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De Novo Synthesis of *Legionella pneumophila* Antigens during Intracellular Growth in Phagocytic Cells

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***Legionella pneumophila* is a gram-negative rod which is able to multiply within phagocytic cells. The process of phagocytosis leads to a rapid environmental change that might require a coordinate regulation of gene expression to ensure intracellular survival. Since there is little information on up- and downregulation of genes during the early phases of phagocytosis, we radiolabeled intracellular *L. pneumophila* at different times after phagocytosis by macrophages of the Mono Mac 6 cell line and immunoprecipitated antigens with anti-legionella sera or monoclonal antibodies. We could identify two antigens which were upregulated, one of which was the Mip protein, three antigens which were downregulated, and three antigens which were not detectable in extracellularly grown *L. pneumophila*. The Mip protein was stained most intensively 4 to 8 h after intracellular infection, suggesting that it is needed during intracellular multiplication rather than initiation of infection. A 44-kDa antigen which was not detectable during extracellular growth was most prominent from 2 to 4 h postinfection when Mono Mac 6 cells were used as phagocytic cells. The 44-kDa antigen was also expressed during growth within *Acanthamoeba castelanii*, MRC-5, and U937 cells but with different kinetics. Synthesis of this antigen was not dependent on protein synthesis of the host cell. Since the 44-kDa antigen could be precipitated by an antiserum produced against a recombinant *Escherichia coli* harboring a plasmid with an *L. pneumophila* insert which also codes for the *mip* gene, we believe that the corresponding gene is within the vicinity of the *mip* gene. We named this protein legionella intracellular growth antigen (LIGA), since it could be found exclusively in intracellularly grown *L. pneumophila*.**

Legionella pneumophila, a gram-negative rod, is a ubiquitous inhabitant of the aquatic environment, where it probably survives and multiplies within protozoa such as amoebae (3, 12, 33). In humans, *L. pneumophila* is a facultative intracellular pathogen causing severe atypical pneumonia called Legionnaires' disease (3, 10, 12, 28). The bacterium survives and proliferates not only within human mononuclear phagocytes (19) and tissue culture cells (11, 39) but also in guinea pig and mouse macrophages (20, 40). Having been engulfed via a coiling process by host cells (18), *L. pneumophila* multiplies in the phagosome surrounded by vesicles, mitochondria, and ribosomes (16). The fusion of the phagosomes with host secondary lysosomes does not occur (17).

Considerable effort has been undertaken to characterize the multistep process of bacterial invasion, intracellular multiplication, and cross talk between legionellae and the phagocyte. Intracellular growth of *L. pneumophila* probably requires a coordinate regulation of different genes. On a genetic level, different loci required for growth in host cells have been identified. The products of the *mip* gene (macrophage infectivity potentiator) and of the *icm* locus are essential for efficient infection and/or intracellular multiplication (8, 9, 23). The *dotA* gene is needed for accurate trafficking of cellular organelles during host cell infection (1).

Kwaik et al. showed by metabolic labeling that more than 35 proteins were induced and at least 32 proteins were repressed during intracellular multiplication of *L. pneumophila* in U937 cells (22). Similarly, Miyamoto et al. found up- or downregulation of several bacterial proteins by infecting guinea pig, hamster, and mouse peritoneal macrophages with *L. pneumophila* (27). Yamamoto et al. reported an induction of protein phosphorylation in macrophages during bacterial infection (41). In addition, sufficient iron utilization (5) and bacterial thymidine synthesis (16) are required for growth of *L. pneumophila*. There is also strong evidence that the major outer membrane protein is important for uptake of the bacteria by macrophages (29).

In the present study, we wanted to further elucidate the early molecular events during infection of different host cells by *L. pneumophila* by use of metabolic radiolabeling. To increase sensitivity and facilitate identification, the radiolabeled products were immunoprecipitated with antisera and antibodies with different specificities.

MATERIALS AND METHODS

Bacterial strains, cell lines, antisera, and antibodies. The *Legionella* strains used in this study were *L. pneumophila* serogroup 1 (JR 32) Philadelphia I and its Mip-negative mutant (JR 32-2) (38).

Legionella strains were cultured on buffered charcoal yeast extract (BCYE) agar plates, supplemented with α-ketoglutaric acid, L-cysteine, and ferric pyrophosphate (Unipath, Wesel, Germany), at 37°C and 5% CO_2, for 5 days, frozen in different caps, and used as a stock. All experiments described in this report were performed with the same batch of legionellae. For each experiment, the bacteria were subcultured twice on BCYE and then processed as described below.

For intracellular propagation of legionellae, we used Mono Mac 6 cells (obtained from H. W. L. Ziegler-Heitbrock, Munich, Germany [42]), U-937 cells (ATCC CRL 1593), MRC-5 cells (ATCC CCL 171), and *Acanthamoeba castelanii* (ATCC 30010).

Polyclonal rabbit anti-legionella sera were used for immunoprecipitation (Table 1). Antisera were prepared in rabbits. Two rabbits per antigen were injected intravenously four times at weekly intervals. One week after the last injection, the rabbits were sacrificed and blood was obtained by heart puncture. The sera were pooled, heated to 56°C for 30 min, and stored at −70°C. The *Escherichia coli* strains used for immunization carried pLAFR 2 as a vector and contained a ca. 20-kbp insert of the *Legionella* genome (37).

We also used mouse monoclonal antibodies directed against Mip protein

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TABLE 1. Polyclonal anti-legionella sera and immunization protocol

Serum no.	Strain	Antigen production procedure	Relevant properties
A3	*L. pneumophila* ATCC 33125	Formalin inactivation	Indirect immunofluorescence titer, 1:400; antibodies against 15-, 19-, 29-, and 60-kDa antigens by use of agar-grown *Legionella* spp.
A4	*L. pneumophila* ATCC 33125	Not treated	Indirect immunofluorescence titer, 1:2,000; antibodies against 15-, 19-, 29-, and 60-kDa antigens by use of agar-grown *Legionella* spp.
A8	*L. pneumophila* ATCC 33125	Boiling for 2 h	Indirect immunofluorescence titer, 1:200; antibodies against 19-, 29-, and 60-kDa antigens by use of agar-grown *Legionella* spp.
A6	*E. coli* K-12 HB101(pBL1920)	Formalin inactivation	Cosmid contains a 20-kbp insert of *L. pneumophila* coding for a 60-kDa heat shock protein
A7	*E. coli* K-12 HB101(pBL3039)	Formalin inactivation	Cosmid contains a 20-kbp insert of *L. pneumophila* coding for a 24-kDa Mip protein

(monoclonal antibody 2D8) (13), legionella heat shock protein (monoclonal antibody 2125; kindly supplied by D. Bitter-Suermann, Hannover, Germany) (34), and vimentin (monoclonal antibody V9; Sigma).

Cell infection. The bacteria were harvested from BCYE plates, washed twice, resuspended in RPMI 1640 serum-free medium, and added to cells at a bacterium/cell ratio of 100:1. This time point was defined as time zero. After an initial centrifugation at 400 × *g* for 20 min, the mixture of cells and legionellae was incubated for 50 to 60 min at 37°C in 5% CO_2. The cells were then washed two times to remove the extracellular bacteria. The flasks with infected cells were incubated for 50 to 60 min at 37°C in cell culture medium with 100 μg of gentamicin per ml to kill any adherent bacteria that had not been ingested by host cells.

To detect antigens which had been synthesized by 1 h postinfection, the bacteria were suspended in a medium without L-methionine and L-cysteine (see below) and added to the Mono Mac 6 cells. After incubation for 30 min, the bacteria were removed by centrifugation and then pulsed for 30 min with [^{35}S]methionine and [^{35}S]cysteine.

In some experiments, the host cells were treated for 4 h with cycloheximide (100 μg/ml) to arrest host cell protein synthesis (4) or 2 h after infection for the next 2 h with erythromycin (10 μg/ml) to kill the intracellular bacteria (25). The efficiency of intracellular bacterial multiplication was determined 2 and 24 h after infection. The infected cells were lysed with 1 ml of distilled water. An aliquot of 0.1 ml of a serial 10-fold dilution of the lysates in buffered yeast extract (BYE) broth was plated in duplicate on BCYE agar plates for determination of bacterial counts. The number of CFU per milliliter was determined after incubation for 10 days at 37°C in 5% CO_2.

Radioactive labeling and immunoprecipitation. For biosynthetic labeling, 2×10^7 to 3×10^7 infected cells were, after the indicated time of infection, washed two times with warm labeling medium without L-methionine and L-cysteine, incubated in the same medium for 30 min at 37°C, and pulsed with [^{35}S]methionine and [^{35}S]cysteine (400 μCi; PRO-MIX; Amersham) for an additional 30 min at 37°C. The adherent cells were removed from the dishes mechanically after incubation with 0.1% trypsin-EDTA in phosphate-buffered saline (PBS; pH 7.4). After three washes with cold PBS, cellular and bacterial proteins were extracted from cell pellets by sonication in lysis buffer (50 mM Tris-HCl [pH 8.3], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1 mg of ABTS per ml, 1 μg of leupeptin per ml, and 1 μg of aprotinin per ml). The lysates were incubated for 15 min at 4°C on a cell mixer and then cleared by centrifugation at 19,000 × *g* for 10 min. The supernatants were either stored at −20°C or used directly for immunoprecipitation.

To determine de novo antigen synthesis during extracellular growth, bacteria were inoculated into BYE broth supplemented with α-ketoglutaric acid, L-cysteine, and ferric pyrophosphate (Unipath) and incubated. The bacteria were radiolabeled at growth phases similar to those of intracellularly grown legionellae. After two washes with BYE broth without yeast extract and L-cysteine, the bacteria were incubated for 30 min in the same medium and then pulsed with [^{35}S]methionine or [^{35}S]cysteine (400 μCi; PRO-MIX) for an additional 30 min. The radiolabeled bacteria were harvested by centrifugation, washed three times with cold water, and lysed by sonication in lysis buffer for 30 to 60 s at 4°C. The lysates were incubated in lysis buffer for 15 min at 4°C and cleared by centrifugation at 19,000 × *g* for 10 min. The supernatants containing the radiolabeled proteins were collected and stored at −20°C or immunoprecipitated directly.

For immunoprecipitation, an aliquot of labeled lysates (10 μCi) was precleared with 5 μl of normal rabbit serum and 25 μl of protein A-Sepharose (Pharmacia, Freiburg, Germany) for 60 min at 4°C. After removing the immune complexes bound to Sepharose beads, the same sample was incubated with specific polyclonal serum (5 μl) or monoclonal antibody (2 μl) for 60 min at 4°C and then for an additional 60 min with protein A-Sepharose (50 μl). The immune complex-bound beads were washed twice in 50 mM Tris-HCl (pH 7.5)–150 mM NaCl–0.1% Nonidet P-40, twice in high-salt buffer (50 mM Tris-HCl [pH 7.5], 500 mM NaCl, 0.1% Nonidet P-40), and finally once in 10 mM Tris-HCl (pH 7.5). Adsorbed antigens were eluted by boiling for 5 min in 50 μl of 50 mM Tris-HCl (pH 6.8) containing 2% sodium dodecyl sulfate (SDS), 10% glycerol, 200 mM dithiothreitol, and a trace of bromphenol blue dye. All samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) with a 12% acrylamide gel system. ^{35}S-labeled proteins were visualized by fluorography after impregnation with Amplify (Amersham).

Determination of the amino-terminal protein sequence of the 44-kDa antigen. Approximately 10^9 infected Mono Mac 6 cells were collected and lysed in lysis buffer. By use of A7 polyclonal rabbit serum, lysed proteins were immunoprecipitated and separated on an SDS–10% polyacrylamide gel until a 31-kDa marker protein (Rainbow protein molecular weight marker; Amersham) had just migrated out of the gel. The proteins were electrotransferred to a polyvinylidene difluoride membrane (Immobilon-PSQ; Millipore). Protein bands were stained with Coomassie brilliant blue. The 44-kDa protein was excised and microsequenced by automated Edman chemistry in a Beckman PI 3000 sequencer. The protein sequence (KVTMTQGPSKVQAAT) was analyzed with the GenBank, EMBL, and Swiss-Prot databases, but no significant homology with known protein sequences was found.

RESULTS

Legionella protein profiles during intracellular growth. A profile of newly biosynthesized proteins of the bacteria grown in Mono Mac 6 cells (Fig. 1b) was compared with either those of broth-grown bacteria (Fig. 1a) or protein profiles of noninfected host cells. As shown in Fig. 1, biosynthesis of three novel proteins of 44, 35, and 15 kDa (Fig. 1b, lanes 1 and 4) could be observed exclusively during intracellular growth of bacteria at 4 h after infection. Antigens in the range of 44 kDa could also be detected in broth-grown bacteria. However, these proteins were regarded to be different from the 44-kDa protein upon further examination, since they could not be precipitated by the A7 antiserum. There were also several proteins whose intracellular biosynthesis was downregulated (Fig. 1b, lanes 1 and 2 [72, 37, and 22 kDa]) or increased (lanes 1 [16 kDa] and 3 [60-kDa legionella heat shock protein]). We could not detect any intracellularly synthesized proteins by use of an antiserum raised against boiled *L. pneumophila*. These changes in protein profiles were reproducible in five individual experiments. The sera used for immunoprecipitation showed no reactions with uninfected cells (data not shown).

Both the Mip protein and a 44-kDa antigen could be detected by use of an antiserum raised against a recombinant *E. coli* harboring a plasmid with a 20-kbp insert of *L. pneumophila*. This antiserum was used since Mip is regarded as a virulence factor contributing to infectivity and intracellular growth and since virulence factors may be genetically organized in pathogenicity islands (30). In the following experi-

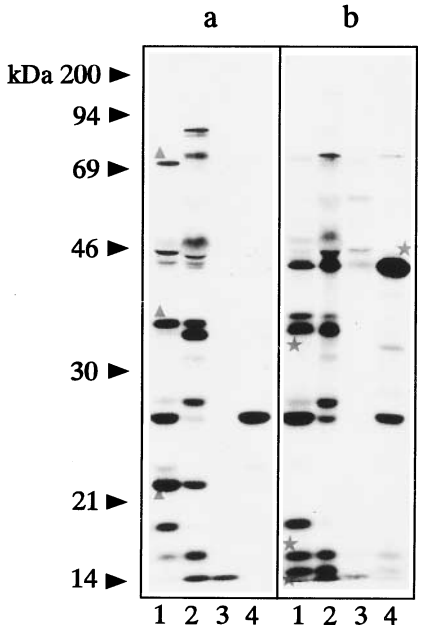

FIG. 1. Autoradiographs of SDS-polyacrylamide gel showing protein profiles of *L. pneumophila* grown in broth (a) and intracellularly 4 h after infection of Mono Mac 6 cell line (b). The immunoprecipitation was done with different anti-legionella sera (A3 [lane 1], A4 [lane 2], A6 [lane 3], and A7 [lane 4]). The same total radioactivity was loaded in each gel. The positions of the molecular mass standards are indicated on the left. Symbols on gels: ★, expression was upregulated; ▲, expression was downregulated.

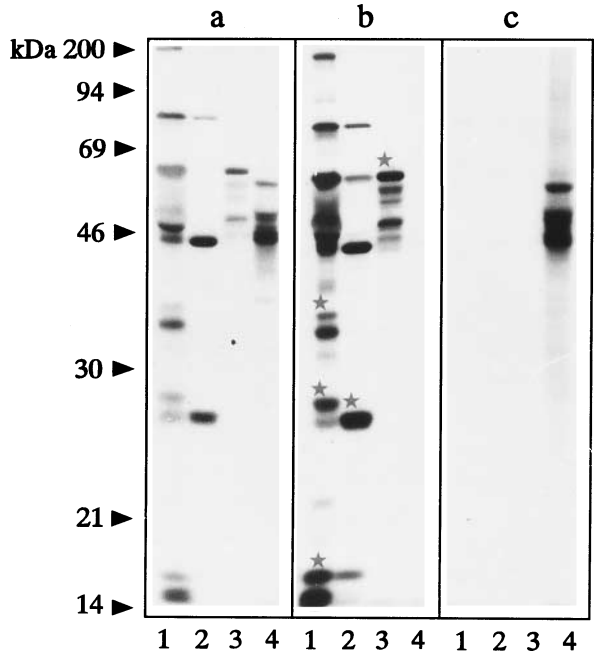

FIG. 3. Autoradiographs of SDS-PAGE of proteins from radiolabeled *L. pneumophila* 4 h after infection of Mono Mac 6 cells (a) in comparison with protein profiles after treatment with cycloheximide (b) and erythromycin (c). The antisera A4 (lane 1), A7 (lane 2), and A6 (lane 3) and V9 anti-vimentin monoclonal antibody (lane 4) were used for immunoprecipitation. Symbol on gel: ★, upregulated after cycloheximide treatment.

ments, we focused on the Mip protein and the related 44-kDa antigen.

Intracellular expression of the Mip protein and the novel 44-kDa protein. In a first set of experiments, the kinetics of the intracellular expression of both antigens was studied by use of the A7 antiserum (Fig. 2). As early as 1 h after infection, synthesis of a 44-kDa antigen was detectable. The expression of the 44-kDa antigen peaked between 2 and 4 h after infection, decreased slowly, and still remained present even at 32 h postinfection. The biosynthesis of the Mip protein differed markedly from that of the 44-kDa protein. During the first hour postinfection, Mip was not newly synthesized. It was expressed most intensively 4 to 8 h after infection, while it was barely visible during the late stage of intracellular bacterial growth.

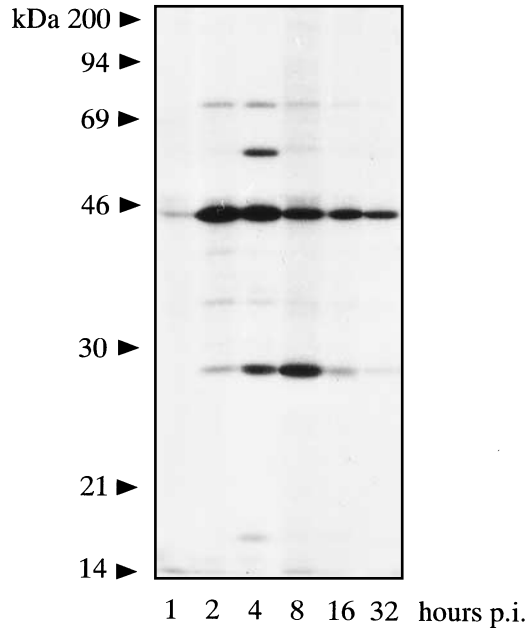

FIG. 2. Autoradiographs of SDS-polyacrylamide gel showing kinetics of intracellular expression of the 44-kDa antigen and the Mip protein (24 kDa) in the Mono Mac 6 cell line. A7 rabbit anti-legionella serum was used for immunoprecipitation. p.i., postinfection.

The 44-kDa protein is a bacterial antigen. To elucidate whether the 44-kDa protein is a prokaryotic or eukaryotic antigen, we treated the macrophages with cycloheximide to inhibit eukaryotic protein synthesis or with erythromycin, which inhibits intracellular growth of the bacteria.

Treatment of legionella-infected macrophages with cycloheximide resulted in the upregulation of some intracellular bacterial antigens as detected by serum A4 (Table 1). During the early phases of the infection, the expression of the 44-kDa protein did not change (Fig. 3b). However, the staining intensity should be interpreted with caution. Since eukaryotic protein synthesis is arrested in cycloheximide-treated cells, most of the counts in the labeled lysates refer to bacterial proteins and could thus be enriched during precipitation with antisera. In nonpretreated cells, a major proportion of the counts may represent eukaryotic proteins which are not precipitated by the antiserum. Therefore, the counts per minute loaded onto the gel differed, although the counts per minute were identical in the labeled lysates. However, it can be concluded that *L. pneumophila* did not require intact host cell functions during intracellular bacterial growth to express the 44-kDa protein. Moreover, cycloheximide treatment of host cells led to an increased

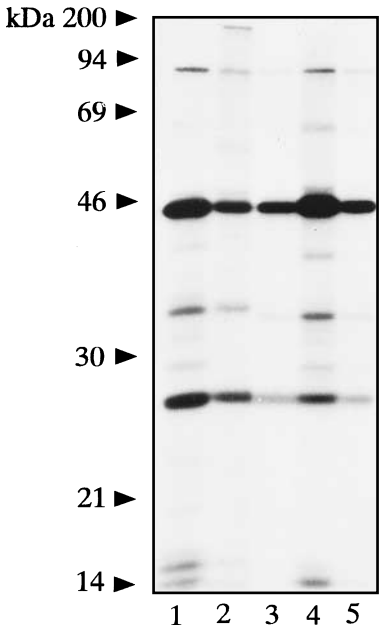

FIG. 4. Autoradiographs of SDS-PAGE showing the expression of the 44-kDa antigen in different host cells 4 h (Mono Mac 6, U937, and HL-60 cells), 2 days (MRC-5 cells), and 3 days (*A. castelanii*) after infection. Immunoprecipitations were done with A7 antiserum. Lanes: 1, Mono Mac 6; 2, U937; 3, HL-60; 4, MRC-5; 5, *A. castelanii.*

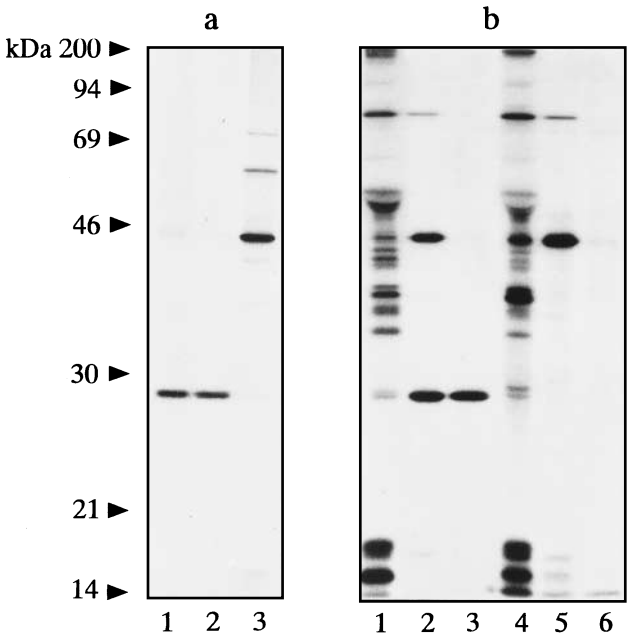

FIG. 5. (a) Autoradiograph of SDS-PAGE showing a sequential immunoprecipitation of intracellular radiolabeled *L. pneumophila* 4 h after infection of Mono Mac 6 cells. Immunoprecipitation was done first with monoclonal antibody 2D8 (lanes 1 and 2) and sequentially with antiserum A7 (lane 3). (b) Autoradiograph of SDS-PAGE showing the intracellular expression of the 44-kDa and Mip proteins in Mono Mac 6 cells 4 h after infection with wild-type *L. pneumophila* (lanes 1 to 3) or the Mip-negative mutant of *L. pneumophila* (lanes 4 to 6). Sera A4 (lanes 1 and 4) and A7 (lanes 2 and 5) and monoclonal antibody 2D8 (lanes 3 and 6) were used for immunoprecipitation.

expression of some legionella proteins like the Mip protein (24 kDa) or heat shock protein (60 kDa) (Fig. 3b, serum A7 and serum A6). However, eukaryotic protein synthesis, as represented by the synthesis of the cell structural protein vimentin, was completely downregulated.

Finally, we tested whether the exposure of infected macrophages to erythromycin 2 h after initiation of infection would affect the observed 44-kDa protein synthesis. The results are shown in Fig. 3c. The synthesis of all bacterial proteins was depressed. The erythromycin treatment did not influence the synthesis of vimentin. These results suggest that the 44-kDa protein does not require an intact host cell protein machinery for its synthesis and that this protein is a bacterial antigen.

The expression of the 44-kDa protein is not dependent on host cells. The expression of the 44-kDa protein could be host cell specific. Therefore, we have infected different monocyte-like cells (U937 and HL-60), fibroblast-like MRC-5 cells, and *A. castelanii* as some of the proposed natural hosts of *Legionella* spp. The immunoprecipitation with serum A7 detected the synthesis of the 44-kDa protein in all tested cell lines (Fig. 4). However, expression occurred at different times after infection and at different levels. The highest levels of 44-kDa antigen expression were similar in all monocyte-like cells (Fig. 4, lanes 1 to 3) and occurred 4 to 8 h after infection, whereas in MRC-5 cells, the highest peak of 44-kDa protein synthesis was 2 days after infection (Fig. 4, lane 4), and in *A. castelanii*, it was 3 days after infection (Fig. 4, lane 5).

The Mip protein does not interfere with the synthesis of the 44-kDa protein. Since the antiserum A7 preferentially detected the Mip protein in legionellae grown on agar, we wanted to show the relatedness between 44-kDa antigen and Mip. We therefore performed a sequential immunoprecipitation of the radiolabeled intracellularly synthesized proteins by use of the monoclonal anti-Mip antibody first and then, afterwards, serum A7 (Fig. 5a, lanes 1 and 2). With this monoclonal antibody, we could precipitate the Mip proteins but not the 44-kDa proteins. Furthermore, the second precipitation of the same cell lysate with the polyclonal polyspecific serum (A7) showed only the 44-kDa proteins (Fig. 5a, lane 3). The same results were obtained by use of nondenatured PAGE. Obviously, there were no physical conditions like covalent binding forces that would allow a coprecipitation of both proteins with the same antibody. This finding suggests that the 44-kDa antigen is immunologically unrelated to the Mip protein. However, it should be kept in mind that the Mip dimer, a proposed functional form of Mip (34), might not be recognized by the monoclonal antibody.

To exclude an association with the Mip protein on the genetic level, we have infected the Mono Mac 6 cells with the Mip-negative mutant of *L. pneumophila* (JR 32-2), which cannot synthesize the Mip protein. Four hours after infection of the macrophages, the biosynthesized proteins were radiolabeled and precipitated with two polyclonal sera (A4 and A7) and anti-Mip monoclonal antibody (Fig. 5b). The Mip-negative legionella mutant did not express the Mip protein (lane 5), but both mutant and wild-type *Legionella* strains expressed the 44-kDa antigen (lanes 2 and 5). In the absence of Mip protein synthesis, the anti-Mip monoclonal antibody did not recognize the 44-kDa protein (lane 6). This indicates a genetic independence of 44-kDa protein from the Mip protein.

DISCUSSION

Different approaches have been used for characterization of the pathogenicity of *Legionella* spp. Molecular epidemiology has led to the identification of legionella metalloprotease (35), legiolysin (37), iron superoxide dismutase (32), major outer

TABLE 2. Legionella antigens regulated by intracellular growth

No. of antigens (size[s])	Type of regulation	Host cell type	Reference
4 (100, 65, 24, and 16 kDa)	Upregulated	Peritoneal macrophages	27
At least 35	Upregulated	U937	22
At least 32	Downregulated	U937	22
1 (40 kDa)	Expressed uniquely intracellularly	U937	22
3 (60 [Hsp], 24 [Mip], and 16 kDa)	Upregulated	Mono Mac 6	This paper
3 (72, 37, and 22 kDa)	Downregulated	Mono Mac 6	This paper
3 (44, 35, and 15 kDa)	Expressed uniquely intracellularly	Mono Mac 6	This paper

membrane protein (14), heat shock protein (15), and major iron-containing protein (24), which, essentially by analogy to other pathogenic microorganisms, may be considered as being virulence associated. The genetic approach attempting to fulfill the so-called molecular Falkow-Koch postulates helped to identify previously unknown genes such as *mip*, *icm*, and *dotA* (1, 2, 7, 20), which in addition might represent a new class of pathogenicity factors. A third, much-less-used strategy is to study the impact of intracellular growth on the synthesis of bacterial proteins. Using this approach, Kwaik et al. (22) and Miyamoto et al. (27) found the changes in intracellular synthesis of bacterial proteins shown in Table 2. To facilitate presumptive identification and to increase the sensitivity and specificity of this method, we combined metabolic radiolabeling with precipitation by antisera and antibodies, and therefore, in contrast to Kwaik et al. (22), were able to detect the bacterial protein synthesis in host cells during early infection. To cover a broad spectrum of detectable antigens, crude bacterial preparations of heat-killed, formalin-killed, and live bacteria were used for immunization. Recombinant *E. coli* harboring plasmids with only partially characterized 20-kbp inserts of *L. pneumophila* was also used as an antigen. It was suspected that in addition to the identified *mip* and *hsp* genes, other virulence-associated genes might be located in their vicinity since virulence genes have been reported to be organized as pathogenicity islands (30). Highly specific monoclonal antibodies were employed to allow identification of certain antigens.

As host cells, Mono Mac 6 cells were used instead of the commonly used myeloblastic U937 and HL-60 cells. This cell line is more differentiated and does not require stimulation by phorbol ester (42). Mono Mac 6 cells, however, handle *L. pneumophila* infections like U937 cells do and respond to infection by cytokine secretion (unpublished data).

With these tools, we could confirm that the intracellular growth of *Legionella* spp. is accompanied by a drastic change of metabolic pathways. In addition to Kwaik et al. (22), we could show that many of the changes had already taken place within the first 4 to 8 h postinfection, reflecting a rapid adaptation process. It was remarkable that the Hsp60 which is induced by higher temperatures (15) is not upregulated in our system.

The Mip protein which contributes to bacterial uptake and intracellular multiplication is upregulated no earlier than 4 to 8 h after infection. This suggests that in this cell system, the Mip protein might play a role during intracellular bacterial growth rather than initiation of infection. When mastocytoma cells transfected with the *mip* gene were used, we could show that the cytosolic Mip was able to support intracellular growth of a Mip-deficient *L. pneumophila* mutant (21). This also suggests that Mip might be dispensable during uptake by some cell types.

Along with Mip, a second antigen of 44 kDa was precipitated with antiserum A7, suggesting that additional antigens might be encoded by the insert. A number of experiments have been undertaken to further characterize this protein. Results have shown that the antigen (i) is an early antigen in cells in which *L. pneumophila* grows rapidly, (ii) is expressed in cell lines of different evolutionary origin, and (iii) does not require active metabolism of the host cell. Because of the absence of molecular weight markers in the report of Kwaik et al. (22), it is difficult to compare our findings with theirs; however, it is suggestive that the 40-kDa protein of Kwaik et al. is the same as our 44-kDa antigen, since both are expressed exclusively in intracellular *Legionella* spp.

Although the 44-kDa antigen might be coded for in the vicinity of *mip* in plasmid pBL3039, it is genetically and immunologically different from Mip since (i) the monoclonal antibody 2D8 did not precipitate or coprecipitate the 44-kDa antigen and (ii) the Mip-negative *L. pneumophila* still expressed the 44-kDa antigen. The lack of sequence homology with the *mip* gene eliminates the possibility that the 44-kDa antigen is a dimeric form of the Mip protein.

Since this antigen is expressed during intracellular growth, we termed it legionella intracellular growth antigen (LIGA).

Whether the intracellular growth regulation indicates that this antigen is a pathogenicity factor is an open question. Preliminary experiments suggest that it is not a pathogenicity factor sensu strictu, since *Legionella* spp. not multiplying in phagocytic cells (e.g., *Legionella steigerwaltii*) also produce this protein (6). However, the same is true for the Mip antigen which is produced by most *Legionella* species regardless of their pathogenicity. A discussion of its role in *Legionella* spp. is, therefore, merely speculative at the moment. The Mip antigen could represent a small stress protein upregulated during the immediate early growth phase or could participate in communication between the bacterium and the host.

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