. C. Kermode, P. H. Naccache, R. Yassin, J. J. Munoz, M. L. Marsh, C. K. Huang, and R. I. Shafai. 1985. Pertussis toxin as a probe of neutrophil activation. Fed. Proc. 45:2151–2155.

14. Bellinger-Kawahara, C., and M. A. Horwitz. 1990. Complement component C3 fixes selectively to the major outer membrane protein (MOMP) of Legionella pneumophila and mediates phagocytosis of liposome-MOMP complexes by human monocytes. J. Exp. Med. 172:1201–1210.

15. Belty, I. F., I. V. Vertiev, I. V. Tartakowskii, I. V. Izepchuk, and S. V. Prozorovskii. 1988. Characteristics of the cytolysin of Legionella pneumophila. Zh. Mikrobiol. Epidemiol. Immunobiol. Feb.(2):4–7.

16. Bender, L., M. Ott, A. Debes, U. Riedst, J. Hessemann, and J. Hacke. 1991. Distribution, expression, and long-range mapping of the cytolysin gene (hyp)1-specific DNA sequences in legionella. Infect. Immun. 59:3333–3336.

17. Benson, R. F., W. L. Thacker, J. A. Lanser, N. Sangster, W. R. Mayberry, and D. J. Bremer. 1991. Legionella adelaidensis, a new species isolated from cooling tower water. J. Clin. Microbiol. 29:1004–1006.

18. Berdal, B. P., K. Bøvre, O. Olsvik, and T. Omland. 1983. Patterns of extracellular proline-specific endopeptidases in Legionella and Flavobacterium spp. demonstrated by use of chromogenic peptides. J. Clin. Microbiol. 17:970–974.

19. Berdal, B. P., O. Olsvik, S. Myhre, and T. Omland. 1982. Demonstration of extracellular chymotrypsin-like activity from various Legionella species. J. Clin. Microbiol. 16:452–457.

20. Beridge, M. J. 1984. Inositol trisphosphate and diacylglycerol as second messengers. Biochem. J. 220:345–360.

21. Bhaduraj, N., T. W. Nash, and M. A. Horwitz. 1986. Interferon-γ-activated macrophages inhibit the intracellular multiplication of Legionella pneumophila. J. Immunol. 137:2662–2669.

22. Black, W. J., F. D. Quinn, and L. S. Tompkins. 1990. Legionella pneumophila zinc metallopeptase is structurally and functionally homologous to Pseudomonas aeruginosae elastase. J. Bacteriol. 172:2608–2613.

23. Blackmon, J. A., F. W. Chandler, W. B. Cherry, A. C. England III, J. C. Feeley, M. D. Hicklin, R. M. McKinney, and H. W. Wilkinson. 1981. Legionellosis in New York. N. Engl. J. Med. 305:465.

24. Blanchard, D. K., J. Y. Djeu, T. W. Klein, H. Friedman, and W. E. Stewart 2d. 1988. Protective effects of tumor necrosis factor in experimental Legionella pneumophila infections of mice via activation of PMN function. J. Leukocyte Biol. 43:29–435.

25. Blanchard, D. K., H. Friedman, T. W. Klein, and J. Y. Djeu. 1989. Induction of interferon-gamma and tumor necrosis factor by Legionella pneumophila: augmentation of human neutrophil bactericidal activity. J. Leukocyte Biol. 45:538–545.

26. Blanchard, D. K., W. E. Stewart 2d, T. W. Klein, H. Friedman, and J. Y. Djeu. 1987. Cytolytic activity of human peripheral blood leukocytes against Legionella pneumophila-infected monocytes: characterization of the effector cell and augmentation by interleukin 2. J. Immunol. 139:551–556.

27. Bland, S. J., R. F. Breiman, and M. A. Horwitz. 1989. A live avirulent mutant Legionella vaccine induces protective immunity against lethal aerosol challenge. J. Clin. Invest. 83:810–815.

28. Bland, S. J., and M. A. Horwitz. 1989. Vaccination with the major secretory protein of Legionella pneumophila induces cell-mediated and protective immunity in a guinea pig model of Legionnaires’ disease. J. Exp. Med. 169:691–705.

29. Bland, S. J., M. A. Horwitz. 1991. Vaccination with the major secretory protein of Legionella pneumophila induces cell-mediated immune responses and protective immunity across different serogroups of Legionella pneumophila and different species of Legionella. J. Immunol. 147:285–291.

30. Bland, S. J., L. Szeto, H. A. Shuman, and M. A. Horwitz. 1990. An immunoprotective molecule, the major secretory protein of Legionella pneumophila, is not a virulence factor in a guinea pig model of Legionnaires’ disease. J. Infect. Invest. 6:817–824.

31. Bokoch, G. M., and A. G. Gilman. 1984. Inhibition of receptor mediated release of arachidonic acid by pertussis toxin. Cell 39:301–308.

32. Breiman, R. F., B. S. Fields, G. N. Sanden, L. Volmer, A. Meier, and J. S. Spika. 1990. Association of shower use with legionnaires’ disease. Possible role of amoebae. JAMA 263:2924–2926.

33. Bremer, D. J., A. G. Steigerwald, and J. E. McDade. 1979. Classification of the legionnaires’ disease bacterium: Legionella pneumophila, genus novus, species nova, of the family Legionellaceae, familia nova. Ann. Intern. Med. 90:656–638.

34. Brubaker, R. R. 1985. Mechanisms of bacterial virulence. Annu. Rev. Microbiol. 39:21–50.

35. Buser, B., J. Summersgill, and R. Miller. 1991. Intracellular free calcium responses in human neutrophils infected with Legionella pneumophila. J. Exp. Biol. 147:291–297.

36. Butler, C. A., E. D. Street, T. P. Hatch, and P. S. Hoffman. 1985. Disulfide-bonded outer membrane proteins in the genus Legionella. Infect. Immun. 48:14–18.

37. Caillon, E., B. Lubochinsky, and D. Rigonier. 1983. Occurrence of diacyl ethereal phospholipids in Stigmatella aurantiaca. J. Bacteriol. 153:1348–1351.

38. Capron, M., and W. Johnson. 1988. Macrophage toxicity and...
complement sensitivity of virulent and avirulent strains of Legionella pneumophila. J. Infect. Dis. 150:377–381.

38. Carpenter, C. L., and L. C. Cantley. 1990. Phosphoinositide kinases. Biochemistry 29:11148–11156.

39. Catrenich, C. E., and W. Johnson. 1989. Characterization of the selective inhibition of growth of virulent Legionella pneumophila by supplemented Mueller-Hinton medium. Infect. Immun. 57:1862–1864.

40. Chandler, F. W., J. A. Blackmon, M. D. Hicklin, R. M. Cole, and C. S. Callaway. 1979. Ultrastructure of the agent of Legionnaires’ disease in the human lung. Am. J. Clin. Pathol. 71:43–50.

41. Chandler, F. W., J. E. McDade, M. D. Hicklin, J. A. Blackmon, B. M. Thomason, and E. F. Ewing, Jr. 1979. Pathologic findings in guinea pigs inoculated with the legionnaires’ disease bacteria. Ann. Intern. Med. 90:67–675.

42. Cianciotto, N. P., J. M. Bangsberg, B. I. Eisenstein, and N. C. Engleberg. 1990. Identification of mip-like genes in the genus Legionella. Infect. Immun. 58:2912–2918.

43. Cianciotto, N. P., B. I. Eisenstein, C. H. Mody, and N. C. Engleberg. 1990. A mutation in the mip gene results in an attenuation of Legionella pneumophila virulence. J. Infect. Dis. 162:121–126.

44. Cianciotto, N. P., B. I. Eisenstein, C. H. Mody, G. B. Toews, and N. C. Engleberg. 1989. A Legionella pneumophila gene encoding a species-specific surface protein potentiates initiation of intracellular infection. Infect. Immun. 57:1255–1262.

45. Ciesielski, C. A., M. J. Blaser, and W. L. Wang. 1986. Serogroup specificity of Legionella pneumophila is related to lipopolysaccharide characteristics. Infect. Immun. 51:397–404.

46. Cohen, H. J., M. E. Chowanec, M. K. Wilson, and P. E. Newburger. 1982. Con-A-stimulated superoxide production by granulocytes: reversible activation of NADPH oxidase. Blood 60:1188–1194.

47. Conlon, J. W., and L. A. E. Ashworth. 1986. The relationship between the serogroup antigen and lipopolysaccharide of Legionella pneumophila. J. Hyg. Camb. 96:39–48.

48. Conlon, J. W., A. Baskerville, and L. A. E. Ashworth. 1986. Separation of Legionella pneumophila proteases and purification of a protease which produces lesions like those of Legionnaires’ disease in guinea pig lung. J. Gen. Microbiol. 132:1565–1574.

49. Conlon, J. W., A. Williams, and L. A. E. Ashworth. 1988. In vivo production of a tissue-destructive protease by Legionella pneumophila. J. Gen. Microbiol. 134:143–149.

50. Conlon, J. W., A. Williams, and L. A. E. Ashworth. 1988. Inactivation of human α-1-antitrypsin by a tissue-destructive protease of Legionella pneumophila. J. Microbiol. 134:481–487.

51. Das, S., A. K. Saha, N. K. Mukhopadhyay, and R. H. Glew. 1986. A cyclic nucleotide-independent protein kinase in Leishmania donovani. Biochem. J. 240:641–649.

52. Das, S., A. K. Saha, A. T. Remaley, R. H. Glew, J. N. Dowling, M. Kajiyoshi, and M. Gottlieb. 1986. Hydrolysis of phosphoproteins and inositol phosphates by cell surface phosphatase of Leishmaniam donovani. Mol. Biochem. Parasitol. 20:143–153.

53. Davis, G. S., W. C. Winn, Jr., D. W. Gump, and H. N. Beaty. 1983. The kinetics of early inflammatory events during experimental pneumonia due to Legionella pneumophila in guinea pigs. J. Infect. Dis. 148:823–835.

54. Davis, G. S., W. C. Winn, Jr., D. W. Gump, J. E. Craighead, and H. N. Beaty. 1982. Legionnaires’ pneumonia after aerosol exposure in guinea pigs and rats. Am. Rev. Respir. Dis. 126:1050–1057.

55. Densen, P., and G. L. Mandell. 1980. Phagocyte strategy vs. microbial tactics. Rev. Infect. Dis. 2:817–838.

56. Donowitz, G., J. Dowling, and D. Focht. 1989. Legionella micdadei inhibits monocyte function. Program Abstr. 29th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 329.

57. Donowitz, G. R., and D. Focht. Unpublished data.

58. Donowitz, G. R., I. Reardon, J. Dowling, L. Rubin, and D. Focht. 1990. Ingestion of Legionella micdadei inhibits human neutrophil function. Infect. Immun. 58:3307–3311.

59. Donowitz, G. R., I. Reardon, and D. Focht. Unpublished data.

60. Donowitz, G. R., I. Reardon, D. Focht, and J. N. Dowling. Unpublished data.

61. Dowling, J. N., J. D. Hempel, and R. H. Glew. Unpublished data.

62. Dowling, J. N., A. W. Pascullie, F. N. Frola, M. K. Zaphyr, and R. B. Yee. 1984. Infections caused by Legionella micdadei and Legionella pneumophila among renal transplant recipients. J. Infect. Dis. 149:703–713.

63. Dowling, J. N., A. K. Saha, and R. H. Glew. Unpublished data.

64. Dowling, J. N., R. S. West, and A. W. Pascullie. 1982. Bactericidal activity of antibiotics against Legionella micdadei (Pittsburgh pneumonia agent). Antimicrob. Agents Chemother. 22:272–276.

65. Dreyfus, L. A. 1987. Virulence associated ingestion of Legionella pneumophila by HeLa cells. Microb. Pathog. 3:45–52.

66. Dreyfus, L. A., and B. H. Iglewski. 1986. Purification and characterization of an extracellular protease of Legionella pneumophila. Infect. Immun. 51:736–743.

67. Ehret, W., and G. Ruckdeschel. 1985. Membrane protein of Legionella. I. Membrane proteins of different strains and serogroups of Legionella pneumophila. Zentralbl. Bakteriol. Mikrobiol. Hyg. Ser. A 259:433–435.

68. Elliott, J. A., and W. C. Winn, Jr. 1986. Treatment of alveolar macrophages with cytochalasin D inhibits uptake and subsequent growth of Legionella pneumophila. Infect. Immun. 51:31–36.

69. Enami, M., and A. Ishihama. 1984. Protein phosphorylation in Escherichia coli and purification of a protein kinase. J. Biol. Chem. 259:526–533.

70. Engleberg, N. C., C. Carter, D. R. Weber, N. P. Cianciotto, and B. I. Eisenstein. 1989. DNA sequence of mip, a Legionella pneumophila gene associated with macrophage infectivity. Infect. Immun. 57:1263–1270.

71. Fields, B. S., J. M. Barbaare, G. N. Sanden, and W. E. Morrill. 1990. Virulence of a Legionella anisa strain associated with Pontiac fever: an evaluation using protozoan, cell culture, and guinea pig models. Infect. Immun. 58:3139–3142.

72. Fields, B. S., E. B. Shotts, Jr., J. C. C. Gorman, and W. T. Newbrough. 1984. Proteorhodopsin of Legionella pneumophila as an intracellular parasite of the ciliated protozoan Tetrahymena pyriformis. Appl. Environ. Microbiol. 47:467–471.

73. Finnerty, W. R., A. A. Makula, and J. C. Feeley. 1979. Cellular lipids of the Legionnaires’ disease bacterium. Ann. Intern. Med. 90:631–634.

74. FitzGeorge, R. B., A. S. R. Featherstone, and A. Baskerville. 1988. Effects of polymorphonuclear leukocyte depletion on the pathogenesis of experimental Legionnaires’ disease. Br. J. Exp. Pathol. 69:105–112.

75. Fraser, D. W., T. R. Tsai, W. Orenstein, W. E. Pardin, H. J. Beecham, R. G. Sharrar, J. Harris, G. F. Mallison, S. M. Martin, J. E. McDade, C. C. Shepard, P. S. Brachman, and the Field Investigation Team. 1977. Legionnaires’ disease: description of an epidemic of pneumonia. N. Engl. J. Med. 297:1195–1197.

76. Friedman, R. L., B. H. Iglewski, and R. D. Miller. 1980. Identification of a cytotoxin produced by Legionella pneumophila. Infect. Immun. 29:271–274.

77. Friedman, R. L., J. E. Lochner, R. H. Bigley, and B. H. Iglewski. 1982. The effects of Legionella pneumophila toxin on oxidative processes and bacterial killing of human polymorphonuclear leukocytes. J. Infect. Dis. 146:328–334.

78. Frisch, C. F., and W. B. Baine. 1984. Production of extracel-
VOL. 94 (Legionella multicomponent H. Legionnaires’ superoxide toxin, Hoffman, I. Ishihara. 1978. Hindahl, Hase, Hedlund, Gabay, Med. Analytical isotachophoresis. Environ. Microbiol. cholerae N. M. S. P. E., 1990. Isolation of Pseudomonas outer lipids of 1985. Isolation of Legionella pneumophila serogroups and other Legionella species. Infect. Immun. 51:94–101.}

90. Hoffman, P. S., C. A. Butler, and F. D. Quinn. 1989. Cloning and temperature-dependent expression in Escherichia coli of a Legionella pneumophila gene encoding a genus-common 60-kilodalton antigen. Infect. Immun. 57:1731–1739.

91. Hoffman, P. S., L. Houston, and C. A. Butler. 1990. Legionella pneumophila htpA/B heat shock operon: nucleotide sequence and expression of the 60-kilodalton antigen in L. pneumophila-infected HeLa cells. Infect. Immun. 58:3380–3387.

92. Hoffman, P. S., L. Pine, and S. Bell. 1983. Production of superoxide and hydrogen peroxide in medium used to culture Legionella pneumophila: catalytic decomposition by charcoal. Appl. Environ. Microbiol. 45:784–791.

93. Homma, J. Y., C. Abe, K. Tanamoto, Y. Hirao, K. Morihara, H. Tsuchiz, R. Yanagawa, E. Honda, Y. Aoi, Y. Fujimoto, M. Gorny, N. Imazeki, H. Noda, A. Goda, S. Takeuchi, and T. Ishihara. 1978. Effectiveness of immunization with single and multicomponent vaccines prepared from a common antigen (OEP), protease and elastase toxoids of Pseudomonas aeruginosa on protection against hemorrhagic pneumonia in mink due to P. aeruginosa. Jpn. J. Exp. Med. 48:111–133.

94. Horwitz, M. A. 1983. Formation of a novel phagosome by the Legionnaires’ disease bacterium (Legionella pneumophila) in human monocytes. J. Exp. Med. 158:1319–1331.

95. Horwitz, M. A. 1983. The Legionnaires’ disease bacterium (Legionella pneumophila) inhibits phagosome-lysosome fusion in human monocytes. J. Exp. Med. 158:2108–2126.

96. Horwitz, M. A. 1984. Phagocytosis of the Legionnaires’ disease bacterium (Legionella pneumophila) occurs by a novel mechanism: engulfment within a pseudopod coil. Cell 36:27–33.

97. Horwitz, M. A. 1987. Characterization of avirulent mutant Legionella pneumophila that survive but do not multiply within human monocytes. J. Exp. Med. 166:1310–1328.

98. Horwitz, M. A. 1988. Phagocytosis and intracellular biology of Legionella pneumophila, p. 283–302. In M. A. Horwitz (ed.), Bacteria-host cell interaction. Alan R. Liss, Inc., New York.

99. Horwitz, M. A., and F. R. Maxfield. 1984. Legionella pneumophila inhibits acidification of its phagosome in human monocytes, J. Cell Biol. 99:1936–1943.

100. Horwitz, M. A., and S. C. Silverstein. 1980. Legionnaires’ disease bacterium (Legionella pneumophila) multiplies intracellularly in human monocytes. J. Clin. Invest. 66:441–450.

101. Horwitz, M. A., and S. C. Silverstein. 1981. Interaction of the legionnaires’ disease bacterium (Legionella pneumophila) with human phagocytes. I. L. pneumophila resists killing by polymorphonuclear leukocytes, antibody, and complement. J. Exp. Med. 153:386–397.

102. Horwitz, M. A., and S. C. Silverstein. 1981. 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. J. Exp. Med. 153:398–406.

103. Horwitz, M. A., and S. C. Silverstein. 1981. Activated human monocytes inhibit the intracellular multiplication of Legionnaires’ disease bacteria. J. Exp. Med. 154:1618–1635.

104. Jacobs, R. F., R. M. Locksley, C. B. Wilson, J. E. Haas, and S. J. Klebanoff. 1984. Interaction of prime alveolar macrophages and Legionella pneumophila. J. Clin. Invest. 73:1515–1523.

105. Jaken, S. 1989. Dicycglycerol: the role of stimulated production in activation of protein kinase C, p. 163–178. In R. H. Michell, A. H. Drummond, and C. P. Downes (ed.), Inositol lipids in cell signaling. Academic Press, Inc., San Diego.

106. Jepras, R. J., and R. B. Fitzgeroge. 1986. The effect of oxygen-dependent antimicrobial systems on strains of Legionella pneumophila of different virulence. J. Hyg. Camb. 97:61–69.

107. Jepras, R. J., R. B. Fitzgeroge, and A. Baskerville. 1985. A comparison of virulence of two strains of Legionella pneumophila based on experimental aerosol infection of guinea-pigs. J. Hyg. Camb. 95:29–38.

108. Katz, S. M., and S. Hashemi. 1982. Electron microscopic examination of the inflammatory response to Legionella pneumophila in guinea pigs. Lab. Invest. 46:24–32.

109. Keen, G. M., and P. S. Hoffman. 1989. Characterization of a Legionella pneumophila extracellular protease exhibiting hemolytic and cytotoxic activities. Infect. Immun. 57:732–738.

110. Kishimoto, R. A., M. D. Kastello, J. D. White, F. G. Shirey, V. G. McGann, E. W. Larson, and K. W. Hedlund. 1979. In vitro interaction between normal cynomolgus monkey alveolar macrophages and legionnaires disease bacteria. Infect. Immun. 25:761–763.

111. Kishimoto, R. A., J. D. White, F. G. Shirey, V. G. McGann, R. F. Berendt, E. W. Larson, and K. W. Hedlund. 1981. In vitro response of guinea pig peritoneal macrophages to Legionella pneumophila. Infect. Immun. 31:1209–1213.

112. Korchak, H. M., L. B. Vosshall, G. Zagon, P. Ljubich, A. M. Rich, and G. Weissmann. 1988. Activation of the neutrophil by calcium-mobilizing ligands. 1. A chemoattractant peptide and the lectin concanavalin A stimulate superoxide anion generation but elicit different calcium movements and phosphoinositide remodeling. J. Biol. Chem. 263:11090–11097.

113. Kuchta, J. M., S. J. States, A. M. McNamara, R. M. Wadowsky, and R. B. Yee. 1983. Susceptibility of Legionella pneumophila to chlorine in tap water. Appl. Environ. Microbiol. 46:1134–1139.

114. Lad, P. M., C. V. Olson, and P. A. Smiley. 1985. Association of...
Polymorphonuclear neutrophils: an effective antimicrobial force. Rev. Infect. Dis. 11:3152-3154.

Staerdaad, R. F., A. Galat, G. L. Verdine, and S. L. Schreiber. 1990. Molecular cloning and overexpression of the human FK506-binding protein FKBP. Nature (London) 346:671-674.

Steifersen, D. O., D. L. Weinbaum, and J. N. Dowling. 1985. Opsin requirements for pHophagocytosis by polymorphonuclear leukocytes. Infect. Immun. 49:695-699.

Styrt, B., R. D. Walker, and J. C. White. 1989. Neutrophil oxidative metabolism after exposure to bacterial phospholipase C. J. Lab. Clin. Med. 114:31-37.

Summersgill, J. T., M. J. Raff, and R. D. Miller. 1988. Interactions of virulent and avirulent Legionella pneumophila with human polymorphonuclear leukocytes. Microb. Pathog. 4:41-47.

Summersgill, J. T., M. J. Raff, and R. D. Miller. 1990. Interactions of virulent and avirulent Legionella pneumophila with human monocytes. J. Leukocyte Biol. 47:31-38.

Szeto, L., and H. A. Shuman. 1990. The Legionella pneumophila major secretory protein, a protease, is not required for intracellular growth or cell killing. Infect. Immun. 58:2585-2592.

Tartakovskii, I. S., I. F. Belyi, I. V. Vertiev, I. G. Nagaev, and S. V. Prozorovskii. 1988. Cytolysin as an immunoserological marker of Legionella pneumophila. Zh. Mikrobio. Epidemiol. Immunobiol. 39:35-43.

Thompson, M. R., R. D. Miller, and B. H. Igleswski. 1981. In vitro production of an extracellular protease by Legionella pneumophila. Infect. Immun. 34:299-302.

Thorpe, T. C., and R. D. Miller. 1981. Extracellular enzymes of Legionella pneumophila. Infect. Immun. 33:632-635.

Tropschugs, M., E. Wachter, S. Mayer, E. R. Schönbrunner, and F. M. Schmid. 1990. Isolation and sequence of an FN 306-binding protein from N. crassa which catalyses protein folding. Nature (London) 346:674-677.

Tyn dall, R. L., and E. L. Domingue. 1982. Cocrystallization of Legionella pneumophila and free-living amoebae. Appl. Environ. Microb. 44:954-959.

Verbrugh, H. A., D. A. Lee, G. R. Elliot, W. F. Keane, J. R. Hoidal, and P. K. Peterson. 1985. Ospomion of Legionella pneumophila in human serum: key roles for specific antibodies and the classical complement pathway. Immunology 54:643-653.

Vergheze, M. W., C. D. Smith, and R. Snyderman. 1985. Potential role for a guanine nucleotide regulatory protein in chemoattractant receptor mediated polyphosphoinositide metabolism, Ca++ mobilization and cellular responses by leukocytes. Biochem. Biophys. Res. Commun. 127:450-457.

Vilco, J. L., P. Rajagopalan, and E. Dournon. 1983. Virulent Legionella pneumophila does not provoke metabolic burst of neutrophils, unlike a nonvirulent strain. Program Abstr. 22nd Intersci. Conf. Antimicrob. Agents Chemother., abstr. 65.

Wadee, A. A., D. Mendelsohn, and A. R. Rabson. 1983. Characterization of a suppressor cell-activating factor (SCAF), released by adherent cells treated with M. tuberculosis. J. Immunol. 130:2266-2270.

Wadowski, R. M., T. M. Wilson, N. J. Kapp, A. J. West, J. M. Kuchta, S. J. States, J. N. Dowling, and R. B. Ye. 1991. Multiplication of Legionella spp. in tap water containing Hartmannella vermiformis. Appl. Environ. Microb. 57:1950-1955.

Wandosell, F., L. Serrano, and J. Avila. 1987. Phosphorylation of a-tubulin carboxyl-terminal tyrosine prevents its incorporation into microtubules. J. Biol. Chem. 262:8208-8217.

Wandosell, F., L. Serrano, M. A. Hernandez, and J. Aliva. 1986. Phosphorylation of tubulin by a calmodulin-dependent protein kinase. J. Biol. Chem. 261:10332-10339.

Wang, J. Y. L., and D. E. Koshland, Jr. 1981. The identification of distinct protein kinases and phosphatases in the prokaryote Salmonella typhimurium. J. Biol. Chem. 256:4640-4648.

Weinbaum, D. L., J. Bailey, R. R. Benner, A. W. Pascule, and J. N. Dowling. 1983. The contribution of human neutrophils and serum to host defense against Legionella micdadei. J.
199. Weinbaum, D. L., R. R. Benner, J. N. Dowling, A. Alpern, A. W. Pasculle, and G. R. Donowitz. 1984. Interaction of Legionella micdadei with human monocytes. Infect. Immun. 46:68–73.

200. Williams, A., A. Baskerville, A. B. Dowsett, and J. W. Conlan. 1987. Immunocytochemical demonstration of the association between Legionella pneumophila, its tissue-destructive protease, and pulmonary lesions in experimental Legionnaires’ disease. J. Pathol. 153:257–264.

201. Winn, W. C., G. S. Davis, D. W. Gump, J. E. Craighead, and H. N. Beaty. 1982. Legionnaires’ pneumonia after intratracheal inoculation of guinea pigs and rats. Lab. Invest. 47:568–578.

202. Winn, W. C., Jr., F. L. Glavin, D. P. Perl, J. L. Keller, T. L. Andres, T. M. Brown, C. M. Coffin, J. E. Sensequa, L. N. Roman, and J. E. Craighead. 1978. The pathology of Legionnaires’ disease. Fourteen fatal cases from the 1977 outbreak in Vermont. Arch. Pathol. Lab. Med. 102:344–350.

203. Winn, W. C., Jr., and R. L. Myerowitz. 1981. The pathology of the Legionella pneumonias. Hum. Pathol. 12:401–422.

204. Wong, K. H., C. W. Moss, D. H. Hochstein, R. J. Arko, and W. O. Schall. 1979. “Endotoxicity” of the Legionnaires’ disease bacterium. Ann. Intern. Med. 90:624–627.

205. Yamamoto, Y., T. W. Klein, C. A. Newton, R. Widen, and H. Friedman. 1988. Growth of Legionella pneumophila in thioglycolate-elicited peritoneal macrophages from A/J mice. Infect. Immun. 56:370–375.

206. Yoshida, S., and Y. Mizuguchi. 1986. Multiplication of Legionella pneumophila Philadelphia-1 in cultured peritoneal macrophages and its correlation to susceptibility of animals. Can. J. Microbiol. 32:438–442.