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Infection of Macrophages with *Legionella pneumophila* Induces Phosphorylation of a 76-Kilodalton Protein

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Infection of peritoneal macrophages from susceptible A/J mice with *Legionella pneumophila* induced phosphorylation of a 76-kDa protein. The phosphorylation occurred when macrophages were infected with a virulent strain of *L. pneumophila* but did not occur when they were infected with an avirulent strain or with other bacteria such as either *Pseudomonas aeruginosa* or *Salmonella typhimurium*. Also, no phosphorylation of this protein was observed when macrophages were stimulated with either lipopolysaccharide or phorbol myristate acetate. However, phosphorylation did occur in macrophages infected with a virulent strain of *L. pneumophila* and treated with either erythromycin to inhibit growth or with cytochalasin D to inhibit uptake of *L. pneumophila* by macrophages. These results support the view that phosphorylation of this protein occurs during the early phases of interaction between *L. pneumophila* and macrophages. The role of this specific protein in the recognition, intracellular uptake, and growth of *L. pneumophila* in permissive macrophages remains to be clarified.

Phosphorylation and dephosphorylation of proteins are recognized as major processes for regulating cellular functions in response to signals such as bacterial lipopolysaccharides (LPS) and growth factors (5, 10, 12). LPS is a powerful regulatory signal for many leukocytes (14, 15) and has been demonstrated to induce phosphorylation of certain cellular proteins in macrophages (19, 22). Growth factors also alter protein phosphorylation in hematopoietic cells following binding to specific cell surface receptors (4, 18, 20). For example, the binding of granulocyte colony-stimulating factor to human myeloid leukemia cells stimulates the phosphorylation of the 22- and 68-kDa proteins on serine and threonine residues, respectively (7, 23). Infection of cells with bacteria alters host cell functions, and protein phosphorylation might be a part of the associated molecular changes. In this regard, Baldwin et al. (1) recently reported on phosphorylation activated by protein kinase C in *Escherichia coli*-infected HEp-2 cells, with the molecular masses of the phosphorylated proteins being 21 and 29 kDa. To our knowledge, however, similar observations have not been reported for macrophages infected with bacteria. In the study reported here, protein phosphorylation was examined in macrophages infected with *Legionella pneumophila* and other bacteria.

L. pneumophila is a facultative intracellular bacterial pathogen and the major cause of Legionnaires' disease (9). This bacterium multiplies in A/J mouse macrophages as well as in human and guinea pig macrophages (25). Recently we observed that infection of cultured macrophages with a virulent strain rather than an avirulent strain of *L. pneumophila* induces both the expression of interleukin 1β mRNA and the activity of cell supernatant interleukin 1 within a few hours following infection (11). These results suggested to us the possibility that the processes of attachment and infection generate a variety of molecular changes within the macrophages. Indeed, in the present studies, we found that a 76-kDa protein is phosphorylated in macrophages infected with a virulent strain of *L. pneumophila* but not in cells exposed to the avirulent strain, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, LPS, or phorbol ester. These results suggest that the phosphorylation of this protein may be a unique event associated with the recognition of *L. pneumophila* by macrophages and may be correlated with other changes in macrophage function following infection.

L. pneumophila serogroup 1, isolated from a patient with fatal legionellosis at Tampa General Hospital, Tampa, Fla., was cultured on buffered charcoal yeast extract agar and prepared exactly as described previously (8). An avirulent strain of *L. pneumophila* was obtained by multiple passages of the virulent parent culture on supplemented Mueller-Hinton agar (3, 24). This strain is relatively nonlethal when injected into mice and guinea pigs (24). *S. typhimurium* LT2 and *P. aeruginosa* were cultured on nutrient agar, and suspensions were prepared by the procedures used for *L. pneumophila*. Thioglycolate-induced peritoneal macrophages were obtained from A/J female mice, 8 to 12 weeks of age, purchased from Jackson Laboratory (Bar Harbor, Maine), and cultured in 24-well culture plates as previously described (25). Phosphorylation of macrophage proteins in response to infection with *L. pneumophila* was detected as previously described (19) with minor modifications. In brief, macrophage monolayers were washed four times with phosphate-free Dulbecco's modified Eagle's minimal essential medium (Sigma Chemical Co., St. Louis, Mo.) and then incubated for 3 h in this medium containing 600 μCi of carrier-free $^{32}P_i$ (Amersham, Arlington Heights, Ill.) per ml. In a few studies, 10% fetal calf serum (FCS) (HyClone Laboratories, Inc., Logan, Utah) was included in minimal essential medium. At the end of this incubation, macrophages were infected with various concentrations of *L. pneumophila*, and the cultures were incubated for 30 min to 6 h at 37°C in 5% CO_2. After incubation, the macrophage monolayers were washed with cold phosphate-buffered saline, solubilized in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (125 mM Tris-HCl [pH 6.8], 4% SDS, 20%

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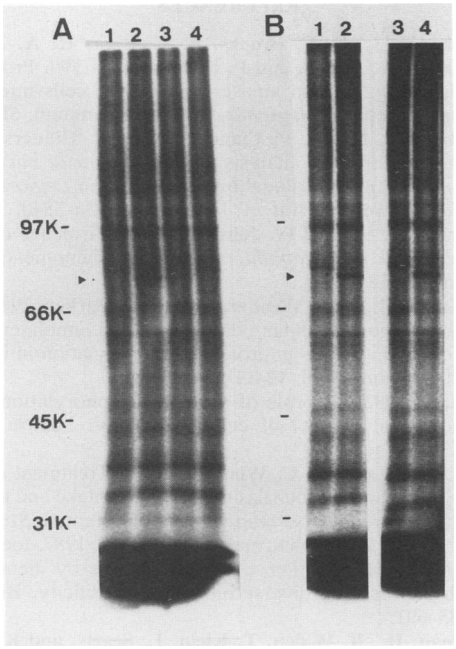

FIG. 1. *Legionella*-induced phosphorylation of A/J mouse macrophage proteins, analyzed by SDS–8% PAGE of ^{32}P-labeled cellular phosphoproteins prepared from macrophage monolayers 2 h after infection with *L. pneumophila*. (A) Effects of different infectivity ratios on protein phosphorylation. Lane 1, uninfected macrophages; lanes 2 through 4, 10^6 macrophages infected with 10^8, 10^7, and 10^6 bacteria, respectively. (B) Effect of serum on protein phosphorylation. Lane 1, uninfected macrophages, without 10% FCS; lane 2, macrophages infected with 10^7 bacteria, without 10% FCS; lane 3, uninfected macrophages, with 10% FCS; lane 4, macrophages infected with 10^7 bacteria, with 10% FCS. Molecular weight markers were phosphorylase *b* (97,400), serum albumin (66,200), ovalbumin (45,000), and carbonic anhydrase (31,000). Arrowheads indicate the 76-kDa phosphoprotein.

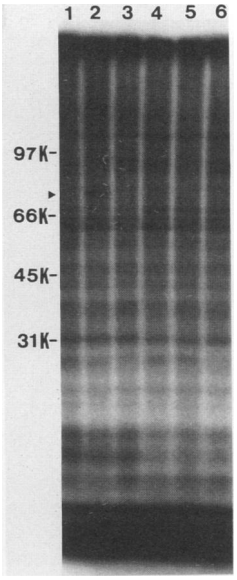

FIG. 2. Protein phosphorylation in macrophages treated with LPS or infected with a virulent or an avirulent strain of *L. pneumophila*, analyzed by SDS–10% PAGE of ^{32}P-labeled cellular phosphoproteins prepared from macrophage monolayers 3 h after treatment. Lane 1, uninfected macrophages; lanes 2 and 3, macrophages infected with 10^7 and 10^6 virulent *Legionella* organisms, respectively; lane 4, macrophages stimulated with 5 μg of *E. coli* LPS per ml; lanes 5 and 6, macrophages infected with 10^8 and 10^7 avirulent *Legionella* organisms, respectively. For molecular weight markers, see the legend to Fig. 1.

glycerol, and 10% 2-mercaptoethanol), and then incubated at 95°C for 10 min. Samples (10^5 cpm) were electrophoresed in 8 or 10% polyacrylamide gels containing SDS (13) and subsequently stained with Coomassie brilliant blue and destained. Dried gels were exposed to Kodak X-OMAT film with intensifying screens to detect changes in the ^{32}P labeling of the various proteins.

Incubation of A/J mouse macrophage monolayers for 3 h with a virulent strain of *L. pneumophila* increased phosphorylation of a 76-kDa protein compared with that of uninfected macrophages (Fig. 1A). The phosphorylation was also observed in studies with two other virulent *Legionella* strains obtained from William Johnson, University of Iowa, and N. Cary Engleberg, University of Michigan, respectively (24a). Phosphorylation of the protein occurred in either the presence or the absence of 10% FCS (Fig. 1B), and therefore, the FCS was left out of subsequent studies to avoid interference with phosphate loading of the cells.

The 76-kDa-protein phosphorylation was not detected at 30 min following infection but was detected by 1 h after infection and was stable for up to 6 h (data not shown). Also, phosphorylation was observed at infectivity ratios of 100 or 10 bacteria per macrophage but not at the ratio of 1 bacterium per macrophage (Fig. 1 and 2). In contrast, infection with the avirulent strain failed to induce phosphorylation at an infectivity ratio as high as 100 bacteria per macrophage (Fig. 2). Stimulation of macrophages with *E. coli* LPS (Sigma Chemical Co.) or phorbol myristate acetate (data not shown) did not induce the 76-kDa-protein phosphorylation in the macrophages (Fig. 2), suggesting that this protein differs from those whose phosphorylation was induced by these two substances in other systems (16, 19, 22).

Treatment of *Legionella*-infected macrophages with erythromycin (Sigma), which inhibits intracellular growth of the bacteria (21), also resulted in phosphorylation of the 76-kDa protein (Fig. 3), as did cytochalasin D (Sigma) treatment (Fig. 3A), which inhibits the uptake but not the attachment of bacteria (6). These results suggest that the phosphorylation of the protein is due to the attachment of *L. pneumophila* to the surface of the macrophage rather than the uptake and growth of the microorganism in the macrophage. Finally, we tested whether the exposure of macrophages to other bacteria would induce the observed protein phosphorylation. Studies with *P. aeruginosa* and *S. typhimurium*, using the same conditions as those with *L. pneumophila*, were performed for comparative purposes. Figure 3B is representative of the data and shows that infection of macrophages with these bacteria was ineffective at inducing the 76-kDa-protein phosphorylation.

Taken together, the data obtained in this study suggest that phosphorylation of the 76-kDa protein might be selectively associated with infection by a virulent *Legionella* strain rather than an avirulent strain, other gram-negative bacteria, or other reportedly active substances such as LPS and phorbol ester. Furthermore, the phosphorylation might be caused by association of a virulence-related *Legionella* ligand with the surface of the macrophage; this ligand is

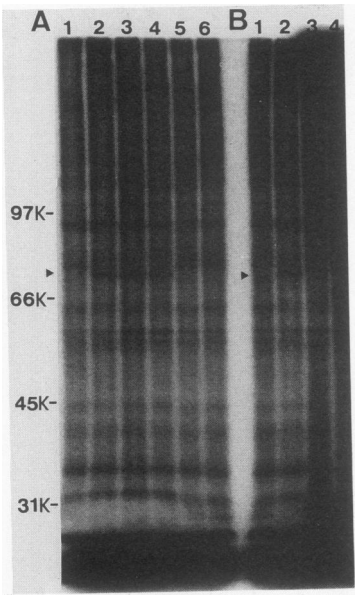

FIG. 3. Effects of erythromycin, cytochalasin D, and infection with *P. aeruginosa* and *S. typhimurium* on protein phosphorylation in macrophages. (A) Effects of inhibitors on protein phosphorylation. Erythromycin treatment (0.5 μg/ml) was performed at the same time as infection with 10^8 virulent *Legionella* organisms, and macrophage monolayers were continuously incubated for 2 h in the presence of erythromycin. Cytochalasin D treatment (1.5 μg/ml) was done 1 h before infection and continued to the end of incubation (2 h). Lane 1, uninfected macrophages; lane 2, infected macrophages; lane 3, macrophages treated with erythromycin and infected; lane 4, macrophages treated with cytochalasin D and infected; lane 5, uninfected macrophages, treated with erythromycin; lane 6, uninfected macrophages, treated with cytochalasin D. (B) Protein phosphorylations in *Pseudomonas*- and *Salmonella*-infected macrophages. Macrophage monolayers were infected with either *P. aeruginosa* or *S. typhimurium* for 1 h, washed to remove nonphagocytized bacteria, and then incubated for 2 h in minimal essential medium containing penicillin (100 U/ml) and streptomycin (100 μg/ml). Lane 1, uninfected macrophages; lane 2, macrophages infected with 10^8 virulent *Legionella* organisms; lane 3, macrophages infected with 10^8 *Pseudomonas* organisms; lane 4, macrophages infected with 10^8 *Salmonella* organisms. For molecular weight markers, see the legend to Fig. 1.

different from LPS and apparently is not displayed on the surface of avirulent bacteria. Alternatively, the phosphorylation could result from the action of *Legionella* kinases similar to those isolated from *Legionella micdadei* (2). However, these kinases have not been reported to exist in *L. pneumophila*, and the molecular masses of the cellular substrates phosphorylated by these kinases were observed to be less than 50 kDa (17). Phosphorylation of the 76-kDa protein and an increase in the steady-state level of interleukin 1β mRNA (11) are two molecular changes associated with early events surrounding infection of macrophages with virulent *L. pneumophila*. Their association with each other and the role of each in the biology of intracellular survival and growth of *L. pneumophila* are currently under investigation.

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