Absence of Tumor Necrosis Factor Alpha, Interleukin-6 (IL-6), and Granulocyte-Macrophage Colony-Stimulating Factor Expression but Presence of IL-1 β , IL-8, and IL-10 Expression in Human Monocytes Exposed to Viable or Killed *Ehrlichia chaffeensis*

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Ehrlichia chaffeensis is a recently isolated minute gram-negative obligatory intracellular bacterium of monocytes/macrophages and is the etiologic agent of human monocytic ehrlichiosis. It is not known how macrophages respond when they encounter ehrlichiae in terms of cytokine production. In this study, we examined cytokine mRNA expression by incubating *E. chaffeensis* with THP-1 cells and performing competitive reverse transcription-PCR (RT-PCR). At 2 h postinfection, the levels of interleukin-1 β (IL-1 β), IL-8, and IL-10 mRNAs were significant but lower than those following *Escherichia coli* lipopolysaccharide (LPS) stimulation. Unlike the situation with *E. coli* LPS stimulation, however, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor alpha (TNF- α) mRNAs were not induced. Time course and dose-response studies confirmed the absence of IL-6, GM-CSF, and TNF- α mRNA induction with *E. chaffeensis*. Viable *E. chaffeensis* organisms were not required for IL-1 β , IL-8, and IL-10 mRNA induction, since heat-killed *E. chaffeensis* induced identical time course responses. IL-1 β , IL-8, and IL-10 mRNAs were detected for up to 21, 21, and 24 h postexposure with *E. chaffeensis*, respectively, which were shut off more rapidly than with LPS stimulation. Although heat treatment of *E. chaffeensis* had no effect, periodate treatment completely abolished the ability of *E. chaffeensis* to induce IL-1 β , IL-8, and IL-10 mRNAs. The capture enzyme-linked immunosorbent assay result corresponded with the RT-PCR results, showing that viable and heat-killed *E. chaffeensis* produced and secreted the same levels of IL-1 β and IL-8. IL-10 production was significantly reduced by heat treatment. Periodate-treated ehrlichiae did not induce production of any of the cytokines tested. Anti-CD14 monoclonal antibody and polymyxin B did not inhibit IL-1 β mRNA expression upon exposure to *E. chaffeensis*. The absence of TNF- α , IL-6, and GM-CSF mRNA induction may delay the development of a protective immune response, thereby allowing *E. chaffeensis* to set up residence in macrophages.

The genus *Ehrlichia* belongs to the family *Rickettsiaceae* and consists of obligatory intracellular bacteria which proliferate in phagosomes in the cytoplasm of leukocytes (36). *Ehrlichia chaffeensis*, which infects blood monocytes/macrophages, has been recently recognized as the etiologic agent of human ehrlichiosis in the United States (2, 13). Serologic studies suggested the existence of *E. chaffeensis* infections in Europe and Africa (22, 31, 45). Since the discovery of this disease in 1986, more than 400 cases of human ehrlichiosis have been reported in 30 states in the United States (14, 16, 41). *E. chaffeensis* is transmitted by ticks. After a median incubation period of 7 to 9 days, patients suddenly develop fever, headache, malaise, thrombocytopenia, leukopenia, and elevated liver enzyme activities (17, 18). The spectrum of the disease ranges from asymptomatic seroconversion to death (17, 18).

Macrophages are potent effector cells that are able to engulf and kill many bacterial invaders. Although several facultative intracellular bacteria, such as *Mycobacterium*, *Listeria*, *Legionella*, and *Salmonella* species, infect macrophages, the infected

macrophages secrete various proinflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor alpha (TNF- α), and IL-6 which alert other cells in the immune system, leading to activation of macrophages and killing of the intracytoplasmic bacteria. Generated cytokines also contribute to inflammatory tissue damage. Recently, evidence indicating that virulent strains of intracellular bacteria do not induce TNF- α has been found (6, 7). Previous data from our laboratory demonstrated that an obligate intramacrophage bacterium, *Ehrlichia risticii*, did not induce strong TNF- α or IL-6 secretion in murine macrophages in vitro (47), so they may remain undetected by the host in the early stages of infection. Whether this inhibition is at the transcriptional or posttranscriptional level was not determined in the study with *E. risticii* (47). In the present study, we examined whether *E. chaffeensis*, which is genetically (16S rRNA base sequence homology, 84.2%) and antigenically divergent from *E. risticii* (2), has a basic profile of human macrophage cytokine production similar to that of *E. risticii* at both the mRNA and protein levels. Additional cytokines (IL-8, IL-10, granulocyte-macrophage colony-stimulating factor [GM-CSF], and transforming growth factor β [TGF- β]) which had not been examined previously were included in this study. Furthermore, whether a protein or carbohydrate component of ehrlichiae is required for or interferes with induction of cytokine mRNAs was examined.

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FIG. 1. Analysis of relative amounts of mRNA by competitive PCR. (A) Total RNA (2 μ g) isolated from unstimulated THP-1 cells was used as the template for cDNA synthesis in each 30- μ l reaction mixture. The cDNA (1 μ l) was then amplified for G3PDH in the presence of 10-fold dilutions of the G3PDH MIMIC. The PCR products (9 μ l) were resolved on 1.8% agarose gels containing EtBr. (B) Quantitative analysis of the competitive PCR experiment shown in panel A. The intensities of MIMIC and target cDNA bands were measured by using a gel video system with image analysis software. The log of the ratios of targets to MIMICs were plotted against the concentration of G3PDH MIMIC added to the PCR mixture. The line was drawn from a linear regression analysis of all datum points. An additional experiment performed in the same manner starting with the reverse transcription step gave a similar result. (C) Different amounts of cDNA prepared in panel A were amplified for G3PDH in the presence of a constant amount of G3PDH MIMIC (5×10^{-2} amol). The PCR products (9 μ l) were resolved on 1.8% agarose gels containing EtBr. (D) Quantitative analysis of the competitive PCR experiment shown in panel C. The intensities of MIMIC and target cDNA bands were measured by using a gel video system with image analysis software. The ratios of targets to MIMICs were plotted against the amount of cDNA added to the PCR mixture. The line was drawn from a linear regression analysis of all datum points. Two additional PCR experiments performed in the same manner gave similar results.

MATERIALS AND METHODS

Monocytic cell line. THP-1 cells were originally derived from a child with acute monocytic leukemia (44) and were obtained from the American Type Culture Collection (Rockville, Md.). Cells were maintained in RPMI 1640 medium (GIBCO, Grand Island, N.Y.) containing 10% heat-inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, Ga.), 2 mM L-glutamine (L-Gln) (GIBCO), and 50 μ M 2-mercaptoethanol (GIBCO) at 37°C in a humidified 5% CO₂ atmosphere.

Human monocyte isolation. Human peripheral blood monocytes were isolated from buffy coats from healthy donors (Ohio Red Cross, Columbus) by centrifugation (800 $\times$ g for 15 min) over Histopaque 1077 (Sigma, St. Louis, Mo.) as described previously (5). The mononuclear cells were resuspended in RPMI 1640 medium and allowed to adhere for 2 h in 100-mm-diameter tissue culture dishes (Corning Glass Works, Corning, N.Y.). After the cells were washed extensively with prewarmed sterile phosphate-buffered saline (PBS) (2.7 mM KCl, 1.8 mM KH₂PO₄, 137 mM NaCl, 10 mM Na₂HPO₄), adherent cells were collected by using a cell scraper, cultured for 1 week at 10^6 cells per ml in RPMI 1640 medium containing 10% FBS, and then used for treatments. Adherent cells prepared in this manner contained about 65% monocytes as assessed by histochemical staining for nonspecific esterase activity (α -naphthyl acetate esterase kit; Sigma) (52).

Preparation of host cell-free *E. chaffeensis*. *E. chaffeensis* Arkansas isolate was originally obtained from J. Dawson (Centers for Disease Control, Atlanta, Ga.). *E. chaffeensis* was cultured in THP-1 cells in RPMI 1640 medium supplemented with 10% FBS and 2 mM L-Gln. When more than 90% of the cells were infected, as determined by examining the cells stained with Diff-Quik (Baxter Scientific Products, Obitz, Ohio), the infected cells were suspended in the culture medium at 10^6 cells per ml, sonicated, and centrifuged at 500 $\times$ g for 5 min. The supernatant was centrifuged at 10,000 $\times$ g for 10 min, and the pellet containing host cell-free organisms was used to infect monocytes. Since ehrlichial organisms are rapidly inactivated once they become extracellular and the host inclusion membranes are difficult to remove without destroying ehrlichiae, further extensive purification was not performed. The uninfected host cell lysate prepared as described above was included in the control.

Treatment of cells. Cells (10^7) in 5 ml of RPMI 1640 medium were treated in each 60-mm-diameter tissue culture dish (Corning) either with lipopolysaccharide (LPS) (*Escherichia coli* O127:B8; Difco, Detroit, Mich.) at 1 μ g/ml, host cell-free *E. chaffeensis* (approximately 100 bacteria per cell), or uninfected THP-1 cell lysate derived from 10^7 cells. For the preparation of heat-killed bacteria, host cell-free *E. chaffeensis* was boiled for 10 min. For the preparation of periodate-treated bacteria, host cell-free *E. chaffeensis* was boiled for 10 min and incubated with 20 mM sodium periodate (Sigma) in 50 mM sodium acetate buffer, pH 4.5, for 1 h at room temperature in the dark. Following a brief rinse with 50 mM sodium acetate, the preparation was then incubated with 50 mM

sodium borohydride (Sigma) in PBS for 30 min at room temperature (50). For the dose-response experiment, the approximate number of *E. chaffeensis* organisms per cell was counted in 100 cells when more than 95% THP-1 cells were heavily infected. The number of infected THP-1 cells containing the appropriate dose of *E. chaffeensis* was calculated, harvested, and sonicated as described above. At the indicated time points after treatment, cells were harvested and used for RNA isolation.

RNA isolation and cDNA synthesis. Total RNA was isolated from the cells by the guanidine thiocyanate method (9). The purity of RNA was assessed on the basis of the A_{260}/A_{280} ratio, and the integrity of RNA was verified by agarose gel electrophoresis. Total cellular RNA (2 μ g) was reverse transcribed in a 30- μ l reaction mixture containing 1 $\times$ reaction buffer (50 mM Tris-HCl [pH 8.3], 75 mM KCl and 3 mM MgCl₂), 0.5 mM deoxynucleoside triphosphate mixture, 1 U of RNasin per μ l, 1.5 μ M oligo(dT) primer, and 10 U of Moloney murine leukemia virus reverse transcriptase (Clontech Laboratories, Inc., Palo Alto, Calif.) per μ l at 42°C for 1 h. The cDNA was heated at 94°C for 5 min and used in the PCR.

PCR analysis. PCR primers and MIMICs (competitive internal standards) used in this study were purchased from Clontech Laboratories. PCR MIMIC for human IL-10 was prepared according to the manufacturer's protocol by using a PCR MIMIC construction kit (Clontech). PCR with the hot start technique was used to reduce nonspecific priming (12). The prepared cDNA (1 to 2 μ l) was amplified in a 50- μ l reaction mixture containing 1 $\times$ PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl₂), 0.2 mM deoxynucleoside triphosphate mixture, 2.0 U of *Taq* DNA polymerase (Life Technologies, Inc., Gaithersburg, Md.), and 0.4 μ M (each) 3' and 5' primers in a DNA thermal cycler (model 480; Perkin-Elmer Corporation, Norwalk, Conn.). The reaction mixture was prepared as a master mixture to minimize reaction variation. One PCR cycle consisted of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 2 min. PCR was conducted for 25 to 30 cycles. The final extension was allowed to continue for 7 min.

Semiquantitative determination of cytokine mRNA level. To compare the relative levels of mRNA expression in different samples, the competitor control DNA containing 5' and 3' primer sequences was used as a standard in the semiquantitative PCR. The amplified product of the competitor was distinguished from that of the target by size. The proper concentration of PCR MIMIC specific for each cytokine was established in preliminary experiments. The concentrations of MIMICs added to the PCR reaction mixtures in the present study are as follows: 1 amol for IL-1 β and TNF- α , 5×10^{-1} or 1 amol for IL-8, 5×10^{-4} or 1×10^{-3} amol for IL-10, and 10^{-1} or 1 amol for glyceraldehyde-3-phosphate dehydrogenase (G3PDH). Following reverse transcription-PCR (RT-PCR), 9 μ l of the reaction mixture was electrophoresed in a 1.8% agarose gel containing 0.5 μ g of ethidium bromide (EtBr). *Hae*III-digested ϕ X174 replica-

FIG. 2. Cytokine mRNA expression in human monocytic cell line THP-1. (A) THP-1 cells were treated with each stimulus at approximately 100 ehrlichiae per cell for 2 h, and total RNA was extracted and subjected to competitive RT-PCR as described in Materials and Methods. A constant amount of PCR

template DNA fragments (GIBCO) were run at 0.5 µg/lane in parallel as molecular size markers providing bands from 1,353 to 72 bp. The identities of the amplified bands were validated by their predicted sizes in the gels. The amounts of PCR products generated by the target and the competitive internal standard were analyzed by using the gel video system (Gel Print 2000i; BioPhotonics Corporation, Ann Arbor, Mich.) and image analysis software (ImageQuant; Molecular Dynamics, Sunnyvale, Calif.). The ratio of target to internal standard was calculated and normalized against G3PDH mRNA in the corresponding sample. The photoprint of the result of a typical experiment is presented.

Enzyme-linked immunosorbent assays (ELISAs) for cytokines. THP-1 cells were seeded at 10^6 cells in 1-ml volumes in 12-well plates (Corning). The cultures were prepared in duplicate and stimulated either with *E. coli* LPS at 1 µg/ml, host cell-free *E. chaffeensis*, or uninfected host cell lysate derived from 10^7 infected cells as described above. After 24 h of incubation, the cultures were collected into microcentrifuge tubes. One culture set was centrifuged at $10,000 \times g$ for 10 min, and then the supernatant (culture supernatant) was assayed for secreted cytokine levels. The other set was sonicated for 5 s and then centrifuged at $10,000 \times g$ for 10 min. The resulting supernatant (culture supernatant plus cell lysate) was assayed for the total amount of intracellular and extracellular cytokine. Cytokine levels were measured according to the manufacturer's instructions by using the human IL-1β, IL-8, and IL-10 immunoassay kits (Quantikine; R&D Systems, Minneapolis, Minn.).

Effects of anti-CD14 MAb and polymyxin B on cytokine mRNA induction. THP-1 cells at 10^7 cells per 5 ml were incubated with each stimulus as described above in the presence or absence of either anti-CD14 monoclonal antibody (MAb) (5 µg/ml; Ancell Corporation, Bayport, Minn.), isotype control mouse immunoglobulin G2b (5 µg/ml; Organon Teknica Corporation, Durham, N.C.), or polymyxin B sulfate (30 µg/ml; Sigma) for 2 h. After incubation, total RNA was isolated and subjected to semiquantitative PCR.

RESULTS

Competitive RT-PCR assay. To analyze the effect of *E. chaffeensis* infection on cytokine mRNA expression in human monocytes, we measured the level of specific cytokine mRNA by the competitive RT-PCR. By using a DNA fragment containing the same primer template sequences as the target which competes for primer binding and amplification, this RT-PCR method provides a sensitive and reliable way to determine the relative level of abundance of a particular mRNA (39). The linearity of RT-PCR assay using various dilutions of target cDNA or MIMIC DNA is shown in Fig. 1. Figure 1 shows that as the concentration of MIMIC decreased, the intensity of the MIMIC-derived band decreased and that of the target-derived band increased. The log of the ratios of target to MIMIC PCR products, when plotted against the concentration of MIMIC input, yielded a linear relationship ($r = 0.99$) (Fig. 1A and B). The ratios of target to MIMIC PCR products, when plotted against the amount of cDNA, also yielded a linear relationship ($r = 0.99$) (Fig. 1C and D). A photograph of the EtBr fluorescence for each gel was obtained by optimizing the exposure so as not to saturate strong bands while sufficiently exposing weak bands.

Cytokine mRNA expression in THP-1 cells and human blood monocytes infected with *E. chaffeensis*. Figure 2 shows the profile of cytokine mRNA expression in THP-1 cells. Cells activated with *E. coli* LPS were included as a positive control for each experiment. *E. coli* LPS is known to induce production of IL-1, IL-6, IL-8, IL-10, GM-CSF, TNF-α, and TGF-β in murine and human monocytes/macrophages (1, 33). Cells

MIMIC was coamplified with cDNA, and then the PCR products were resolved on 1.8% agarose gels. No MIMIC was included for GM-CSF, IL-6, and TGF-β, since GM-CSF and IL-6 were not detected in the samples except in LPS-treated cells. TGF-β was constitutively expressed in all samples. The data presented are from one of three independent experiments that gave similar results. (B) The intensities of bands were determined by using a gel video system with image analysis software, and the ratios of targets to MIMICs were normalized against G3PDH mRNA in the corresponding samples and shown in the graph. Data are presented as means $\pm$ standard deviations of three independent experiments. Bars: 1, medium; 2, THP-1 lysate; 3, viable *E. chaffeensis*; 4, heat-killed *E. chaffeensis*; 5, periodate-treated *E. chaffeensis*; 6, *E. coli* LPS.

FIG. 3. Cytokine mRNA expression in human peripheral blood monocytes. Human peripheral blood monocytes at 10^7 cells were treated with either *E. coli* LPS, host cell-free *E. chaffeensis* at approximately 100 ehrlichiae per cell, or uninfected THP-1 cell lysate. After 2 h, total RNA was extracted and subjected to competitive RT-PCR as described in Materials and Methods. A constant amount of PCR MIMIC was coamplified with cDNA, and then the PCR products were resolved on 1.8% agarose gels. No MIMIC was included for GM-CSF, IL-6, and TGF- β , since GM-CSF and IL-6 were not detected in the samples except in LPS-treated cells. TGF- β was constitutively expressed in all samples. The data presented are from one of three independent experiments that gave similar results.

treated with medium alone or lysates of uninfected cells were used as negative controls. The absence of contamination of RNA with genomic DNA was monitored by verifying the PCR product from a "minus-reverse transcriptase" negative control. This control cDNA was prepared by assembling a reaction mixture that contains RNA and all other reagents except for reverse transcriptase. No PCR product was generated in the negative control for each sample with specific primers for each mRNA used in this study. The optimum cycle number for each

mRNA was used to assay PCR products during the exponential phase of the amplification. In THP-1 cells stimulated with *E. coli* LPS at 1 μ g/ml, mRNAs for IL-1 β , IL-6, IL-8, IL-10, TNF- α , and GM-CSF were induced within 2 h (Fig. 2). TGF- β mRNA was constitutively expressed in both infected and uninfected cells, in agreement with the previous report using human blood monocytes and monocytic cell lines (48). The expression of constitutively expressed G3PDH mRNA served as a control for the amount of input RNA across the samples for each experiment. All samples showed comparable levels of expression for G3PDH. In cells infected with *E. chaffeensis*, significant but lower levels of IL-1 β , IL-8, and IL-10 were expressed than with *E. coli* LPS at 2 h postincubation (Fig. 2). However, IL-6 and GM-CSF mRNAs were not detected in *E. chaffeensis*-infected cells, even with the increase in the number of PCR cycles and the amount of input cDNA. The mRNA expression was also determined at 6 and 18 h postinfection for all cytokines. IL-1 β , IL-8, and IL-10 mRNAs were induced, but IL-6, GM-CSF, and TNF- α mRNAs were not induced at either 6 or 18 h (data not shown). A low level of constitutive TNF- α message was detected in nontreated THP-1 cells as reported previously (19), but the TNF- α mRNA levels were not elevated in *E. chaffeensis*-infected cells compared with cells treated with medium alone or THP-1 cell lysate. Similar results were obtained with human peripheral blood monocytes. IL-1 β , IL-8, and IL-10 mRNAs were induced, but IL-6, TNF- α , and GM-CSF mRNAs were not detected in peripheral blood monocytes infected with *E. chaffeensis* for 2 h (Fig. 3). The induction of IL-6, GM-CSF, and TNF- α mRNAs in peripheral blood monocytes was also absent at 6 and 18 h postinfection with *E. chaffeensis* (data not shown).

Ehrlichial carbohydrate component is required for IL-1 β , IL-8, and IL-10 mRNA induction in THP-1 cells. LPS is not readily detectable in ehrlichiae by conventional methods (36). To determine whether viable ehrlichiae and which ehrlichial component(s) are required in cytokine response in monocytes or whether any ehrlichial component is blocking cytokine mRNA induction, we compared the effects of heat-killed and periodate-treated ehrlichiae to that of viable ehrlichiae. In cells treated with heat-killed *E. chaffeensis*, cytokine mRNA induction was similar to that with viable organisms (Fig. 2). In contrast, periodate-treated ehrlichial preparation failed to induce any of the cytokine mRNAs examined. Mild periodate oxidation at acidic pH has been reported to destroy carbohy-

TABLE 1. Cytokine production by THP-1 cells infected with *E. chaffeensis*^a

Treatment	Fraction ^b	Concn (pg/ml)		
		IL-1 β	IL-8	IL-10
Medium	S	<0.3	<3.0	<2.0
	S+L	<0.3	<3.0	<2.0
THP-1 lysate	S	<0.3	<3.0	<2.0
	S+L	<0.3	<3.0	<2.0
Viable <i>E. chaffeensis</i>	S	216 $\pm$ 19	14,322 $\pm$ 1342	948 $\pm$ 138
	S+L	342 $\pm$ 37	20,849 $\pm$ 2721	1,440 $\pm$ 118
Heat-killed	S	170 $\pm$ 11	13,936 $\pm$ 2755	252 $\pm$ 23
	S+L	313 $\pm$ 35	14,268 $\pm$ 3258	319 $\pm$ 69
Periodate-treated	S	<0.3	<3.0	<2.0
	S+L	<0.3	<3.0	<2.0
<i>E. coli</i> LPS	S	776 $\pm$ 84	26,505 $\pm$ 1770	2,052 $\pm$ 371
	S+L	780 $\pm$ 87	24,871 $\pm$ 2550	2,197 $\pm$ 369

^a Cytokine levels were measured by the capture ELISA (see Materials and Methods). THP-1 cells were incubated with *E. chaffeensis* at 1,000 organisms per cell. The detection limits of IL-1 β , IL-8, and IL-10 were 0.3, 3.0, and 2.0 pg/ml, respectively. Results are expressed as the means $\pm$ standard deviations of three independent experiments.

^b S, culture supernatant; S+L, culture supernatant plus cell lysate.

FIG. 4. Dose-dependent IL-1 β , IL-8, and IL-10 mRNA expression in THP-1 cells in response to *E. chaffeensis*. (A) THP-1 cells were stimulated with various numbers of host cell-free *E. chaffeensis* for 2 h. The numbers of ehrlichiae per cell correspond to those of original infected cells used to prepare host cell-free ehrlichiae. The prepared cDNA was coamplified with a constant amount of PCR MIMIC, and then the PCR products were loaded on 1.8% agarose gels. The data presented are from one of two independent experiments that gave similar results. (B) The intensities of bands were determined by using a gel video system with image analysis software, and the ratios of targets to MIMICs were normalized against G3PDH mRNA in the corresponding samples and shown in the graph.

dehydrate without altering protein or lipid structure (50). Neither treatment could induce cytokine mRNAs which were not induced by live ehrlichiae. Therefore, these results indicate that viable organisms are not required, but ehrlichial carbohydrate is required for IL-1 β , IL-8, and IL-10 mRNA induction. Neither viable ehrlichiae nor ehrlichial protein or carbohydrate appears to interfere with cytokine mRNA expression, since none of the levels of cytokine mRNA expression were increased.

Cytokine secretion in THP-1 cells and human peripheral blood monocytes exposed to *E. chaffeensis*. To examine the cytokine protein synthesis and secretion, we measured cytokine level by a capture ELISA. As noted in Table 1, the results corresponded with RT-PCR data. Both viable and heat-killed *E. chaffeensis* could increase IL-1 β , IL-8, and IL-10 production and secretion in THP-1 cells. In cells stimulated with *E. chaffeensis* or LPS, the proportions of extracellular and intracellular cytokines were similar. This indicates that the secretion of synthesized cytokines in *E. chaffeensis*-infected cells was not altered. IL-10 production by heat-killed *E. chaffeensis* was three- to fourfold lower than that by viable bacteria despite the presence of identical levels of IL-10 mRNA induction by heat-killed and viable *E. chaffeensis*. This result suggests that viable ehrlichiae or ehrlichial protein component(s) might be required for efficient IL-10 protein synthesis. IL-1 β , IL-8, and IL-10 were also produced in human peripheral blood monocytes in response to approximately 100 *E. chaffeensis* per cell (data not shown). Although human peripheral blood monocytes incubated with medium or THP-1 lysate expressed variable low levels of these three cytokine mRNAs, IL-1 β or IL-10 protein was not detectable in the culture medium. IL-8 protein was detected in the medium control which was increased approximately twofold upon exposure to *E. chaffeensis* or *E. coli* LPS.

Dose-dependent IL-1 β mRNA expression in THP-1 cells infected with *E. chaffeensis*. Since the levels of IL-1 β , IL-8, and IL-10 mRNA expression were significantly lower than those with *E. coli* LPS (1- μ g/ml) stimulation, we examined whether approximately 100 bacteria per cell used was the optimum for stimulation. As shown in Fig. 4, IL-1 β mRNA was maximally induced at approximately 100 bacteria per cell. For IL-8 and IL-10, 100 bacteria per cell also induced maximum mRNA.

Time course analysis of IL-1 β mRNA expression in THP-1 cells. To examine if cytokine induction and turnoff are different in viable and heat-killed ehrlichiae, we performed a time course analysis. Figure 5 indicates that the expression of IL-1 β and IL-8 mRNAs by viable *E. chaffeensis* peaked at 6 h of

infection and then decreased back to the 0-h level at 24 h. Identical time course results of IL-1 β and IL-8 mRNA expression were obtained with heat-killed *E. chaffeensis*. IL-10 mRNA induction peaked at 12 h, and the mRNA was not detected after 48 h of postinfection with either viable or heat-killed *E. chaffeensis*. This suggests that proliferation and protein components of ehrlichiae do not influence the induction or stability of mRNAs of these three cytokines. Next, to confirm the rapid shutoff of cytokine mRNA expression by ehrlichial stimulation, we measured IL-1 β and IL-8 mRNA levels at 18, 21, and 24 h. As shown in Fig. 5C, the levels of IL-1 β and IL-8 mRNA expression were still high at 24 h with LPS stimulation but almost disappeared at 21 h of *E. chaffeensis* stimulation.

Effects of anti-CD14 MAb and polymyxin B on *E. chaffeensis*-mediated IL-1 β induction. CD14, which is a differentiation antigen of mature monocytes, binds the complex of LPS and LPS-binding protein (LBP) (23, 51) and is responsible for cytokine gene induction by LPS (11). To evaluate whether *E. chaffeensis* induces IL-1 β mRNA primarily through CD14, we compared the inhibitory effect of anti-CD14 MAb on the expression of mRNAs for IL-1 β in THP-1 cells. As indicated in Fig. 6, treatment with 5 μ g of anti-CD14 MAb per ml significantly inhibited LPS-induced IL-1 β mRNA expression as previously described (11). In contrast, *E. chaffeensis*-mediated IL-1 β mRNA induction was not significantly affected by anti-CD14 MAb. Isotype-matched control monoclonal antibody did not have an effect on IL-1 β mRNA induction. We also examined the effect of polymyxin B which is known to bind to LPS and block LPS action (32, 46). In the presence of polymyxin B at 30 μ g/ml, LPS-mediated IL-1 β induction was dramatically blocked, while *E. chaffeensis*-mediated IL-1 β response was not inhibited. The concentration of polymyxin B used was the maximum concentration that did not cause toxic effects to mammalian cells (42). Taken together, these results suggest that *E. chaffeensis* might not contain typical enteric bacterium-type LPS and employ receptor(s) different from CD14 for signal transduction in IL-1 β gene expression.

DISCUSSION

In our study we found that *E. chaffeensis* did not induce TNF- α , IL-6, and GM-CSF and induced lower levels of IL-1 β , IL-8, and IL-10 than *E. coli* LPS in THP-1 cells did. Previously, we have shown that *E. risticii* induces IL-1 α production in murine peritoneal macrophages in vitro. However, the levels of TNF- α , IL-6, and prostaglandin E₂ produced were significantly lower than those induced by *E. coli* LPS stimulation (47). Thus, at both mRNA and protein levels, *Ehrlichia* spp. do not appear to induce strong proinflammatory cytokine production. This absence of strong activation of monocytes may aid the survival of organisms during the early stages of infection in delaying overall activation of host immune system. The absence of TNF- α , IL-6, and GM-CSF mRNA induction at 2 h postinfection was not due to delayed response of these cytokine mRNA induction, since these mRNAs were not detected either at 6 or 18 h postinfection. The number of *E. chaffeensis* used was optimum for IL-1 β , IL-8, and IL-10 mRNA induction. Therefore, the absence of these cytokine mRNAs was not due to the presence of insufficient or excess numbers of *E. chaffeensis*. Since peripheral blood monocytes responded to *E. chaffeensis* in a similar manner, the result obtained with THP-1 cells is relevant to understanding the responses of normal human monocytes to *E. chaffeensis*. It is not clear why *E. chaffeensis* does not induce either TNF- α , IL-6, or GM-CSF mRNA. It may be simply that *E. chaffeensis* lacks a component(s) which is

FIG. 6. Effects of anti-CD14 MAb and polymyxin B on IL-1 β mRNA induction. (A) THP-1 cells were incubated at approximately 100 *E. chaffeensis* per cell, 1 μ g of *E. coli* LPS per ml, or control lysate in the presence or absence of either monoclonal anti-CD14, isotype control mouse immunoglobulin G2b (IgG), or polymyxin B sulfate for 2 h. After incubation, total RNA was extracted and subjected to semiquantitative RT-PCR in the presence of 1 amol of IL-1 β MIMIC. The PCR products were resolved on 1.8% agarose gels. The data presented are from one of two independent experiments that gave similar results. Molecular size standards (ϕ X174 DNA digested with *Hae*III) are shown in the unlabeled, leftmost lane. (B) Histogram showing the results after the intensities of bands were analyzed by a gel video system with image analysis software. The ratios of targets to MIMICs were normalized against G3PDH mRNA in the corresponding samples and shown in the graph.

present in *E. coli* LPS and induces these cytokine mRNAs. Alternatively, *E. chaffeensis* may induce a suppressive factor which selectively suppresses induction of these mRNAs. Our preliminary data showed that when *E. chaffeensis* and *E. coli* LPS were added simultaneously to THP-1 cells, LPS-mediated mRNA induction of TNF- α or IL-6 was not impaired. This suggests the former possibility. However, our previous study demonstrated that when mouse peritoneal macrophages were incubated with live or heat-killed *E. risticii* for 24 h, TNF- α production in response to *E. coli* LPS was significantly reduced (47), suggesting the possible delayed induction of a suppressive factor.

A gram-negative facultative intracellular bacterium, *Legionella pneumophila*, induces IL-1 α , IL-1 β , IL-6, TNF- α , and GM-CSF in murine peritoneal macrophages with 1 h of exposure (53). A gram-positive facultative intracellular bacterium, *Mycobacterium tuberculosis*, induces transcription and secretion of TNF- α , IL-1 β , and IL-6 in THP-1 cells within 1 and 4 h,

respectively, thus inducing a strong cell-mediated immune response (20, 54). *Listeria monocytogenes* also induces expression of IL-1 α , IL-1 β , IL-6, and TNF- α mRNAs at 4 h postinfection (26). Obligate intracellular bacteria *Rickettsia* and *Chlamydia* spp. have been reported to induce both TNF- α and IL-1 production by macrophages (28, 38, 49). The absence of TNF- α and IL-6 mRNA induction in macrophages by *E. chaffeensis* is unique among known intracellular bacteria. This may be related to the obligate intramonozytic nature of *Ehrlichia* spp. They may need more-stringent inhibition of macrophage activation in order to survive. Our results also suggest that clinical signs of ehrlichiosis are not caused by the simple interaction of macrophages and ehrlichiae. Our preliminary work suggests that additional immune components are required in inducing strong proinflammatory cytokines in *Ehrlichia*-infected macrophages.

Although weaker than with *E. coli* LPS, *E. chaffeensis* could induce IL-1 β , IL-8, and IL-10 mRNAs and proteins in human macrophages. Viable ehrlichiae are not required for this induction, as is IL-1 α production in murine macrophages by *E. risticii* (47). The early shutoff of mRNA expression appears to be another unique nature of ehrlichial cytokine gene induction. Our results show that *E. chaffeensis*-induced IL-1 β and IL-8 mRNA expression was turned off more rapidly than LPS-induced expression. IL-10 mRNA induction by *E. chaffeensis* which peaked at 12 h and decreased almost to the background level also appears to be transient compared with IL-10 induction by LPS shown in the previous report (15). This is also distinct from cytokine mRNA induction by other intracellular bacteria. IL-1 α mRNA in mouse spleen cells infected with *L. monocytogenes* has been detected at 120 h postinfection (24). *M. tuberculosis*-induced strong transcription of mRNA of cytokines including IL-1 β , IL-8, and IL-10 was identified at 24 h postinfection in human blood monocytes (4). Although the mechanism by which *Ehrlichia* organisms induce early turnoff of cytokine mRNA expression is not clear at this point, we speculate that *Ehrlichia* organisms may reduce cytokine mRNA stability.

None of these cytokines generated apparently suppressed the growth of *E. chaffeensis* in macrophages at all. IL-8 is known to attract neutrophils and T cells (3). IL-8 may temporarily elicit the influx of neutrophils to the site of infection; this may serve as one of the earliest but short-lived host protective responses to *E. chaffeensis* infection. In support of this, we have previously demonstrated that *E. risticii* can be taken up by equine neutrophils and killed in vitro (29). Since IL-10 deactivates macrophages (30), IL-10 may play an immunosuppressive role in *E. chaffeensis* infection, which is in agreement with our earlier result demonstrating the hypimmune response in mice infected with *E. risticii* (37). IL-1 β production may be correlated with early clinical signs, such as fever and depression.

Both viable and heat-killed *E. chaffeensis* induced the same levels of IL-1 β , IL-8, and IL-10 mRNAs, and these levels of cytokine mRNA expression were not different over an 120-h incubation period whether *E. chaffeensis* was viable or heat killed. Similarly, both viable and heat-killed *L. monocytogenes* and nonhemolytic strains of *L. monocytogenes* induce IL-1 β in P388D₁ macrophages, suggesting that hemolysin and/or listerial proliferation is not required for IL-1 β mRNA induction (26). Like *E. chaffeensis*, heat-killed and viable *E. risticii* induced essentially the same cytokine responses in murine peritoneal macrophages (47). Thus, ehrlichial proliferation inside macrophages is not a prerequisite for expression of these cytokine mRNAs. *Brucella suis* generates a protein factor which inhibits TNF- α production (7). Since neither heat nor perio-

date treatment enhanced ehrlichial cytokine mRNA expression, ehrlichiae do not appear to generate such an inhibitor.

Heat shock proteins of *Mycobacterium* spp., *L. pneumophila*, and *E. coli* and outer membrane proteins from *Chlamydia trachomatis* are capable of inducing proinflammatory cytokines (21, 27, 34, 49). LPS from *C. trachomatis* (25), *E. coli*, and *Salmonella* species and lipoarabinomannan from *Mycobacterium* sp. (4) are potent inducers of proinflammatory cytokines. All of these bacterial components can induce both TNF- α and IL-1, whereas *Ehrlichia* spp. did not induce TNF- α . Lipoteichoic acid from pneumococcal cell wall has also been shown to induce production of IL-1 but not TNF- α in human monocytes (35). Although *E. chaffeensis* has a GroEL homolog (40), our study showed that this did not appear to induce cytokine mRNA. In the case of *E. chaffeensis*, carbohydrate, rather than heat-labile protein, was required for IL-1 β , IL-8, and IL-10 mRNA induction. This carbohydrate may be a part of LPS of *E. chaffeensis*. Although for *E. chaffeensis*, the presence of LPS has not been yet demonstrated, *E. risticii* appeared to contain LPS of an extremely low endotoxic activity (8).

LPS by itself has a limited capacity to bind to CD14, but when serum LBP is present, LPS-LBP complexes bind to CD14, leading to induction of several proinflammatory cytokines (11, 51). LBP binds to lipid A portion of LPS (43). CD14 is not, however, the only LPS receptor that is involved in the cytokine gene expression in LPS-stimulated cells. CD11 and CD18, nonspecific binding sites which mediate hydrophobic interactions of LPS with the monocyte membrane and an unidentified molecule which mediate the serum-dependent binding of the purified polysaccharide of LPS from *Salmonella minnesota* R7 were reported (10). A recent study showed that lipoarabinomannan but not a 65-kDa heat shock protein or culture filtrate protein induces cytokine of *M. tuberculosis* gene expression in THP-1 cells through CD14-like LPS (54). Although a LPS-like substance of *E. chaffeensis* may be responsible for IL-1 β , IL-8, and IL-10 induction, the lack of inhibition of IL-1 β mRNA expression by polymyxin B and anti-CD14 antibody suggests that *E. chaffeensis* acts through non-CD14 receptor(s) on the surfaces of macrophages. This may be the reason why the cytokine expression is different from that with *E. coli* LPS. Further studies on the mechanisms of cytokine gene regulation in response to ehrlichial stimuli could provide valuable insight into the host immune and inflammatory responses to ehrlichial infection.

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