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Anti-*Ehrlichia chaffeensis* Antibody Complexed with *E. chaffeensis* Induces Potent Proinflammatory Cytokine mRNA Expression in Human Monocytes through Sustained Reduction of IκB-α and Activation of NF-κB

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***Ehrlichia chaffeensis* is an obligatory intracellular bacterium that infects monocytes and macrophages and is the etiologic agent of human ehrlichiosis in the United States. Our previous studies showed that the exposure of human monocytes to *E. chaffeensis* induces the expression of interleukin-1β (IL-1β), IL-8, and IL-10 genes in vitro but not the expression of tumor necrosis factor alpha (TNF-α) and IL-6 mRNAs. In this study, the effect of anti-*E. chaffeensis* antibody complexed with *E. chaffeensis* on the expression of major proinflammatory cytokines in human monocytes was examined. Human monocytic cell line THP-1 was treated with *E. chaffeensis* which had been preincubated with human anti-*E. chaffeensis* serum for 2 h, and the levels of cytokine mRNAs were evaluated by competitive reverse transcription-PCR. Anti-*E. chaffeensis* antibody complexed with *E. chaffeensis* significantly enhanced mRNA expression of IL-1β in THP-1 cells. The expression of TNF-α and IL-6 mRNAs was also induced. The levels of secreted IL-1β, TNF-α, and IL-6 during 24 h of stimulation were comparable to those induced by *Escherichia coli* lipopolysaccharide at 1 μg/ml. Fab fragment of anti-*E. chaffeensis* immunoglobulin G complexed with *E. chaffeensis* did not induce any of these three cytokines, indicating that ehrlichial binding is required for IL-1β mRNA expression and that binding of the immune complex to the Fcγ receptor is required for TNF-α and IL-6 mRNA expression and enhanced IL-1β mRNA expression. Furthermore, prolonged degradation of IκB-α and activation of NF-κB were demonstrated in THP-1 cells exposed to anti-*E. chaffeensis* serum and *E. chaffeensis*. This result implies that development of anti-*E. chaffeensis* antibody in patients can result in the production of major proinflammatory cytokines, which may play an important role in the pathophysiology of ehrlichiosis and immune responses to it.**

Ehrlichia chaffeensis, a gram-negative obligately intracellular bacterium that can infect monocytes and macrophages (32), is recognized as the etiologic agent of human monocytic ehrlichiosis in the United States (1, 11). Since the discovery of the disease in 1986, more than 400 cases of human ehrlichiosis have been reported in 30 states in the United States (14). A PCR assay detected *E. chaffeensis* DNA in the Lone Star tick, *Amblyomma americanum*, implying that this tick is a vector of the disease (2). Seven to nine days after a tick bite, patients develop fever, headache, malaise, thrombocytopenia, leukopenia, and elevated liver enzyme activities and C-reactive-protein levels (16). These clinical signs were similar to those mediated by proinflammatory cytokines, including interleukin-1β (IL-1β), tumor necrosis factor alpha (TNF-α), and IL-6. However, we were puzzled, since our previous results showed that *E. chaffeensis* induces IL-1β but not TNF-α or IL-6 in the human monocytic cell line THP-1 and human peripheral blood monocytes (22). *E. chaffeensis*-infected patients develop a high titer of antibody; therefore, a serologic test has been used as the primary diagnostic method (12). Although in human monocytic ehrlichiosis no correlation of clinical signs and antibody development has been reported, in experimental *Ehrlichia canis* infection of dogs and *Ehrlichia risticii* infection of horses the development of clinical signs coincides with formation of the antibody to *E. canis* and *E. risticii*, respectively (20, 34). Cross-linking of Fc receptors on monocytes by anti-Fc receptor antibody or immobilized immunoglobulin G (IgG) was reported to induce TNF-α, IL-1β, IL-6, and IL-8 production in human monocytes (13, 21, 25, 26, 31). Therefore, we examined whether the complex of anti-*E. chaffeensis* antibody and *E. chaffeensis* induces a strong proinflammatory cytokine response in human monocytes and whether this response is mediated through Fcγ receptors. The mechanism by which cross-linking Fc receptors induce cytokines in monocytes is unknown; however, transcription factor NF-κB is known to be involved in induction of cytokine gene expression by various stimuli, including lipopolysaccharide (LPS) (10, 18, 23). We therefore further examined whether the complex of *E. chaffeensis* and anti-*E. chaffeensis* antibody induces NF-κB activation in human monocytes. This study revealed an important new role of antibody for host defense and pathogenesis during intracellular bacterial infection.

MATERIALS AND METHODS

***E. chaffeensis*.** *E. chaffeensis* Arkansas was cultured in THP-1 cells as previously described (22) in RPMI 1640 medium supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, Ga.) and 2 mM L-Gln (GIBCO, Grand Island, N.Y.). When more than 90% of the cells were infected, as determined by examining cells stained with Diff-Quik (Baxter Scientific Products, Obetz, Ohio), the infected cells were sonicated and centrifuged at 500 × *g* for 5 min. The supernatant was centrifuged at 10,000 × *g* for 10 min, and the pellet, containing host cell-free *E. chaffeensis* organisms, was used to infect THP-1 cells.

Treatment of cells. For competitive reverse transcription-PCR (RT-PCR) analysis, 10^7 THP-1 cells in 5 ml of medium were treated with each stimulus for 2 h and harvested for RNA isolation. For electrophoretic mobility shift and Western immunoblot assays, 10^6 cells in 1 ml of medium were incubated with each stimulus for the periods indicated in the figures and nuclear and cytoplasmic

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extracts were prepared. Human anti-*E. chaffeensis* serum was kindly provided by J. R. Harkens and J. Kudlak of the Oklahoma State Department of Health (homologous-antibody titer = 1:640 by the indirect fluorescent-antibody [IFA] test) (33). Dog anti-*E. chaffeensis* serum was developed as previously described (IFA titer = 1:5,120) (6). Normal dog serum was negative for *E. chaffeensis* by the IFA test. Normal human serum was purchased from Sigma Chemical Co. (St. Louis, Mo.) and negative by the IFA test. The sera were preabsorbed with 10^6 uninfected THP-1 cells per ml by incubation at 37°C for 1 h and used at 0.2 ml per ml of incubation medium. IgG and Fab fragment were purified from preabsorbed normal or dog anti-*E. chaffeensis* serum by using an ImmunoPure Fab preparation kit (Pierce Chemical Co., Rockford, Ill.) and used at 0.1 mg per ml of incubation medium. Preparation of Fab fragments was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunofluorescence microscopy using fluorescein isothiocyanate-conjugated anti-dog IgG as previously described (28).

Competitive RT-PCR analysis. Total RNA was isolated from the cells by the guanidinium thiocyanate method (9). Total cellular RNA (2 μg) was reverse transcribed in a 30-μl reaction mixture by using a kit (first-strand cDNA synthesis kit; Clontech Laboratories Inc., Palo Alto, Calif.). The prepared cDNA (1 to 2 μl) was amplified in a 50-μl reaction mixture. Conditions for PCR were as follows: 0.4 μM each primer, 0.2 mM deoxynucleoside triphosphate mixture (Life Technologies, Inc., Gaithersburg, Md.), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, and 2.0 U of *Taq* DNA polymerase (Life Technologies). Primers and nonhomologous internal standards (PCR MIMICs) for IL-1β and TNF-α and primers for IL-6 were purchased from Clontech Laboratories. The PCR MIMIC for IL-6 was constructed by using a PCR MIMIC construction kit (Clontech). The amounts of MIMICs added to the PCR mixtures in the present study were 1 amol for IL-1β, TNF-α, and glyceraldehyde-3-phosphate dehydrogenase and 10^{-3} amol for IL-6. Reaction mixtures were incubated in a DNA thermal cycler (model 480; Perkin-Elmer) for 30 cycles (denaturation at 94°C for 45 s, annealing at 60°C for 45 s, extension at 72°C for 2 min). Aliquots of reaction products were electrophoresed in 1.8% agarose gels containing 0.5 μg of ethidium bromide per ml. The amounts of PCR products generated by the target and competitive internal standard were analyzed by a gel video system (Gel Print 2000i; BioPhotonics Corp., Ann Arbor, Mich.) and image analysis software (ImageQuaNT; Molecular Dynamics, Sunnyvale, Calif.). The ratio of target to internal standard was calculated and normalized against glyceraldehyde-3-phosphate dehydrogenase mRNA in the corresponding sample.

Nuclear- and cytoplasmic-extract preparation. The nuclear extract for the NF-κB assay and the cytoplasmic extract for the IκB-α Western blot assay were prepared from the same experimental sample by the method of Schreiber et al. (37). Briefly, after the treatment described above, cells were centrifuged, washed with Tris-buffered saline (pH 7.9), and then suspended in 400 μl of cold buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μM aprotinin, 14 μM leupeptin, 1 μM pepstatin, 80 μg of benzamidine per ml). After being incubated on ice for 15 min, the cells were lysed by the addition of Nonidet P-40 (final concentration of 0.6%). Lysis was completed by vigorous vortexing for 10 s. The homogenate was centrifuged at 13,000 × *g* for 30 s in a microcentrifuge, the supernatant was saved for cytoplasmic extract, and the nuclear pellet was resuspended in 50 μl of cold buffer C (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μM aprotinin, 14 μM leupeptin, 1 μM pepstatin, 80 μg of benzamidine per ml). After centrifugation at 13,000 × *g* for 30 s, the resulting supernatant of nuclear extract was stored in small aliquots at −80°C. Protein concentrations were determined by using the bicinchoninic acid protein assay reagent (Pierce).

EMSA. Electrophoretic mobility shift assays (EMSAs) were performed by using a gel shift assay system (Promega Corp., Madison, Wis.) according to the manufacturer's protocol. The NF-κB consensus oligonucleotide (22 bp) provided in the kit was end labeled with [γ-^{32}P]ATP with T4 nucleotide kinase, and 100,000 cpm of the probe was used to assess NF-κB binding. The reaction mixture for DNA-protein interaction contained 2 μg of the nuclear extract in 10 mM Tris-HCl (pH 7.5)–1 mM $MgCl_2$–0.5 mM EDTA–0.5 mM dithiothreitol–50 mM NaCl–0.05 μg of poly(dI-dC) per μl in 4% glycerol in a final volume of 25 μl. The radioactive DNA probe was added to this mixture and incubated at room temperature for 20 min. For competition assays, a 50-fold-excess amount of unlabeled SP1 consensus oligonucleotide (22 bp; Promega) or NF-κB consensus oligonucleotide was added to the reaction mixture with the labeled oligonucleotide. For supershift assay, 1 μl of rabbit antibody to p50 (NF-κB1) or rabbit antibody to p65 (RelA) (Rockland, Gilbertsville, Pa.) was added to the extracts before incubation with the labeled oligonucleotide. After incubation, the samples were first loaded onto a 4% polyacrylamide gel (0.5× Tris-borate-EDTA) that had been prerun for 30 min and were then electrophoresed at 100 V for 4 h. The gel was exposed to an X-ray film (X-Omat AR; Kodak, Rochester, N.Y.) at −80°C overnight. After autoradiography, the gel was dried onto a piece of Whatman 3MM paper under vacuum at room temperature for 2 h and analyzed with a PhosphorImager (Molecular Dynamics).

Western immunoblot analysis. The cytoplasmic extracts (30 μg each) prepared as described above were fractionated by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Schleicher & Schuell Inc., Keene, N.H.) by using a semidry electroblotting apparatus. Membranes were blocked overnight in Tween–phosphate-buffered saline (PBS-Tween) containing 5% nonfat milk powder. The membranes were then incubated with rabbit antiserum to IκB-α (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.) at 1 μg/ml in blocking solution at room temperature for 45 min and washed in PBS-Tween. Blots were then incubated with a 1:1,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham Life Science Inc., Arlington Heights, Ill.) in blocking solution for 30 min and washed in PBS-Tween. The antigen was detected by using an enhanced chemiluminescence Western blotting detection system according to the procedure recommended by the manufacturer (Amersham). The same membrane was reprobed with a 1:1,000 dilution of mouse monoclonal anti-α-tubulin (Cedarline Lab. Limited, Hornby, Ontario, Canada) to detect α-tubulin as an internal control.

Cytokine assays. THP-1 cells or human peripheral blood monocytes isolated as previously described (22) were incubated at 10^6 cells per ml in 12-well culture plates with each stimulus for 24 h, and culture supernatants were assayed for each cytokine level according to the manufacturer's protocol by using human cytokine immunoassay kits (Quantikine; R&D systems, Minneapolis, Minn.).

RESULTS

***E. chaffeensis* organisms preincubated with human or dog anti-*E. chaffeensis* serum induce strong TNF-α, IL-6, and IL-1β mRNA expression.** The conditions of the competitive RT-PCR were optimized as described elsewhere (22). We previously showed that THP-1 cell lysate used as a control for host cell substance contamination in *E. chaffeensis* preparation had no influence on cytokine mRNA expression in THP-1 cells (22). To examine the effect of anti-*E. chaffeensis* serum on the mRNA expression of major proinflammatory cytokines, THP-1 cells were treated with *E. chaffeensis* preincubated with medium, normal human serum, or anti-*E. chaffeensis* patient serum and TNF-α, IL-6, and IL-1β mRNA expression was analyzed by competitive RT-PCR. Figure 1 shows that *E. chaffeensis* preincubated with human anti-*E. chaffeensis* serum (the titer of immunofluorescent antibody against *E. chaffeensis* was 1:640) induced strong TNF-α and IL-6 mRNA expression, which was not induced by *E. chaffeensis* alone or with normal human serum. IL-1β mRNA expression by *E. chaffeenis* was enhanced in the presence of antiserum but not in the presence of normal human serum (about fourfold). Because of the limited availability of human anti-*E. chaffeensis* serum, we examined whether dog anti-*E. chaffeensis* serum (the titer of immunofluorescent antibody against *E. chaffeensis* was 1:5,120) has a effect on cytokine mRNA induction similar to that of human antiserum and the optimum dosage of the dog immune serum in inducing cytokine mRNA expression. As shown in Fig. 2, dog anti-*E. chaffeensis* serum also strongly induced IL-1β, IL-6, and TNF-α mRNA expression. The maximum response was seen with a 10-fold dilution of dog anti-*E. chaffeensis* serum.

Cytokine mRNA expression with anti-*E. chaffeensis* serum is mediated by the Fcγ receptor. Previously, cross-linking of Fcγ receptors with anti-Fcγ receptor monoclonal antibody was reported to induce IL-1β, IL-6, IL-8, and TNF-α from human peripheral blood monocytes (13, 21, 25, 26, 31). We previously showed that *E. risticii* complexed with anti-*E. risticii* antibody binds to $P388D_1$ macrophages and that the Fab fragment of anti-*E. risticii* IgG blocks ehrlichial binding to either ehrlichial receptor or Fc receptor on $P388D_1$ cells (28). We speculated that (i) anti-*E. chaffeensis* antibody bound to *E. chaffeensis* interacts with Fcγ receptors that are on the monocyte surface and that (ii) this interaction is responsible for IL-6, TNF-α, and enhanced IL-1β mRNA expression. To test this hypothesis, THP-1 cells were exposed to *E. chaffeensis* preincubated with the dog immune serum, IgG, or Fab fragment purified from the immune serum, and cytokine mRNA expression was analyzed by RT-PCR. As shown in Fig. 3, the induction of TNF-α, IL-6, and IL-1β mRNA expression in cells treated with *E. chaffeensis* in the presence of anti-*E. chaffeensis* IgG was as high as that in cells treated with *E. chaffeensis* in the presence of anti-*E. chaffeensis* serum. In contrast, *E. chaffeensis* prein-

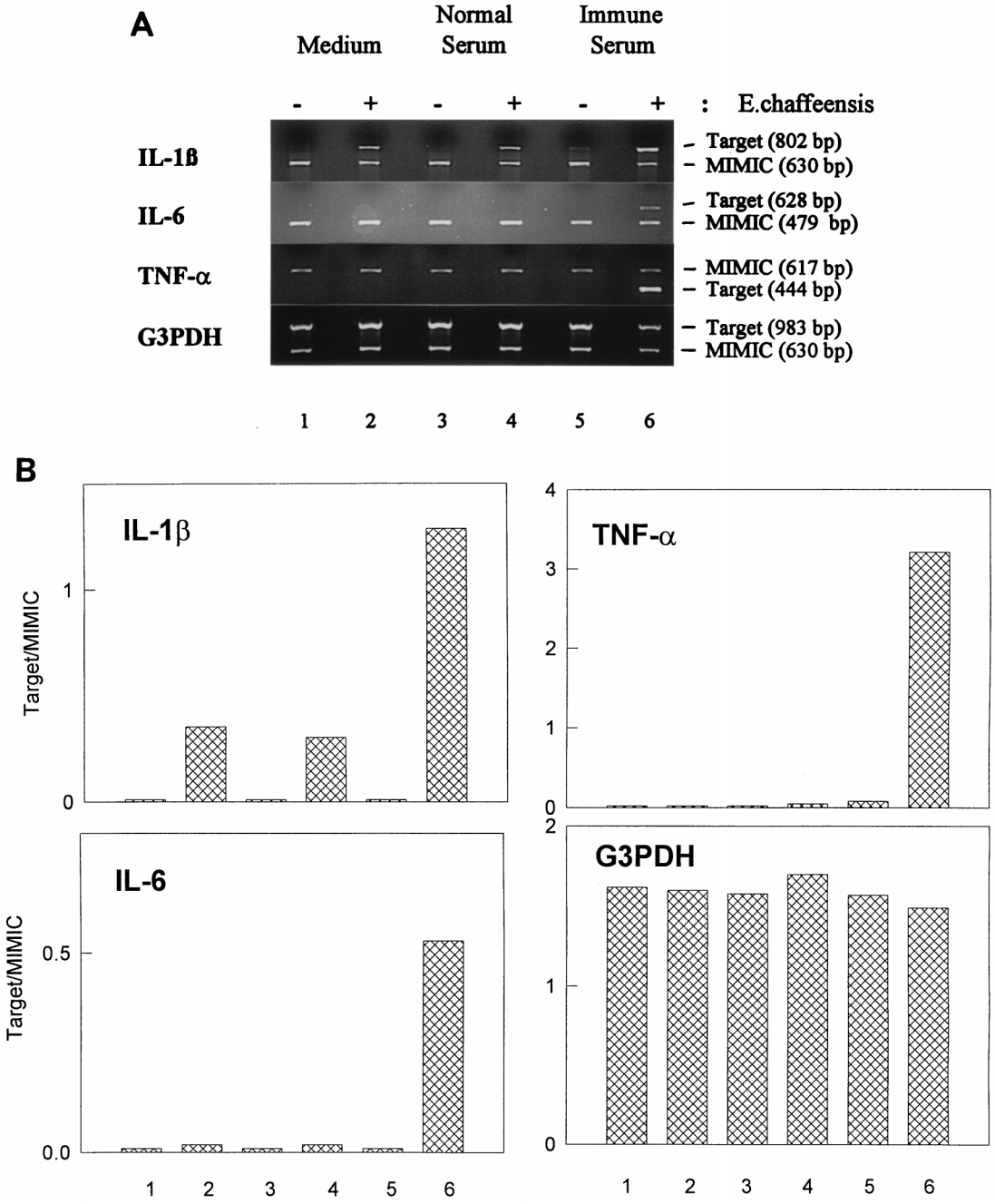

FIG. 1. Effect of human anti-*E. chaffeensis* serum on mRNA expression of IL-1β, IL-6, and TNF-α in THP-1 cells exposed to *E. chaffeensis*. (A) Medium alone, normal human serum, and human anti-*E. chaffeensis* serum were incubated at 37°C for 30 min with or without host cell-free *E. chaffeensis* and added to THP-1 cells. After 2 h, total RNA was extracted and subjected to competitive RT-PCR as described in Materials and Methods. A constant amount of the PCR MIMIC was coamplified with cDNA, and then the PCR products were resolved on 1.8% agarose gels. The data presented are from one of two independent experiments that gave similar results. (B) The intensities of the bands were determined by using a gel video system with image analysis software, and the ratios of targets to MIMICs are shown in the graph. G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

cubated with the Fab fragment of the immune IgG did not induce any of three cytokines examined. This may be due to the blockage of binding of the ehrlichial carbohydrate component, which was shown to be required for IL-1β induction (22) by the Fab fragment of anti-*E. chaffeensis* antibody. IL-1β mRNA induction in the presence of anti-*E. chaffeensis* antibody, therefore, most likely occurs independently from the receptor(s) for the ehrlichial carbohydrate. This result indicates that interaction of anti-*Ehrlichia* IgG with Fcγ receptors on monocytes is required for induction of TNF-α and IL-6 mRNA and for enhanced IL-1β mRNA expression.

Secretion of IL-1β, IL-6, and TNF-α by THP-1 cells. To examine the protein synthesis and secretion of cytokines, we determined cytokine levels secreted by THP-1 cells by capture enzyme-linked immunosorbent assay (ELISA). As shown in Table 1, normal serum did not have an effect on *E. chaffeensis*-mediated secretion of IL-1β, IL-6, and TNF-α. The immune serum induced secretion of TNF-α and IL-6 in THP-1 cells in

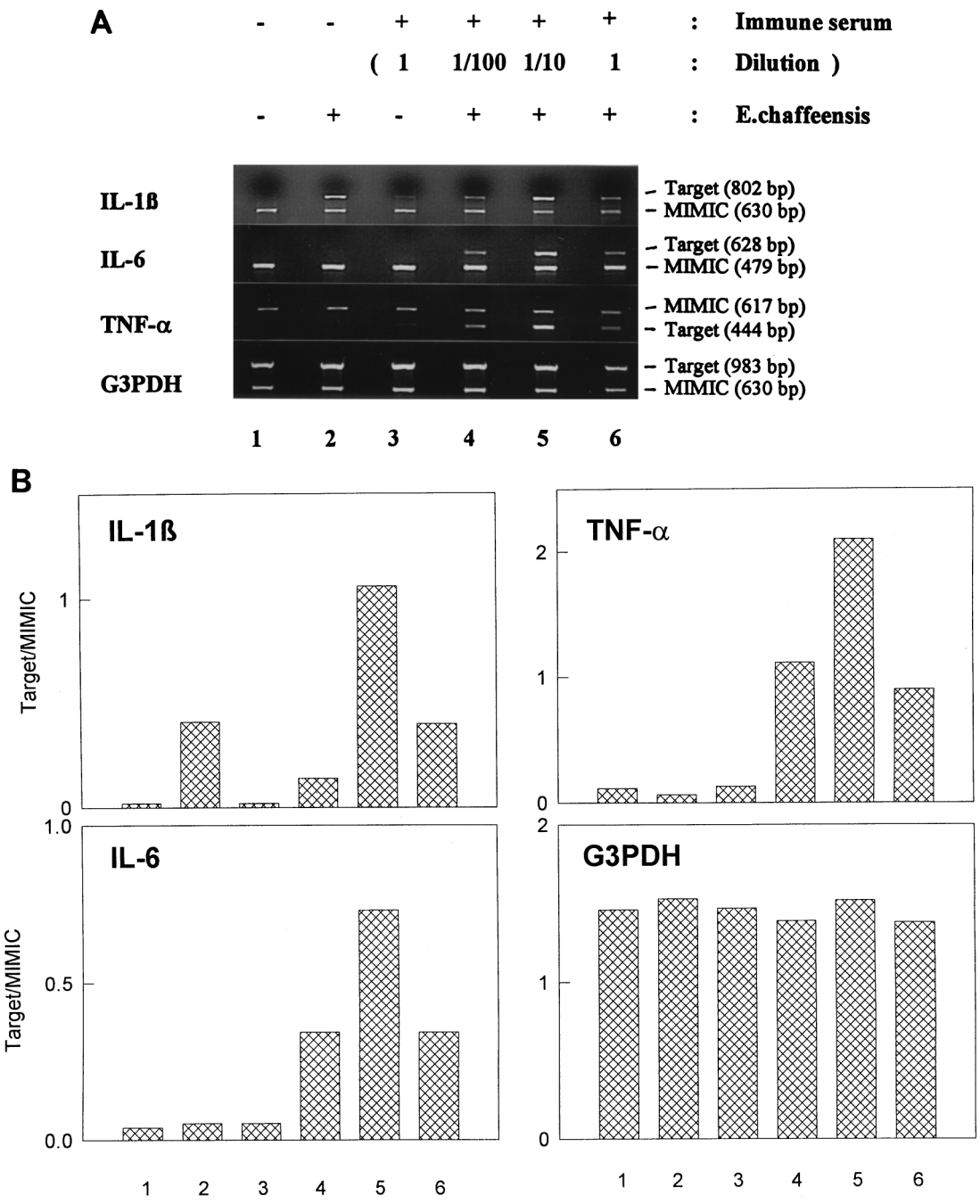

FIG. 2. Effect of dog anti-*E. chaffeensis* serum on expression of IL-1β, IL-6, and TNF-α mRNA in THP-1 cells exposed to *E. chaffeensis*. (A) Host cell-free *E. chaffeensis* organisms were incubated with either medium alone or different dilutions of dog anti-*E. chaffeensis* serum at 37°C for 30 min and added to THP-1 cells. After 2 h, total RNA was extracted and subjected to RT-PCR as described in Materials and Methods. A constant amount of the PCR MIMIC was coamplified with cDNA, and then the PCR products were resolved on 1.8% agarose gels. Similar data were obtained in two independent experiments. (B) The intensities of the bands were determined by using a gel video system with image analysis software, and the ratios of targets to MIMICs are shown. G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

the presence of *E. chaffeensis*. The secretion of IL-1β was also enhanced in the presence of the immune serum. Anti-*E. chaffeensis* IgG had similar effects on cytokine secretion. The levels of secreted cytokines in cells stimulated by immune serum or the anti-*E. chaffeensis* IgG were comparable to those in cells stimulated with *E. coli* LPS. However, immune Fab fragment failed to induce the secretion of any of these cytokines. These results corresponded with the RT-PCR results shown above. Similar results were obtained with normal human peripheral blood monocytes as shown in Table 2, supporting the data obtained with THP-1 cells.

Prolonged NF-κB activation is involved in cytokine gene expression in THP-1 cells exposed to anti-*E. chaffeensis* serum and *E. chaffeensis*. The transcription factor NF-κB consists of a family of dimeric factors and is known to play an important role in the transcriptional regulation of many cytokine genes,

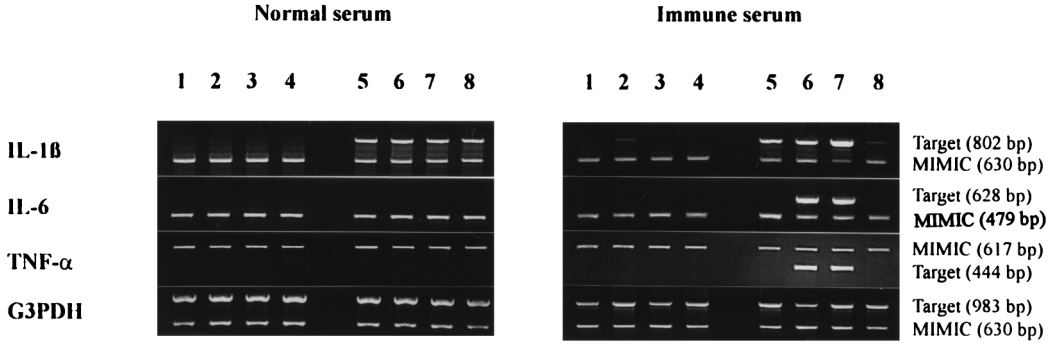

FIG. 3. Effect of IgG or the Fab fragment of dog anti-*E. chaffeensis* serum on mRNA expression of IL-1β, IL-6, and TNF-α in THP-1 cells exposed to *E. chaffeensis*. Total RNA was prepared from THP-1 cells treated for 2 h with medium (lane 1), serum (dilution, 1/10) (lane 2), IgG (lane 3), Fab fragment (lane 4), medium plus *E. chaffeensis* (lane 5), serum plus *E. chaffeensis* (lane 6), IgG plus *E. chaffeensis* (lane 7), or Fab fragment plus *E. chaffeensis* (lane 8). RT-PCR was performed as described in Materials and Methods. A constant amount of the PCR MIMIC was coamplified with cDNA, and then the PCR products were resolved on 1.8% agarose gels. Data shown are from one of two independent experiments with similar results. G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

including those for IL-1β, IL-6, IL-8, TNF-α, and granulocyte-macrophage colony-stimulating factor, by extracellular signals such as LPS (4, 10). The heterodimer p65/p50 is the prototypical and most abundant form which has been reported to be involved in transcriptional activation of various immune and inflammatory genes, while the homodimer p50/p50 is constitutively present (4, 10). We examined whether NF-κB activation is involved in the cytokine induction in THP-1 cells exposed to dog anti-*E. chaffeensis* serum and *E. chaffeensis*. In cells exposed to *E. chaffeensis* only, NF-κB (p65/p50) activation peaked at 30 min postincubation and decreased thereafter back to the control level at 4 h. In cells exposed to anti-*E. chaffeensis* serum and *E. chaffeensis*, NF-κB (p65/p50) activation peaked at 2 h and lasted for up to 8 h postincubation (Fig. 4A). Quantitation results of p65/p50 complexes confirmed that anti-*E. chaffeensis* serum and *E. chaffeensis* induced stronger and more prolonged NF-κB activation in THP-1 cells than *E. chaffeensis* alone (Fig. 4B). The induced NF-κB activity was composed of p65/p50 heterodimer, as confirmed by supershift assays (Fig. 4C, lanes 8 and 9). The faster-migrating complex constitutively present in both unstimulated and stimulated cells was composed of p50/p50 homodimers, as confirmed by supershifting (Fig. 4C, lanes 8 and 9). Binding of NF-κB complexes to the NF-κB consensus oligonucleotide was specific, since it was competitively inhibited by the nonlabeled specific oligonucleotide (Fig. 4C, lane 4) but not by a nonspecific oligonucleotide (Fig. 4C, lane 3).

IκB-α degradation correlates with NF-κB activation in THP-1 cells exposed to anti-*E. chaffeensis* serum and *E. chaffeensis*. IκB-α is a cytoplasmic protein (37 kDa) that binds to NF-κB and inhibits the binding of NF-κB to its cognate binding site by masking the nuclear localization signal of NF-κB (3, 5). Upon activation of cells with various extracellular signals such as LPS, phorbol myristate acetate (PMA), IL-1, or TNF-α, IκB-α is rapidly degraded, allowing NF-κB to translocate into the nucleus and bind to an NF-κB-responsive DNA element (19). We examined the changes in cytoplasmic IκB-α protein levels by Western blot analysis to determine whether IκB-α degradation correlates with NF-κB activation. As shown in Fig. 5, transient IκB-α degradation was demonstrated at 15 min to 1 h in THP-1 cells exposed to *E. chaffeensis* alone. In cells exposed to dog anti-*E. chaffeensis* serum and *E. chaffeensis*, prolonged IκB-α degradation was observed for up to 4 h, which generally corresponds to prolonged NF-κB activation, as observed in Fig. 4.

TABLE 1. Secretion of IL-1β, IL-6, and TNF-α by THP-1 cells infected with *E. chaffeensis* in the absence or presence of anti-*E. chaffeensis* serum[a]

Treatment	Concn (pg/ml)		
	IL-1β	IL-6	TNF-α
Medium alone	<0.3	<0.7	<4.4
Medium + *E. chaffeensis*	330 ± 21	<0.7	6 ± 3
Medium + *E. coli* LPS	836 ± 17	675 ± 105	2,924 ± 668
Normal serum + medium	<0.3	<0.7	<4.4
Normal serum + *E. chaffeensis*	339 ± 33	<0.7	8 ± 3
Immune serum + medium	<0.3	<0.7	9 ± 2
Immune serum + *E. chaffeensis*	728 ± 159	597 ± 73	2,982 ± 140
Immune IgG + medium	<0.3	<0.7	<4.4
Immune IgG + *E. chaffeensis*	754 ± 44	675 ± 120	2,778 ± 174
Immune Fab + medium	<0.3	<0.7	<4.4
Immune Fab + *E. chaffeensis*	13 ± 2	<0.7	5 ± 3

[a] Cytokine levels were measured by capture ELISA (see Materials and Methods). The detection limits of IL-1β, IL-6, and TNF-α were 0.3, 0.7, and 4.4 pg/ml, respectively. Results are the means ± standard deviations of three independent experiments.

DISCUSSION

This study demonstrated, for the first time, that antibody complexed with bacteria is responsible for proinflammatory cytokine production. Monocytic *Ehrlichia* spp. induce a strong antibody response (32). Antibody has been shown to play a

TABLE 2. Secretion of IL-6 and TNF-α by human peripheral blood monocytes infected with *E. chaffeensis* in the absence or presence of anti-*E. chaffeensis* serum[a]

Treatment	Concn (pg/ml)	
	IL-6	TNF-α
Medium alone	<0.7	44 ± 2.7
Medium + *E. chaffeensis*	<0.7	45 ± 4.2
Medium + *E. coli* LPS	746 ± 89	2,601 ± 180
Normal serum + medium	<0.7	40 ± 4.2
Normal serum + *E. chaffeensis*	<0.7	51 ± 4.2
Immune serum + medium	<0.7	43 ± 6.4
Immune serum + *E. chaffeensis*	736 ± 105	2,445 ± 125

[a] Cytokine levels were measured by capture ELISA. The detection limits of IL-6 and TNF-α were 0.7 and 4.4 pg/ml, respectively. Results are the means ± standard deviations of three independent experiments.

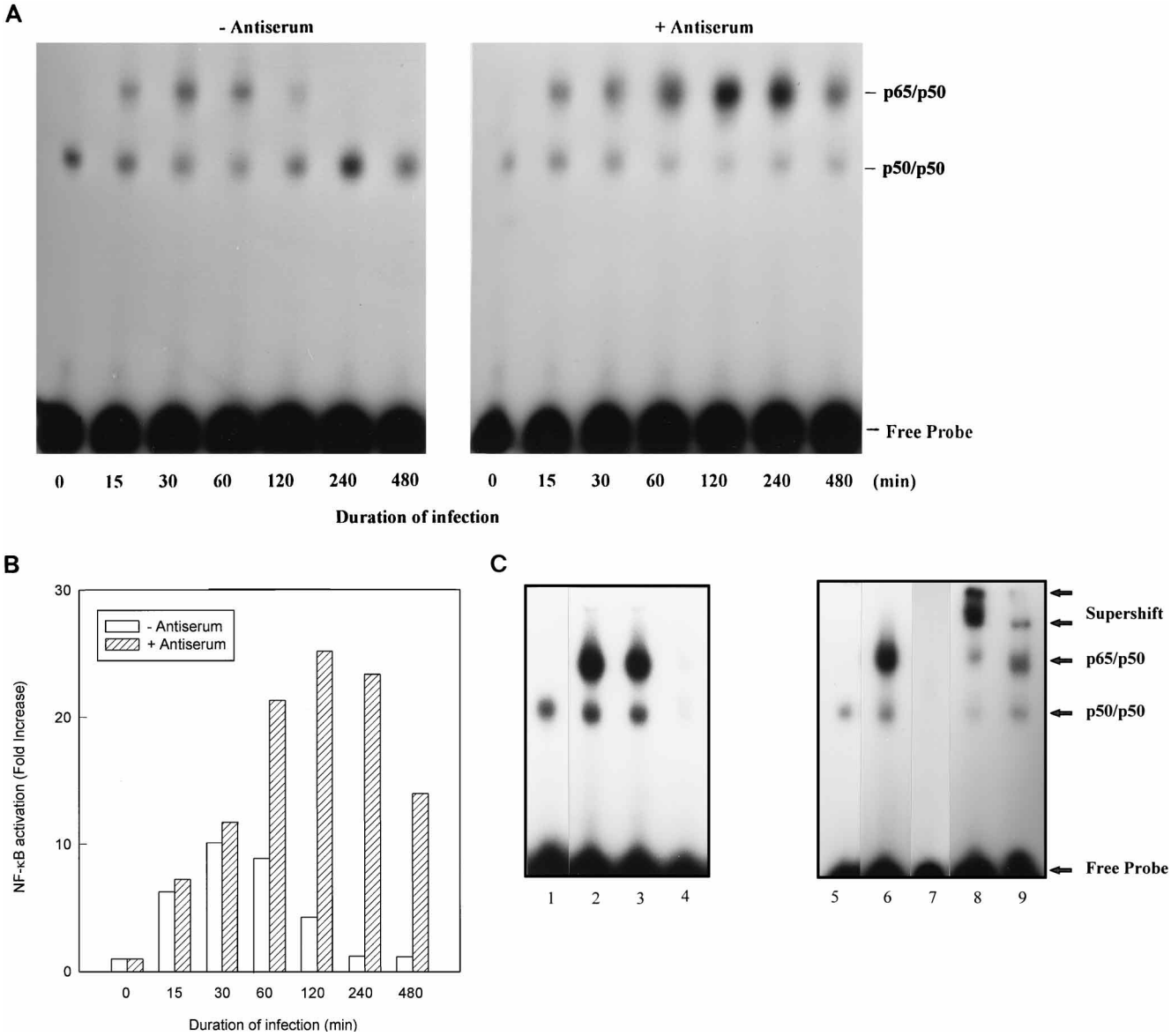

FIG. 4. Effect of dog anti-*E. chaffeensis* serum on the activation of NF-κB in THP-1 cells exposed to *E. chaffeensis*. (A) THP-1 cells were treated with host cell-free *E. chaffeensis* in the presence or absence of dog anti-*E. chaffeensis* serum. At the indicated times, nuclear extracts were prepared and levels of active NF-κB were assessed in EMSAs as described in Materials and Methods. These autoradiograms are from one of two independent experiments with similar results. The positions of p65/p50, p50/p50, and free probe are marked. (B) The gels used for panel A were dried onto 3MM papers, and the intensities of the bands were analyzed with a PhosphorImager. Levels of activated NF-κB (p65/p50) were corrected against the 0-h value. (C) Nuclear extracts were prepared from THP-1 cells stimulated for 1 h with medium (lanes 1 and 5) or *E. chaffeensis* preincubated with anti-*E. chaffeensis* serum (lanes 2 to 4, 6, 8, and 9). EMSA was performed on nuclear extracts preincubated with no reagents (lanes 1, 2, 5, and 6), unlabeled SP1 consensus oligonucleotide (lane 3), unlabeled NF-κB consensus oligonucleotide (lane 4), anti-p50 antibody (lane 8), or anti-p65 antibody (lane 9). Anti-p50 antibody incubated with radioactive NF-κB probe without nuclear extract was included as a negative control (lane 7).

significant role in host immune defense in infectious diseases as well as in ehrlichial infection through antibody-dependent cell-mediated cytotoxicity (27) and in vitro neutralization of infection (28, 35). The role of antibody in proinflammatory cytokine induction in infectious diseases has, however, been overlooked because in most infections, the agent (virus, bacterium, or protozoan) itself causes a significant cytokine production.

Our results also demonstrate that antibody complexed with ehrlichias enhances proinflammatory cytokine mRNA expression through the Fcγ receptor. It was already known that Fc receptor cross-linking leads to the production of IL-1β, IL-6, IL-8, and TNF-α in human monocytes (13, 21, 25, 26, 31). However, the signaling mechanism of cytokine production by Fcγ receptor cross-linking in human monocytes was not known, although a recent report showed that stimulation of a mouse macrophage cell line with immobilized IgG2a leads to NF-κB activation (29). NF-κB regulates the expression of genes involved in the immune and inflammatory responses, including many cytokine genes, such as those for TNF-α, IL-1β, IL-6, and IL-8. NF-κB becomes activated in response to viruses, bacteria, inflammatory cytokines, and PMA (4). Several recent studies examined the time-course effects of these stimuli on NF-κB activation and IκB-α degradation. In THP-1

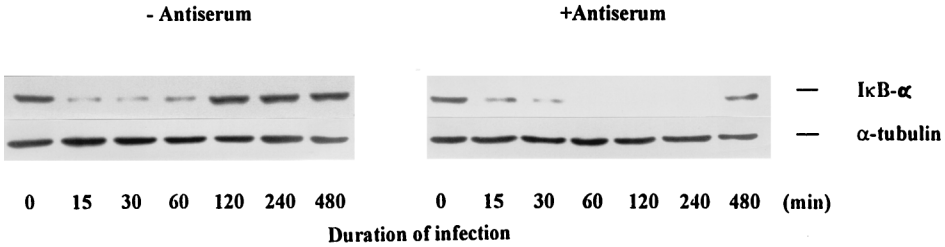

FIG. 5. Effect of dog anti-*E. chaffeensis* serum on the degradation of IκB-α in THP-1 cells exposed to *E. chaffeensis*. THP-1 cells were treated with host cell-free *E. chaffeensis* in the presence or absence of dog anti-*E. chaffeensis* serum. At the indicated times, cytoplasmic extracts were prepared and levels of IκB-α were assessed by Western blot assays as described in Materials and Methods. The levels of α-tubulin were included as an internal control.

cells stimulated with TNF-α or LPS, IκB-α was rapidly degraded, but after 1 h of stimulation, IκB-α protein reappeared (36). In U937 cells activated by TNF-α or PMA plus ionomycin, IκB-α was rapidly decayed within 10 min but reappeared at between 20 and 40 min (7). In the pre-B-cell line 70Z/3, both PMA and IL-1 caused rapid decay of IκB-α within 5 min, and IκB-α protein began to reappear after 40 min of stimulation with PMA (19). The decay of IκB-α coincided with the appearance of NF-κB DNA-binding activity in these studies. Interestingly, our data show that the degradation of IκB-α and the activation of NF-κB in cells exposed to anti-*E. chaffeensis* antibody and *E. chaffeensis* persisted longer than the other stimulations described above. The sustained NF-κB activation may play a role in the induction of major proinflammatory cytokines by anti-*E. chaffeensis* antibody and *E. chaffeensis*. We previously showed that *E. risticii* organisms induced significantly lower levels of IL-6, TNF-α, and prostaglandin E_2 in mouse peritoneal macrophages than *Escherichia coli* LPS induces (38). We also showed that IL-1β, IL-8, and IL-10 but not TNF-α and IL-6 are induced in human monocytes and THP-1 cells infected with *E. chaffeensis* (22). Thus, weak proinflammatory cytokine production might be advantageous for ehrlichiae by allowing them to remain undetected and establish an infection; however, once the host manages to make antiehrlichial antibodies, ehrlichiae may be eliminated not only by antibody-dependent cell-mediated cytotoxicity and neutralization but also by inducing large amounts of proinflammatory cytokines through the sustained activation of NF-κB.

Induction of cytokines by LPS is known to be mediated through the activation of NF-κB (10, 18, 23). The present data also show that interaction of the complex of anti-*E. chaffeensis* antibody and *E. chaffeensis* with the Fcγ receptor activates NF-κB. Although the precise mechanisms of activation of the common transcription factor by two different receptors have not been documented, several research groups recently reported that LPS induces activation of several protein kinases, such as mitogen-activated protein kinases (MAPK), protein kinase C, and cyclic-AMP-dependent protein kinase (39). A tyrosine kinase inhibitor inhibits LPS-induced expression of IL-1, TNF-α, and IL-6 (17). In addition, herbimycin A inhibits LPS-induced NF-κB complex formation in Jurkat T lymphoma cells (24). MAPK has also been reported to be involved in signal transduction through the FcγRI receptor in human monocytic cells (15). This result suggests that the CD14 receptor and the Fcγ receptor may have a common upstream pathway, such as MAPK activation leading to NF-κB activation.

E. chaffeensis in the absence of immune serum can induce NF-κB translocation. Recent reports have shown that in some pathogenic bacteria, cell wall components other than LPS promote NF-κB activation in a monocytic or macrophage cell line. For example, lipoarabinomannans from different strains of *Mycobacterium tuberculosis* have been demonstrated to differentially stimulate the activation of NF-κB in murine macrophages (8). *Treponema pallidum* and *Borrelia burgdorferi* lipoproteins were shown to induce both NF-κB activation and cytokine production in THP-1 cells, and this induction pathway was proposed to be distinct from the CD14-dependent pathway employed by LPS, since transfection of a CD14-negative pre-B-cell line with the human CD14 gene imparted responsiveness to LPS but not to the lipopeptides of *T. pallidum* and *B. burgdorferi* (30). Although the receptor(s) for *Ehrlichia* organisms has not been identified yet, our previous results showed that *E. chaffeensis*-mediated IL-1β induction in THP-1 cells does not involve CD14 (22). Data from the present study suggest that both the ehrlichial receptor and the Fcγ receptor are able to induce NF-κB activation but that signals mediated by the ehrlichial receptor produce a different kinetics of NF-κB activation, i.e., activation that is much weaker and shorter than that mediated by the Fcγ receptor.

The levels of secreted proinflammatory cytokines induced by anti-*E. chaffeensis* antibody and *E. chaffeensis* in THP-1 cells were comparable to those in cells stimulated with LPS. Therefore, in the presence of anti-*E. chaffeensis* antibody the effect of *E. chaffeensis* on the host can be as detrimental as endotoxic shock. This generation of proinflammatory cytokines may cause temporary damage in the host but concurrently contribute to antiehrlichial mechanisms by activating protective immune responses against ehrlichiae. The data presented in this paper show that the antibody against ehrlichiae has an additional significant role in immune protection and pathology in ehrlichial infection.

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