Induction of Cytokine Granulocyte-Macrophage Colony-Stimulating Factor and Chemokine Macrophage Inflammatory Protein 2 mRNAs in Macrophages by *Legionella pneumophila* or *Salmonella typhimurium* Attachment Requires Different Ligand-Receptor Systems

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The attachment of bacteria to macrophages is mediated by different ligands and receptors and induces various intracellular molecular responses. In the present study, induction of cytokines and chemokines, especially granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage inflammatory protein 2 (MIP-2), was examined, following bacterial attachment, with regard to the ligand-receptor systems involved. Attachment of *Legionella pneumophila* or *Salmonella typhimurium* to cultured mouse peritoneal macrophages increased the steady-state levels of cellular mRNAs for the cytokines interleukin 1 β (IL-1 β), IL-6, and GM-CSF as well as the chemokines MIP-1 β , MIP-2, and KC. However, when macrophages were treated with α -methyl-D-mannoside (α MM), a competitor of glycopeptide ligands, induction of cytokine mRNAs was inhibited, but the levels of chemokine mRNAs were not. Pretreatment of the bacteria with fresh mouse serum enhanced the level of GM-CSF mRNA but not the level of MIP-2 mRNA. In addition, serum treatment reduced the inhibitory effect of α MM on GM-CSF mRNA. These results indicate that bacterial attachment increases the steady-state levels of the cytokine and chemokine mRNAs tested by at least two distinct receptor-ligand systems, namely, one linked to cytokine induction and involving mannose or other sugar residues and the other linked to chemokine induction and relatively α MM insensitive. Furthermore, opsonization with serum engages other pathways in the cytokine response which are relatively independent of the α MM-sensitive system. Regarding bacterial surface ligands involved in cytokine mRNA induction, evidence is presented that the flagellum may be important in stimulating cytokine GM-CSF message but not chemokine MIP-2 message. Analysis of cytokine GM-CSF and chemokine MIP-2 signaling pathways with protein kinase inhibitors revealed the involvement of calmodulin and myosin light-chain kinase in GM-CSF but not MIP-2 mRNA induction, adding further evidence that several distinct receptor systems are engaged during the process of bacterial attachment and induction of cytokines and chemokines, such as GM-CSF and MIP-2, respectively.

It is widely recognized that cytokine production by macrophages during infection is important and serves to regulate inflammatory responses (4). In this regard, various studies have reported cytokine induction by bacterial antigens as well as during bacterial infections (16, 19, 24, 26). Bacterial lipopolysaccharide (LPS) is one of many potential cytokine-inducing ligands and has been shown to engage specific receptors and stimulate defined signaling pathways (20, 23). However, even though LPS is a major outer membrane component of gram-negative bacteria, it may not contribute to initial macrophage interaction since the biologically active lipid A is buried in the membrane and not exposed under normal physiological conditions.

The infection of macrophages with intracellular bacteria involves three distinct steps, including attachment to the macrophage surface, internalization, and endosomal processing and migration (46). All of these steps probably contribute to the formation of cytokines during infection, and recently we have shown the importance of the attachment phase for cytokine induction (48). The receptors or binding sites on macrophages

which might be involved in this process are complement and immunoglobulin Fc receptors (14, 33). However, attachment and infection of macrophages by some bacteria occur (albeit at a reduced level) even in the absence of complement and antibody (14). Thus, ligand-receptor systems other than complement and Fc might facilitate infection, especially in environments with low serum components, such as alveolar spaces; however, little is known concerning these interactions.

Macrophages produce another class of proinflammatory agents referred to as chemokines (27, 32, 40). These chemokines include members of C, C-C and C-X-C supergene families which have been isolated from a variety of activated and transformed cell types and appear to play a critical role in mobilization and activation of various cell types (2, 7, 18, 38, 44). The signaling mechanisms controlling chemokine induction during infection is poorly understood, similar to the case with cytokine induction. In the present study, we examined proinflammatory cytokines interleukin-1 β (IL-1 β), IL-6, and granulocyte-macrophage colony-stimulating factor (GM-CSF) and chemokines macrophage inflammatory protein 1 β (MIP-1 β), MIP-2, and KC cellular mRNA levels in macrophages following the attachment phase of infection with two different facultative intracellular pathogens, namely, *Legionella pneumophila* and *Salmonella typhimurium*. KC and MIP-2 belong to C-X-C chemokine family and are the mouse homolog of the human *gro* (25). MIP-1 β was originally identified as an LPS-

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inducible heparin-binding protein (45) and is a member of the C-C chemokine family. These chemokines produced by macrophages in response to invading microorganisms might contribute to the migration of neutrophils and monocytes as well as lymphocytes in infection sites, but the role of individual chemokines in infection is not clear. Therefore, in this study, cytokine and chemokine induction was examined in detail in terms of the cytokine GM-CSF and the chemokine MIP-2 mRNAs as a representative cytokine and chemokine gene, respectively, since both mRNAs showed minimum constitutive expression in control macrophages and good responses upon bacterial attachment to the macrophages.

MATERIALS AND METHODS

Macrophages. Thioglycolate-elicited peritoneal macrophages were obtained from 8- to 12-week-old female BALB/c mice (Jackson Laboratory, Bar Harbor, Maine) as described previously (47). Macrophage monolayers (10^6 cells per well; 24-well tissue culture plates) were cultured overnight in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (HyClone Laboratories, Logan, Utah) and then used as described previously for mRNA studies (49).

Bacteria. *L. pneumophila* M124, serogroup 1, was cultured on buffered charcoal-yeast extract agar (Difco Laboratories, Detroit, Mich.) for 2 days at 37°C (8). *S. typhimurium* LT2 was obtained from the American Type Culture Collection, Rockville, Md. Transposon-insertion mutants of *S. typhimurium* which are flagellum and fimbria negative [χ 4308; Δ (*fim-ahp*)-391 *flh*-8007::Tn10] or fimbria negative only [χ 4253; Δ (*fim-ahp*-11::Tn10)-391] and the parental strain [χ 4252; Δ (*ahp*-11::Tn10)-251] (22) were kindly provided by R. Curtiss III, Washington University, St. Louis, Mo. Another transposon-insertion flagellum-negative mutant of *S. typhimurium* (kk1012) and the parent strain (kk1004) (21) were kindly provided by K. Kutsukake, Hiroshima University, Hiroshima, Japan. These *S. typhimurium* strains were cultured on nutrient agar for 1 day at 37°C. All bacterial suspensions were prepared in 10% fetal calf serum-RPMI 1640 medium after determining the concentration by spectrophotometry (8). Opsonized bacteria were prepared by incubation in RPMI 1640 medium containing 20% fresh BALB/c mouse serum for 30 min at 37°C.

Radioisotope-labeled bacteria. *S. typhimurium* χ 4252, χ 4308, and χ 4253 were labeled with ^{14}C . In brief, bacteria were cultured in tryptose phosphate broth (Difco) with 50 μCi of ^{14}C -amino acid mixture (Amersham, Arlington Heights, Ill.) per ml for 18 h at 37°C. The resulting cultures were washed with phosphate-buffered saline (PBS; Sigma Chemical Co., St. Louis, Mo.) and resuspended in PBS, and the concentration of bacteria was determined by spectrophotometry. The radioactivities of labeled bacteria were 12,495 to 18,945 cpm/ 10^8 bacteria.

Isolation of flagella. *Salmonella* flagella were isolated from a culture of the fimbria-negative mutant (χ 4253) as described previously (15). Further purification of flagella was performed with an anion-exchange membrane (D15X; Sartorius Co., Edgewood, N.Y.) as described in the manufacturer's protocol. The purity of the isolated flagellin was determined by sodium dodecyl sulfate (SDS)-8% polyacrylamide gel electrophoresis. The LPS content in the flagellin preparation was determined by *Limulus* amoebocyte lysate assay (QCL-1000; BioWhittaker, Walkersville, Md.). The flagellin was diluted in 10% fetal calf serum-RPMI 1640 medium supplemented with 20 μg of polymyxin B (Sigma) per ml to neutralize endogenous LPS (34). In some experiments, the flagellin was digested with trypsin (250 U/ml; Sigma) in PBS for 30 min at 37°C. The trypsin was inactivated by heating at 95°C for 30 min.

Protein kinase, TYK, and CaM inhibitors. The tyrosine kinase (TYK) inhibitor genistein was purchased from ICN Biochemicals, Cleveland, Ohio. The protein kinase inhibitors K252a, K252b, K5720, and KT5926 were kindly provided by S. Nakanishi, Kyowa Hakko Kogyo Co., Tokyo, Japan. The cyclic AMP-dependent protein kinase (PKC) inhibitor H89 and calmodulin (CaM) inhibitor W-7 were obtained from Calbiochem, La Jolla, Calif.

Macrophage treatment. The macrophage monolayers were pretreated with 1 μg of cytochalasin D (CyD; Sigma) per ml for 30 min and then incubated with or without bacteria (100 bacteria per macrophage) or the flagellum preparation (1 $\mu\text{g}/\text{ml}$) for 1 h in the presence of CyD. The CyD treatment did not make a significant change in the macrophage morphology nor in the mRNA induction by LPS under the experimental conditions used (48). In some experiments, 0.1 M α -methyl-D-mannoside (αMM ; Sigma) was also added. In others, the macrophage monolayers were pretreated with 1 μg of CyD per ml and protein kinase (K252a, 0.4 to 2 μM ; K252b, 10 μM ; K5720, 10 μM ; KT5926, 2 to 10 μM ; H89, 25 μM), TYK (genistein, 10 μM), or CaM (W-7, 25 μM) inhibitors for 30 min and then stimulated with bacteria in the presence of both CyD and inhibitors for 1 h.

Attachment of bacteria to macrophages. The macrophage monolayers were treated with or without 1 μg of CyD per ml for 30 min and then incubated with ^{14}C -bacteria for 1 h in the presence or absence of CyD. The infectivity ratio was 100 bacteria per macrophage. After incubation, the monolayers were washed with Hanks' balanced salt solution (Sigma) and then lysed in 0.1% saponin (Sigma). The radioactivity of macrophage lysates was measured by a scintillation counter.

Quantitative RT-PCR. RNA isolation from macrophages and quantitative

FIG. 1. Profile of cytokine and chemokine mRNAs induced by bacterial attachment to macrophages. Macrophage monolayers were pretreated with 1 μg of CyD per ml for 30 min and then stimulated with *L. pneumophila* (Lp) or *S. typhimurium* (Sal.) for 1 h in the presence of CyD. The infectivity ratio was 100 bacteria per macrophage. Total RNA was isolated and subjected to RT-PCR. BMG was used as an endogenous standard. PCR products were visualized by electrophoresis in agarose gel containing ethidium bromide.

reverse transcription (RT)-PCR (RT-PCR) were performed as described previously (49). In brief, total RNA was isolated from macrophages (approximately 10^6 cells) by the single-step method with TRI REAGENT (Molecular Research Center, Cincinnati, Ohio). Reverse transcription of total RNA was performed with avian myeloblastosis virus reverse transcriptase in a commercial reaction mixture (Reverse Transcription System; Promega, Madison, Wis.). The cDNAs were subjected to differential PCR (29) with primers for β_2 -microglobulin (BMG), IL-1 β , IL-6, GM-CSF, MIP-1 β , MIP-2, and KC. The primers for IL-1 β , IL-6, and GM-CSF were purchased from Stratagene, La Jolla, Calif. The other primers were synthesized on a DNA synthesizer (PS250; Cruachem, Dulles, Va.) by using published sequences (6, 13, 39). The PCR was performed in a Minicycler (MJ Research, Watertown, Mass.) for 30 cycles. The first cycle consisted of a 5-min denaturation at 94°C, a 5-min annealing at 60°C, and then 30 cycles each of 1.5 min at 72°C, 45 s at 94°C, and 45 s at 60°C, with a final extension of 10 min at 72°C. The PCR products were visualized by electrophoresis in 2% agarose gels containing ethidium bromide or quantified by high-performance liquid chromatography (HPLC) with a TSK DEAE-NPR column (TosoHaas, Montgomeryville, Pa.). Peak absorbance (260 nm) was computer analyzed and normalized relative to the BMG amplicon, which was coamplified in each sample (49). There was no significant difference in BMG (housekeeping gene) mRNA levels as shown by the amount of primer-specific products between control and experimental groups.

Statistical analysis. Statistical analysis was performed with the two-tailed Student *t* test.

RESULTS

Increases in cytokine and chemokine mRNAs levels by bacterial attachment. When macrophages were exposed to *L. pneumophila* or *S. typhimurium* (100 bacteria per macrophage) for 1 h in the presence of CyD, which prevents uptake but permits attachment of bacteria (48), mRNA levels for all of the cytokines (IL-1 β , IL-6, and GM-CSF) and chemokines (MIP-1 β , MIP-2, and KC) tested were increased over that in control cultures (Fig. 1). Control cultures had for the most part low levels of cytokine and chemokine mRNAs, with the exception of a relatively high level of MIP-1 β mRNA, which has been reported previously (42). From these results, it appears that attachment of bacteria to macrophage surfaces provides signals sufficient to increase the steady-state levels of mRNAs of both the cytokines and chemokines that were tested.

Role of various ligands-receptors in mRNA induction. Bacterial attachment to macrophages and endocytosis are partially

FIG. 2. Effect of α MM on cytokine (left) and chemokine (right) induction by *S. typhimurium* attachment. Macrophage monolayers were pretreated with 1 μ g of CyD per ml for 30 min and then stimulated with *S. typhimurium* (+Sal.) in the presence of CyD and the presence or absence of 0.1 M α MM (AMM) for 1 h. The infectivity ratio was 100 bacteria per macrophage. Isolated total RNA was subjected to quantitative RT-PCR, and the resulting PCR products were analyzed by HPLC as described in Materials and Methods. Data were normalized relative to an endogenous standard (BMG) by comparing the ratios of PCR products and expressed as cytokine or chemokine levels relative to the BMG level. Each bar represents the mean $\pm$ standard deviation for three to five experiments. $\star$, $p < 0.05$ compared with α MM-untreated group.

mediated by cell surface sugars and lectins (30). Such interactions can be blocked by α MM and other carbohydrates, and we tested whether this competitor could modulate cytokine and chemokine mRNA levels following bacterial attachment. Macrophage cultures were treated with CyD and α MM and infected with either *S. typhimurium* or *L. pneumophila*. Figure 2 shows that α MM treatment greatly reduced the level of IL-1 β , IL-6, and GM-CSF mRNAs in response to *S. typhimurium* attachment but had no effect on mRNAs for the chemokines tested, which were increased following bacterial attachment. Similar results were also observed in *L. pneumophila*-infected macrophages. That is, *L. pneumophila* attachment induced increased levels of GM-CSF (relative level of mRNA, 0.74 ± 0.04 [mean $\pm$ standard deviation]), but 0.1 M α MM treatment significantly suppressed the levels of GM-CSF (relative level of mRNA, 0.25 ± 0.05 ; $P < 0.05$ compared with that α MM-nontreated group). In contrast, MIP-2 induction was not affected by α MM treatment (relative level of mRNA, 1.66 ± 0.13 for bacterium-attached macrophages versus 1.57 ± 0.21 for bacterium-attached and α MM-treated macrophages). α MM treatment alone had no effect on the levels of cytokine or chemokine mRNAs tested in control macrophages (data not shown). These results suggested that the α MM-sensitive receptor system might be implicated in bacterial induction of cytokine but not chemokine mRNAs.

Fc and complement receptors are known to be activated during infection (14, 33). To determine whether α MM could modulate cytokine and chemokine induction by these receptors, experiments were performed with *S. typhimurium* pretreated with fresh mouse serum. When macrophages were stimulated with opsonized bacteria, an enhancement of GM-CSF mRNA induction compared with that of macrophages treated with nonopsonized bacteria (Fig. 3) was observed, while MIP-2 mRNA levels were increased little by treatment with opsonized bacteria. The increase in the GM-CSF mRNA level by opsonized bacteria did not appear to be due totally to an α MM-inhibitable mechanism because α MM treatment inhibited the effect of opsonized bacteria by only 33.8%, whereas the competitor inhibited the nonopsonized bacteria effect by

87.2% (Fig. 3). As before, α MM had no effect on MIP-2 mRNA. On the basis of these results with the carbohydrate competitor, it appears that several receptor systems might be involved in cytokine GM-CSF and chemokine MIP-2 mRNA regulation.

Effects of protein kinase inhibitors on cytokine GM-CSF and chemokine MIP-2 mRNA induction. The results described above suggest that several receptor systems might be involved in the regulation of cytokine and chemokine mRNA. To determine whether several different signaling pathways were also involved, cultures were retested for mRNA levels following treatment with various protein kinase inhibitors. For this purpose, the TYK inhibitor genistein, cyclic AMP-dependent protein kinase (PKA) inhibitors K5720 (17) and H89 (9), the PKC inhibitor K252b (17), the myosin light-chain kinase (MLCK) inhibitor KT5926 (28), wide-range inhibitor K252a for protein kinases (17), and the CaM inhibitor W-7 (12) were used. Macrophages were pretreated with CyD and each inhibitor for 30 min and then stimulated with *S. typhimurium* for 1 h in the presence of both CyD and inhibitor. The total RNA was isolated from the macrophages, and the levels of GM-CSF and MIP-2 mRNAs were measured. Control macrophages were treated with CyD and stimulated with or without bacteria or treated with CyD and inhibitor without bacteria. The treatment with kinase inhibitors did not result in any changes of message levels of GM-CSF and MIP-2 in control macrophages without bacteria. Figure 4 shows that the wide-range protein kinase inhibitor K252a, at 2 μ M, strongly inhibited both GM-CSF and MIP-2 mRNA induction by bacterial attachment. Even when a lower concentration of K252a, such as 0.4 μ M, was used, it also inhibited both GM-CSF and MIP-2 mRNA (58 and 44% inhibition, respectively; data not shown). Also, treatment with the MLCK inhibitor KT5926 showed marked inhibition of GM-CSF mRNA induction, but inhibition of MIP-2 mRNA was minimal at the 10 μ M concentration used. A lower concentration of KT5926 (2 μ M) still showed 31% inhibition of GM-CSF mRNA induction (data not shown). Furthermore, the CaM inhibitor W-7 also showed a selective inhibition of GM-CSF mRNA induction but not MIP-2. However, specific inhibitors for TYK, PKA, and PKC did not sig-

FIG. 3. Effect of opsonization on the levels of GM-CSF (left) and MIP-2 (right) mRNAs induced by *S. typhimurium* attachment. Macrophage monolayers were pretreated with CyD for 30 min and then stimulated with *S. typhimurium* bacteria (+Sal.) opsonized with fresh serum or unopsonized bacteria for 1 h in the presence of CyD and the presence or absence of 0.1 M α MM (AMM). See the legend to Fig. 2 for details. Each bar represents the mean $\pm$ standard deviation for three experiments. $\star$, $P < 0.05$ compared with α MM-untreated group.

nificantly affect the induction levels of either GM-CSF or MIP-2 mRNAs at the tested concentrations. These data indicate that different signaling pathways, which have different sensitivities to kinase inhibitors, may be involved in GM-CSF and MIP-2 mRNA induction by bacterial attachment.

Induction of cytokine GM-CSF and chemokine MIP-2 mRNAs by *Salmonella* mutants. To analyze the bacterial ligands responsible for GM-CSF and MIP-2 induction by bacterial attachment, surface-structure-deficient mutants of *S. typhimurium* were used. When the flagellum- and fimbria-negative mutant χ 4308 was used for cytokine and chemokine mRNA induction, GM-CSF mRNA levels were significantly lower when compared with those of the parent strain (χ 4252), but MIP-2 mRNA was unaffected (Fig. 5). Similar results were obtained when a different flagellum-deficient mutant strain, kk1012, was used; that is, the level of GM-CSF mRNA was significantly lower than in cultures induced by the parent strain kk1004, while levels of MIP-2 were comparable to those of the parent strain (Fig. 5). Thus, it appears that at the very least, flagella might serve as a ligand for cytokine GM-CSF mRNA regulation. The possible involvement of other surface structures, such as fimbriae, for induction of GM-CSF and other cytokines by bacterial attachment is not clear, since the flagellum-positive and fimbria-negative mutant χ 4253 did not consistently result in induction of cytokines such as GM-CSF and IL-6 (data not shown).

The phagocytosis of *S. typhimurium* mutants by macrophages as well as attachment to macrophages was measured with 14 C-labeled bacteria, since the lack of flagella seems to cause a reduction of attachment as a result of reduced motility. As shown in Table 1, there was significant reduction of phagocytosis as well as attachment of the flagellum-negative mutant χ 4308 as compared with that of the parent strain χ 4252. Use of the fimbria-negative mutant χ 4253 did not result in any significant change in phagocytosis or attachment to the macrophages.

Induction of GM-CSF and MIP-2 mRNAs by *Salmonella* flagella. Even though use of the flagellum-negative mutant reduced attachment to macrophages, the possible involvement of flagella in GM-CSF induction by bacterial attachment was still possible. Therefore, GM-CSF and MIP-2 mRNA induction

by isolated flagella was examined. For this purpose, the flagellum-positive, fimbria-negative mutant χ 4253 was used for isolation of flagella. The isolated flagellin obtained showed high purity by SDS-polyacrylamide gel electrophoresis and little

FIG. 4. Effect of TYK, protein kinase, and CaM kinase inhibitors on the levels of GM-CSF and MIP-2 mRNAs induced by *S. typhimurium* attachment. Macrophage monolayers were pretreated with CyD and with or without inhibitors as described in the text for 30 min and then stimulated with bacteria for 1 h in the presence of CyD and the presence or absence of inhibitors. The concentrations of the inhibitors were as follows: genistein, 10 μ M; K5720, 10 μ M; H89, 25 μ M; K252a, 2 μ M; K252b, 10 μ M; KT5926, 10 μ M; W-7, 25 μ M. Total RNA was isolated from macrophages and subjected to quantitative RT-PCR. The levels of mRNA were expressed as the percentages of inhibitor-untreated macrophages stimulated with bacteria. Each bar represents the mean $\pm$ standard deviation for three experiments. MPs, macrophages; $\star$, $P < 0.05$ compared with inhibitor-untreated macrophages.

FIG. 5. Effect of flagellum-negative mutants on the induction of GM-CSF and MIP-2 mRNAs. Macrophage monolayers were pretreated with CyD for 30 min and then stimulated with either the mutant or parent strain of *S. typhimurium* for 1 h in the presence of CyD. Total RNA was isolated from macrophages and then subjected to quantitative RT-PCR. The levels of mRNA were expressed as the percentages of parent strain-stimulated macrophages. Each bar represents the mean $\pm$ standard deviation for three experiments. x4252, parent strain; x4308, flagellum- and fimbria-negative mutant; kk1004, parent strain; kk1012, flagellum-negative mutant. *, $P < 0.05$ compared with parent strain-stimulated macrophages.

contaminated LPS (≤ 1 ng/ μ g of flagellin). Treatment of macrophages with 1 μ g of the flagellin protein per ml in the presence of polymyxin B markedly induced GM-CSF mRNA, but MIP-2 mRNA was not induced (Fig. 6). Trypsin digestion of flagella diminished the induction of GM-CSF mRNA (data not shown), whose engaged cytokine mRNA induction is due to the flagellin protein, not contaminated LPS. Furthermore, when macrophages were treated with flagellin in the presence of α MM, levels of GM-CSF mRNA showed significant inhibition.

DISCUSSION

The induction of increased steady-state levels of cytokine mRNAs by bacterial attachment to macrophages has been

FIG. 6. Induction of GM-CSF and MIP-2 mRNAs by *Salmonella* flagella. Macrophage monolayers were pretreated with CyD for 30 min and then stimulated with 1 μ g of flagella (Fla.) in the presence of CyD, 20 μ g of polymyxin B per ml, and the presence or absence of 0.1 M α MM (AMM) for 1 h. See the legend to Fig. 2 for details. Each bar represents the mean $\pm$ standard deviation for three experiments. *, $P < 0.05$ compared with flagellum-only-treated macrophages. -, control.

demonstrated previously by us (48), and it was studied further in terms of chemokine mRNAs in the present study. The results indicate that the initial attachment of bacteria to macrophages is sufficient to generate a signal for induction of not only cytokine but also chemokine messages, and such a signal may be mediated by certain bacterial ligand and macrophage receptor interactions. It seems likely that many bacterial ligands-macrophage receptors are involved in the attachment of whole bacteria to macrophages. However, what ligand and receptor may be involved in induction of specific cytokines and chemokines in macrophages following attachment of bacteria is unknown.

In the present study, the possible ligand-receptor system involved in various cytokines and chemokines induced by bacterial attachment was examined by use of an inhibitor for carbohydrate-protein interaction, surface structure mutants of bacteria, and protein kinase inhibitors. Since lectinophagocytosis of macrophages to bacteria in a serum-free environment has been reported (30), an involvement of a bacterial lectin or macrophage lectin-like receptor in cytokine and chemokine induction seems likely. α MM has marked affinity for certain lectins, such as concanavalin A and type 1 fimbrial lectin of bacteria and other proteins such as mannose receptors on macrophages, and therefore acts as an inhibitor of these proteins (30). Treatment with α MM significantly inhibited induction of cytokines but not chemokine messages induced by bacterial attachment. Treatment with other carbohydrates, such as α -methyl-D-galactoside, α -methyl-D-glucoside, and *N*-acetyl-D-galactosamine, also had some inhibitory effect on GM-CSF induction, but the inhibitory potencies were relatively weak compared with that of α MM (data not shown). Since α MM can bind to fimbriae and mannose receptors which are present in bacteria and macrophages, respectively, the present results indicate a possible involvement of these proteins in bacterium-mediated cytokine induction but not chemokine induction. Involvement of the complement receptor CR3 in induction of cytokine mRNA by the bacteria as a result of a lectin-like property, similar to involvement of β -glucan binding to CR3 (37), is also possible. Nevertheless, blocking experiments using α MM clearly indicated that the cytokine and chemokine mRNAs tested may be induced separately by different ligand-receptor

TABLE 1. Phagocytosis of *S. typhimurium* by macrophages

Strain (phenotype)	% of bacteria phagocytized ^a	
	+CyD	-CyD
x4252 (Fim ⁺ Fla ⁺)	4.40 $\pm$ 1.10	10.50 $\pm$ 1.20
x4253 (Fim ⁻ Fla ⁺)	2.45 $\pm$ 0.35	10.75 $\pm$ 1.45
x4308 (Fim ⁻ Fla ⁻)	0.76 $\pm$ 0.23 ^b	1.70 $\pm$ 0.11 ^b

^a Macrophage monolayers (10^6 cells) were incubated with or without 1 μ g of CyD per ml for 30 min and then infected with 14 C-labeled bacteria (x4252, 12,990 cpm/ 10^8 bacteria; x4253, 18,945 cpm/ 10^8 bacteria; x4308, 12,495 cpm/ 10^8 bacteria) for 1 h in the presence or absence of CyD. The monolayers were washed to remove nonphagocytized bacteria and lysed in saponin solution. The labeled bacteria in macrophage lysates were measured by a scintillation counter. The percentage of bacteria phagocytized was calculated as follows: (counts per minute of macrophage lysate/counts per minute of bacteria added) $\times$ 100. The data represent the means $\pm$ standard deviations for three experiments.

^b $P < 0.05$ compared with x4252 values.

systems of macrophages following bacterial attachment. That is, cytokine IL-1 β , IL-6, and GM-CSF mRNA induction was due to an α MM-sensitive ligand-receptor interaction, but the chemokine MIP-1 β , MIP-2, and KC mRNA induction was due to an α MM-insensitive ligand-receptor system.

The treatment of *S. typhimurium* with fresh serum induced higher levels of cytokine GM-CSF message than those of untreated bacteria; however, a similar effect on chemokine MIP-2 was not observed. In the case of *L. pneumophila*, neither GM-CSF nor MIP-2 messages showed an enhancement of induction by fresh serum treatment (data not shown). This latter finding may correlate with previous reports showing that the surface of *L. pneumophila* organisms tends to promote low complement deposition (5). Nevertheless, the enhanced induction of cytokine GM-CSF messages, but not chemokine MIP-2 messages, by opsonized *S. typhimurium* indicates that complement and/or Fc receptors may be involved in GM-CSF induction, but not in MIP-2 induction, in the presence of fresh serum, which supports the involvement of distinct ligand-receptor systems for certain cytokine versus chemokine induction. The lower sensitivity of GM-CSF induction by opsonized *S. typhimurium* to α MM treatment, compared with that of unopsonized *S. typhimurium*, also adds further evidence to support distinct ligand-receptor systems in cytokine versus chemokine induction. Thus, these data suggest the possibility that at least three different receptors might be involved in mediating cytokine GM-CSF and chemokine MIP-2 message levels. One of these is independent of opsonin and carbohydrate binding and mediates MIP-2 message levels. The other two involve either opsonins or carbohydrates and modulate GM-CSF messages.

The involvement of distinct receptor systems is also supported by studies with protein kinase, TYK, and CaM inhibitors. The use of the wide-range kinase inhibitor K252a (17) markedly inhibited both GM-CSF and MIP-2 induction by *S. typhimurium* at a concentration used for inhibition of protein phosphorylation in platelets (28). However, use of specific inhibitors of PKA, PKC, and TYK, such as K252b, H89, K5720, and genistein, did not inhibit induction of either GM-CSF or MIP-2 mRNAs at concentrations which have been used for experiments with intact cells (9, 17, 41). In contrast, use of inhibitors like the MLCK inhibitor KT5926 and the CaM inhibitor W-7 selectively inhibited induction of GM-CSF mRNA but not of MIP-2 mRNA, suggesting that different signaling pathways are involved in cytokine GM-CSF and chemokine MIP-2 induction (Fig. 5). Involvement of CaM in cytokine induction has been reported previously in LPS-induced cytokine gene expression (31); however, the signaling pathway for LPS may differ from the one observed in our studies because LPS cytokine gene induction also depends on PKC (20), which was not required for GM-CSF induction by *S. typhimurium* attachment (Fig. 5). Although these inhibitor studies implicate CaM-MLCK in attachment-induced signaling for GM-CSF induction, further studies are required, especially ones involving the measurement of CaM-MLCK activity in the cells. Furthermore, the role of TYKs in this process cannot be formally ruled out since negative results with inhibitors are not absolute because of the different susceptibilities of these enzymes to inhibitors in various cell types. Nevertheless, the inhibitor studies showed the different susceptibilities of MIP-2 and GM-CSF induction to CaM and MLCK inhibitors, indicating that two distinct signaling pathways are required for cytokine GM-CSF and chemokine MIP-2 induction by bacterial attachment.

Regarding the ligands on the bacterial surface responsible for the increased levels of mRNAs observed in our studies, we emphasized flagella and fimbriae, since *L. pneumophila* (36) and *S. typhimurium* both display these structures. By using

flagellum- and fimbria-negative mutants, we observed the importance of flagella in the induction of GM-CSF mRNA but not that of MIP-2 (Fig. 5). These results indicate several possibilities about involvement of flagella in cytokine GM-CSF and chemokine MIP-2 induction by bacterial attachment. Because bacterial flagella are mainly involved in motility (43), flagellum-negative mutants are not motile. Therefore, it seems possible that the reduction of GM-CSF mRNA following treatment with flagellum-negative mutants may be due to the reduced interaction between bacteria and macrophages because of lack of motility. In fact, treatment with the flagellum- and fimbria-negative mutant χ 4308 significantly reduced the attachment to macrophages compared with that of the flagellum- and fimbria-positive parent strain, as shown by the radiolabeled bacterial uptake assay (Table 1). Only the fimbria-negative mutant χ 4253 did not result in a significant reduction of attachment to macrophages.

To determine whether bacterial flagella have direct cytokine-inducing activity as a ligand, the induction activity of isolated flagella was examined. The results clearly showed that flagellum treatment increased the level of GM-CSF mRNA, but MIP-2 mRNA was not induced. Furthermore, GM-CSF mRNA induction by flagella was significantly blocked by α MM. Although the precise mechanism is unclear, the binding of flagella to macrophages is a possibility, because it has been suggested that besides motility, flagellar filaments may carry adhesins for attachment to intestinal mucosa (3, 35). Thus, it seems likely that the lower cytokine GM-CSF message induction by the flagellum-negative mutant may be caused by at least two factors, i.e., the lower attachment and the fewer ligands available for GM-CSF induction. In contrast, chemokine MIP-2 induction may be caused by other ligands and may not require a high density of certain ligands, since the lower attachment of mutant bacteria still induced the same level of MIP-2 as that induced by the control parent bacteria. The involvement of fimbriae as a potential ligand in cytokine and chemokine induction is still possible, because certain types of fimbriae have been shown to activate macrophages or uroepithelial cells to induce cytokines (1, 10, 11).

In summary, *L. pneumophila* and *S. typhimurium* binding to macrophages increases the steady-state levels of cytokine and chemokine mRNAs tested. It appears that cytokine GM-CSF and chemokine MIP-2 are induced by different ligand-receptor and signaling systems in bacterium-attached macrophages. Bacterial flagellum is one of the ligands involved in cytokine induction but not chemokine induction by *S. typhimurium* attachment. Since the genetic structure of bacterial flagellum and the phenomenon of phase variation differ with different bacteria, the possible involvement of flagella in cytokine induction should be studied with other bacteria, including *L. pneumophila*. Nevertheless, these results support the possibility that cytokine and chemokine responses of macrophages to invading microorganisms may be regulated by different receptor groups.

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