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Differential Effects of Virulent versus Avirulent *Legionella pneumophila* on Chemokine Gene Expression in Murine Alveolar Macrophages Determined by cDNA Expression Array Technique

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The cDNA expression array technique is a powerful tool to determine, at one time from many genes, specific gene messages modulated by infection. In the present study, we identified genes modulated in response to virulent versus avirulent *Legionella pneumophila* infection of the alveolar macrophage cell line MH-S by the cDNA expression array technique. Many macrophage genes were found to be modulated after 5 h of in vitro infection with *L. pneumophila*. In particular, it was found that the monocyte chemotactic protein 3 (MCP-3) gene expression was significantly induced by infection with virulent *L. pneumophila* but not with avirulent *L. pneumophila*. In contrast, other chemokine genes, such as macrophage inflammatory protein (MIP) 1α, were induced by both virulent and avirulent *L. pneumophila*. Reverse transcription (RT)-PCR assay of total RNA isolated from macrophages infected with the bacteria for 5 or 24 h confirmed the differential induction of the chemokine genes by virulent versus avirulent *L. pneumophila*. Thus, the cDNA expression array technique readily revealed differential induction by *L. pneumophila* infection of select chemokine genes of macrophages from more than 1,100 genes. These results also indicate that certain chemokine genes may be selectively induced by virulent bacteria.

Legionella pneumophila is the causative agent of Legionnaires' disease, a severe form of pneumonia and sometimes a systemic infection, especially in immunocompromised individuals with defective immune response mechanisms (4). *L. pneumophila* is a gram-negative bacillus and grows preferentially in macrophages and other phagocytic cells. Development of cell-mediated immunity is known to be essential in host defense to *L. pneumophila* infection (6). Specific cytokines are considered key factors in host immunity to intracellular microorganisms, especially those produced by macrophages which help to regulate development of cellular immunity. Although numerous studies concerning *L. pneumophila* infection have been conducted, the interaction between this organism and alveolar macrophages is still not well understood. The newly developed cDNA expression array technique with membranes can differentially detect more than 1,100 expressed genes at one time and is considered an excellent method to determine gene messages which can be expressed by cells (9). Since identifying genes that are modulated by infection may provide important insights into the key molecular changes in the pathogenesis of infection, this relatively new array technique to detect the expression of different genes was used to investigate *L. pneumophila* infection in alveolar macrophages.

For these experiments, the MH-S murine alveolar macrophage cell line, which was derived from BALB/c mouse alveolar macrophages (7), was used as the source of target cells for *L. pneumophila* infection. MH-S cells obtained from the American Type Culture Collection, Manassas, Va., were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (Hyclone Laboratories, Logan, Utah). The MH-S cells were adhered to a tissue culture dish (100 by 20 mm; BD Falcon, Franklin Lakes, N.J.) at a concentration of 10×10^6 cells/dish for 2 h in 5% CO_2 at 37°C and then used for the experiments. Virulent *L. pneumophila* M124 (3) and avirulent *L. pneumophila* M124-Av, which was prepared by multiple passage of M124 (11), were cultured on buffered-charcoal yeast extract medium (Gibco Laboratories, Madison, Wis.) for 3 days at 37°C, as described previously (3). The virulent *L. pneumophila* M124 strain was lethal for genetically susceptible strain A/J mice, whereas avirulent *L. pneumophila* strain M124-Av was not lethal for the strain A/J mice infected intraperitoneally (11). The growth of the bacteria in macrophages was also different between virulent and avirulent *L. pneumophila* strains (11). That is, virulent *L. pneumophila* M124 showed a more than 100-fold increase in the number of viable bacteria in susceptible mouse strain A/J peritoneal macrophages during 48 h of culture, but avirulent strain M124-Av did not. The MH-S cells were infected with either *L. pneumophila* M124 or M124-Av for 30 min at a concentration of 100 bacteria per cell, washed to remove noninfected bacteria with Hank's balanced salt solution, and then incubated in RPMI 1640 medium with 10% fetal calf serum. The number of viable bacteria in macrophage lysates prepared with 0.1% saponin (Sigma Chemical Co., St. Louis, Mo.) was determined by standard plate counts on buffered-charcoal yeast extract medium, as described previously (13).

Total cellular RNA from cultured cells was extracted with the Atlas Pure Total RNA labeling system (Clontech, Palo Alto, Calif.) at 5 h postinfection, and quantification of extracted RNA was performed with the RiboGreen RNA quan-

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TABLE 1. *L. pneumophila* growth in MH-S alveolar macrophage cell line

Time after infection (h)	Mean growth[a] ± SD of *L. pneumophila* strain[a]	
	M124 (virulent)	M124-Av (avirulent)
0	$3.4 \times 10^2 \pm 0.5 \times 10^2$	$2.4 \times 10^2 \pm 0.4 \times 10^2$
5	$5.3 \times 10^2 \pm 0.4 \times 10^2$	$2.5 \times 10^2 \pm 0.6 \times 10^2$
24	$6.7 \times 10^4 \pm 0.8 \times 10^4$	$3.9 \times 10^2 \pm 0.9 \times 10^2$
48	$1.8 \times 10^5 \pm 0.2 \times 10^5$	$4.4 \times 10^2 \pm 0.5 \times 10^2$

[a] Number of viable bacteria (CFU) in MH-S cell lysates measured at indicated time after infection by plate count method. Data are from triplicate cultures and represent three experiments.

titation kit (Molecular Probe, Eugene, Oreg.) in an Fmax fluorometer (Molecular Probe). Since the macrophage response to bacteria is complicated due to involvement of multiple factors in the bacteria-macrophage interaction, analysis of gene expression at an early time point of interaction, such as 5 h postinfection, was chosen for this study. To determine gene expression, the membrane-based microtechnique with an Atlas cDNA expression array (mouse 1.2 array; Clontech) was performed in accordance with the manual provided. The array included 1,176 mouse cDNAs and 9 housekeeping control cDNAs and negative controls immobilized on a nylon membrane. The cDNAs on a membrane are divided into 22 categories: (i) 25 cDNAs for cell surface antigens, (ii) 290 cDNAs for transcription factors and DNA-binding proteins, (iii) 45 cDNAs for cell cycle regulators, (iv) 55 cDNAs for cell adhesion receptors and proteins, (v) 4 cDNAs for extracellular transporters, (vi) 81 cDNAs for oncogenes and tumor suppressors, (vii) 20 cDNAs for stress response proteins, (viii) 36 cDNAs for ion channels and transport proteins, (ix) 2 cDNAs for extracellular matrix proteins, (x) 1 cDNA for trafficking and targeting protein, (xi) 12 cDNAs for metabolic pathways, (xii) 2 cDNAs for posttranslational modification and folding, (xiii) one cDNA for translation, (xiv) 60 cDNAs for apoptosis-associated proteins, (xv) 97 cDNAs for receptors, (xvi) 116 cDNAs for extracellular cell signaling and communication, (xvii) 160 cDNAs for modulators, effectors, and intracellular transducers, (xviii) 39 cDNAs for protein turnover, (xix) 57 cDNAs for cytoskeleton and motility proteins, (xx) 48 cDNAs for DNA synthesis, repair, and recombination proteins, (xxi) 25 cDNAs for other, and (xxii) 9 cDNAs for housekeeping genes.

The purified RNA, which was analyzed for genomic DNA contamination by PCR with primers specific for β-actin, was processed with the gene-specific CDS primer mix (Clontech), deoxynucleoside triphosphate, [^{32}P]dATP, and reverse transcriptase for preparation of cDNA. The ^{32}P-labeled cDNA was purified through a Chroma Spin-200 column (Clontech). The labeled cDNA in a solution of ExpressHyb (Clontech) with heat-denatured, sheared-salmon-testes DNA was then hybridized overnight to the Atlas array membrane at 68°C. The membrane was washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) with 1% sodium dodecyl sulfate, 0.1× SSC with 0.5% sodium dodecyl sulfate, and 2× SSC, sequentially, and then exposed to a PhosphorImager (Storm 860; Molecular Dynamics, Sunnyvale, Calif.). Results of the gene expression were analyzed by computer with Atlas image software (Clontech).

L. pneumophila readily infected the MH-S cells, as shown in Table 1, and 5 h after infection, the bacteria numbers minimally increased in both virulent- and avirulent-*L. pneumophila*-infected cultures. However, by 24 h after infection, it was obvious that the virulent *L. pneumophila* strain grew remarkably, but the avirulent strain did not, similar to previous results concerning growth of virulent versus avirulent *L. pneumophila* in primary peritoneal macrophages from genetically susceptible strain A/J mice (13).

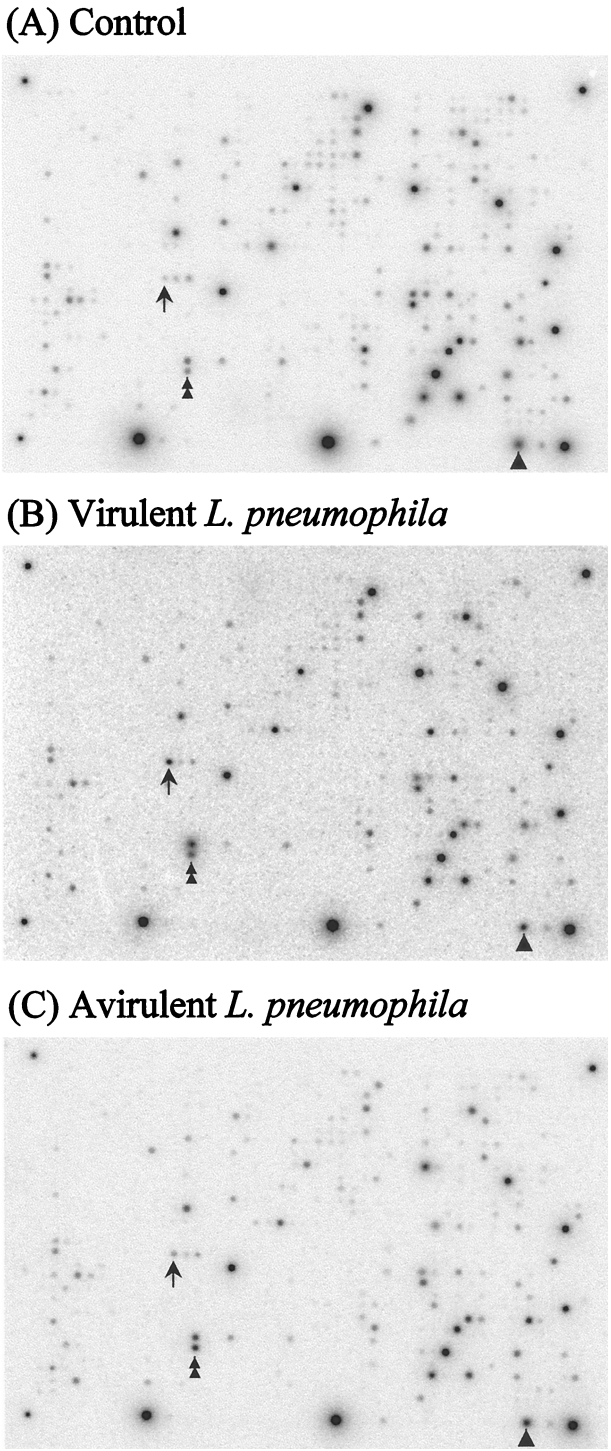

FIG. 1. Phosphorimages of cDNA expression array membranes for MH-S cells infected with or not infected with *L. pneumophila*. The total RNA was extracted from cells infected with either virulent (B) or avirulent (C) *L. pneumophila* and cells not infected with either strain (A) as the control at 5 h postinfection and subjected to cDNA expression array assay. Arrowheads, double arrowheads, and arrows indicate β-actin, MIP-1β, and MCP-3 cDNA, respectively.

The MH-S cells infected with either *L. pneumophila* M124

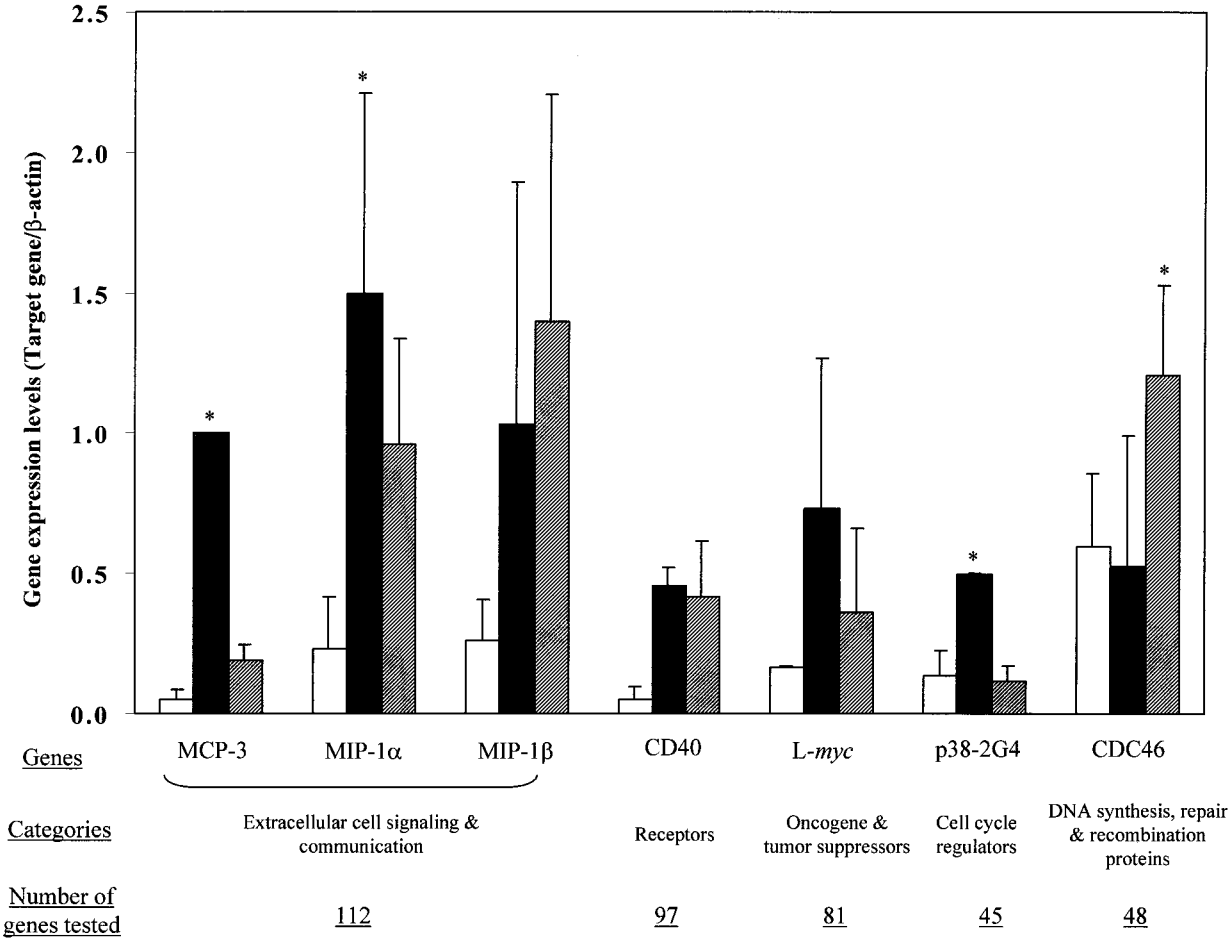

FIG. 2. Gene expression levels for selected genes in MH-S cells infected with *L. pneumophila* at 5 h postinfection or not infected. Results represented are means plus standard deviations from three independent experiments. *, $P < 0.05$ compared to noninfected control analyzed by Student's *t* test. Open column, noninfected control; closed column, infected with virulent *L. pneumophila* (M124); gray column, infected with avirulent *L. pneumophila* (M124-Av).

or M124-Av were analyzed for gene expression by the cDNA array technique at an early-infection time point, such as 5 h postinfection, as compared to gene expression in noninfected control cells. Comparison of phosphorimages of DNA from the control cultures not infected with *L. pneumophila* as compared to cultures infected with virulent *L. pneumophila* M124 showed that only select genes were significantly modulated (Fig. 1). Figure 2 shows a semiquantitative analysis of select genes as the ratio of target gene expression versus housekeeping β-actin genes, which was stable between control versus infected cells. Since there were many genes affected by infection in terms of expression level, stable up-regulated genes were specifically assessed between experiments after infection. As was apparent by analysis of specific gene expression levels in comparison to β-actin gene expression, there were several genes which were markedly induced by the virulent but not by the avirulent bacteria (Fig. 2). It should be noted that the lower expression levels of genes varied between experiments. However, the expression of several genes related to inflammation was significantly modulated by infection with *L. pneumophila*. In particular, infection with the virulent *L. pneumophila* M124 strain significantly up-regulated gene expression for monocyte chemotactic protein 3 (MCP-3) and macrophage inflammatory protein 1α (MIP-1α), and *p38-2G4* (the gene specifically for cell cycle-modulated nuclear protein [8]). Other genes, such as those for MIP-1β and CD40 and the L-*myc* gene, were induced by the virulent *L. pneumophila* strain in some experiments; however, there was no significant difference between infected and noninfected groups due to a high variation in gene expression levels between experiments. It is important to note that modulation of the gene for MCM5 DNA replication licensing factor (the CDC46 homolog), which is involved in the initiation of cell-cycle-specific DNA replication and expression at the late G1 to S phase (5), seemed to occur readily in the alveolar macrophage cells infected with avirulent *L. pneumophila*, but not in cells infected with the virulent bacteria. However, the pathophysiological role of this gene in *L. pneumophila* infection is not known.

Since an effective host defense against bacterial invasion is characterized by the vigorous recruitment and activation of inflammatory cells, chemokine production is considered a critical event during infection (10). In this regard, chemokine MCP-3 and MIP-1β messages in *L. pneumophila*-infected cells were further investigated by reverse transcription-PCR (RT-PCR). RNA extraction and RT-PCR were performed as described previously (12). The PCR primers for β_2-microglobulin (housekeeping gene), MIP-1β, and MCP-3 were designed from GenBank cDNA sequences using a website program Primer 3 (http://www.path.cam.ac.uk/cgi-bin/primer3.cgi). The PCR was performed in a Minicycler (MJ Research, Watertown, Miss.) for either 25 cycles (β_2-microglobulin and MIP-1β) or 30 cycles (MCP-3) and at a 60°C annealing temperature. PCR products

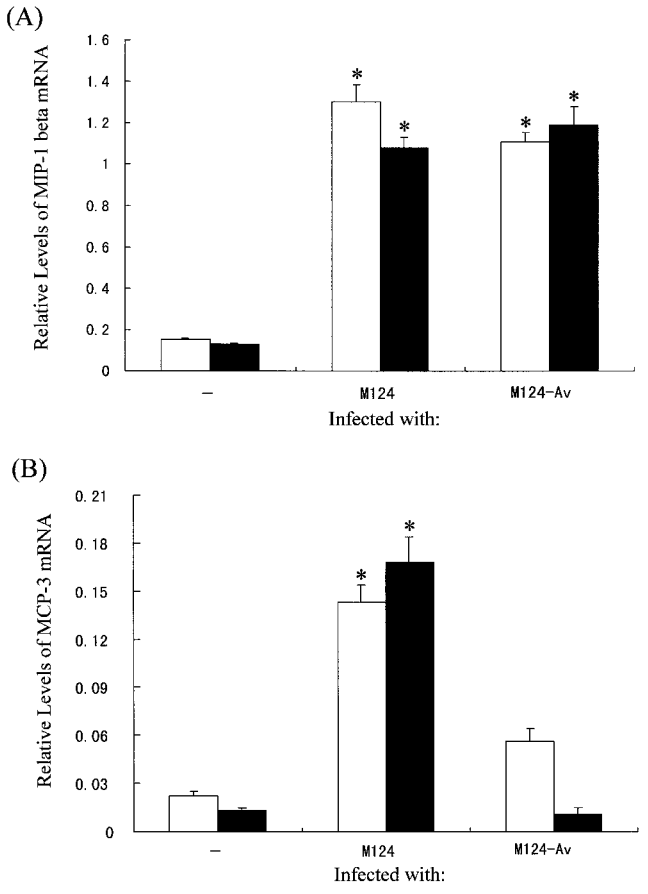

FIG. 3. Levels of MIP-1β (A) and MCP-3 (B) mRNA in MH-S cells infected with virulent (strain M124) or avirulent (strain M124-Av) *L. pneumophila*. RNA was extracted from the cells at 5 h (open column) or 24 h (closed column) postinfection. The mRNA expression for chemokines was determined by RT-PCR and normalized to β_2-microglobulin using densitometry readings. The data are presented as the ratio (mean plus standard deviation) of chemokines to β_2-microglobulin densities from three independent experiments. —, noninfected control; *, $P < 0.05$ compared to noninfected control.

were analyzed on ethidium bromide-stained 2% agarose gels, semiquantitated, and normalized to β_2-microglobulin using densitometry readings.

Both MCP-3 and MIP-1β belong to the CC subfamily of chemokines and are involved in early inflammatory responses, including infection (1). In previous studies, we observed induction of MIP-1β and other chemokines, such as MIP-2 and KC, by *L. pneumophila* infection of macrophages (12, 14). However, chemokine induction by virulent versus avirulent *L. pneumophila* infection has not yet been reported. The cDNA expression array experiments revealed that the virulent *L. pneumophila* strain induced MCP-3 messages but avirulent bacteria did not at 5 h postinfection. As shown in Fig. 3, the results of the cDNA expression array experiments were confirmed by RT-PCR. That is, the virulent *L. pneumophila* M124 strain markedly induced MCP-3 messages, and this was evident even at 24 h after infection. In contrast, the avirulent *L. pneumophila* M124-Av strain did not induce any significant level of MCP-3 messages during infection ($P < 0.05$ compared with noninfected control group or virulent-bacteria-infected group). Furthermore, MIP-1β induction also was stimulated at a similar level by both virulent and avirulent *L. pneumophila* strains. Thus, the virulent *L. pneumophila* strain induced both MCP-3 and MIP-1β, but the avirulent bacteria induced only MIP-1β.

The mechanism of selective chemokine induction by the virulent bacteria is not clear. MCPs are known to down-regulate interleukin 12 induction induced by bacteria such as *Staphylococcus aureus* (2). Other current studies also showed interleukin 12 down-regulation by virulent *L. pneumophila*, but not by avirulent organisms (K. Matsunaga, T. W. Klein, H. Friedman, and Y. Yamamoto, unpublished data). Therefore, it can be speculated that MCP induced by virulent bacteria plays an immunoregulatory role in infection. Nevertheless, the results obtained showed that alveolar macrophages respond to virulent *L. pneumophila* infection differently than to infection with avirulent bacteria. Particularly, the differential regulation of chemokine MCP-3 induction by *L. pneumophila* was revealed in this study. Thus, it is apparent that the cDNA expression array technique is a powerful tool for analysis of host cell responses to infection by *L. pneumophila*, and the results obtained confirm that important modulations of gene expression following exposure to infectious agents can be readily screened by this technique.

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