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Rickettsia rickettsii Infection of Cultured Human Endothelial Cells Induces NF-κB Activation

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Rickettsia rickettsii*, the etiologic agent of Rocky Mountain spotted fever, is an obligate intracellular bacterial organism that infects primarily the vascular endothelial cells (EC). A component of the EC response to infection is transcriptional activation, which may contribute to the thrombotic and inflammatory consequences of disease. In this study, we explore *R. rickettsii*-induced activation of the nuclear factor-κB/Rel (NF-κB) family of transcription factors involved in early transcriptional responses to injurious stimuli. Two NF-κB species were activated by infection and reacted with a double-stranded oligonucleotide probe corresponding to the κB binding domain of the murine kappa light-chain gene enhancer. Gel supershift analysis demonstrated the reactivity of these complexes with antibodies against p65 and p50, and the induced species were tentatively identified as p50-p50 homodimers and p50-p65 heterodimers. Semiquantitative reverse transcription-PCR analysis revealed dramatic increases in the steady-state levels of mRNA coding for the inhibitory subunit of NF-κB (IκBα), transcription of which is enhanced by the binding of NF-κB within the IκBα promoter region. NF-κB activation was first detected 1.5 h following infection and was biphasic, with an early peak of activation at approximately 3 h, a return to baseline levels at 14 h, and even higher levels of activation at 24 h. It is likely that NF-κB activation requires cellular uptake of *R. rickettsii*, since treatment of EC with cytochalasin B during infection to block entry inhibited activation by only 70% at 3 h. *R. rickettsii*-induced activation of NF-κB may be an important controlling factor in the transcriptional responses of EC to infection with this obligate intracellular organism.

Activation of the nuclear factor κ-B/Rel (NF-κB) family of transcription factors is a critical step in the response of vascular endothelial cells (EC) to injurious and inflammatory agents such as cytokines, growth factors, endotoxin, viral infection, and oxidant stress (1). NF-κB resides in the EC cytoplasm as homodimers and heterodimers of specific subunits, including p50 (NF-κB1), p65 (RelA), and c-Rel (1). It exists as a preformed but inactive pool bound to an inhibitory subunit (IκB) or containing an inhibitory prosequence (p105), and its activation follows the phosphorylation and degradation of these inhibitory components after cell stimulation (16). Following activation, NF-κB is translocated to the nucleus, whereupon it influences transcription by binding to specific sites within promoter region enhancer elements of various genes (5). For example, EC NF-κB activation is involved in the inducible expression of the genes for interleukin-8 (11), interleukin-6 (27), E-selectin (37), vascular cell adhesion molecule-1 (1), tissue factor (13), and urokinase (6).

Activation of host cell NF-κB has been demonstrated in response to infection by numerous viruses including human cytomegalovirus (23, 38), human immunodeficiency virus (21), and human T-cell leukemia virus (22), as well as some invasive bacteria and intracellular parasites. *Shigella flexneri* infection of HeLa cells results in rapid activation of NF-κB and other transcription factors which is linked to invasion, since noninvasive strains induce only low levels of activation (3). NF-κB activation in a macrophage cell line occurs within minutes of exposure to *Listeria monocytogenes* and apparently precedes cellular invasion (7). Infection of T lymphocytes with the intracellular parasite *Theileria parva* induces sustained NF-κB activation by a mechanism which is believed to involve enhanced synthesis of NF-κB (9).

The vascular EC is a primary target of infection by *Rickettsia rickettsii*, the etiologic agent of Rocky Mountain spotted fever, and several studies demonstrate that intracellular *R. rickettsii* infection alters the pattern of EC gene expression. Upon entry of *R. rickettsii* into the cytoplasm, the vascular EC rapidly undergoes a transcriptional response which results in increased expression of proteins involved in the expression of procoagulant and proinflammatory molecules including tissue factor (29, 33), E-selectin (30), plasminogen activator inhibitor-1 (2, 26), and interleukin (10, 31). In the present study, we demonstrate that infection of cultured human EC with *R. rickettsii* results in NF-κB activation which exhibits sustained, biphasic kinetics and is probably mediated by intracellular uptake of organisms. Such activation may be a central event in the transcriptional response of the host cell to infection.

MATERIALS AND METHODS

EC culture and infection. Human umbilical vein EC were cultured as previously described (4, 34) with umbilical cords collected within 48 h of delivery. The cells were cultured in McCoy's 5a medium (Flow Laboratories, McLean, Va.) containing 20% fetal bovine serum, EC mitogen (50 μg/ml; Collaborative Research, Inc., Bedford, Mass.), heparin (100 μg/ml; Sigma Chemical Co., St. Louis, Mo.), and insulin (25 μg/ml; Sigma). Cells at passage 2 were used in the experiments and were plated so as to achieve 80 to 90% confluence after 5 to 7 days in culture. *R. rickettsii* Sheila Smith organisms were obtained as a plaque-purified seed stock (1×10^7 to 5×10^7 PFU/cm^2) prepared in Vero (African green monkey kidney) cells (American Type Culture Collection, Rockville, Md.) (28), diluted for use in culture medium. EC were infected with approximately 6×10^4 PFU per cm^2 of cell culture area. Infection was monitored by using EC plated on

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Thermanox coverslips (Ted Pella Inc., Tustin, Calif.) and stained by immunofluorescence with antibody against *R. rickettsii* (kindly provided by Theodore Tzianobos, Centers for Disease Control, Atlanta, Ga.) as previously described (29, 34). Cytochalasin B (Sigma) was used at a final concentration of 10 μM.

Nuclear isolation and extraction. Following infection with *R. rickettsii*, nuclei were isolated and nuclear proteins were extracted as previously described (17, 19) with approximately 5×10^6 EC per experimental condition. The protein concentration in the nuclear extract was measured with Bradford reagent. This procedure typically yielded protein concentrations of between 1 and 2 mg/ml. HeLa cell nuclear extracts used as controls were obtained from Promega Corp., Madison, Wis.

Gel shift assays. Gel shift assays were performed with the gel shift assay system (Promega), as specified by the manufacturer, and with 1 to 2 μg of protein obtained from the nuclear extractions for each gel shift reaction. Sequences of double-stranded consensus oligonucleotides used in the gel shift reactions were as follows: NF-κB (Promega), 5′-AGT TGA GGG GAC TTT CCC AGG C-3′; AP-1 (Promega), 5′-CGC TTG ATG AGT CAG CCG GAA-3′; SP-1 (Promega), 5′-ATT CGA TCG GGG CGG GGC GAG C-3′; and mutant NF-κB (Santa Cruz Biotechnologies, Inc., Santa Cruz, Calif.), 5′-AGT TGA GGC GAC TTT CCC AGG C-3′. Probe labelling was carried out as specified by the manufacturer with [γ-^{32}P]ATP (3,000 Ci/mmol; 10 mCi/ml) (DuPont NEN Research Products, Boston, Mass.). Competition studies were performed with a 10-fold molar excess of unlabelled oligonucleotides added to reaction mixtures prior to the addition of radiolabelled oligonucleotides. Reaction mixtures were analyzed on 4% nondenaturing polyacrylamide gels prepared with 0.5× TBE buffer (89 mM Tris-HCl [pH 8.0], 89 mM boric acid, 2 mM EDTA) as the running buffer. The gels were electrophoresed at 100 V for 3 h and subjected to autoradiographic exposure for 12 to 18 h.

Gel supershift assays. Antibodies targeted against p65 and p50 (100 μg/0.1 ml) were purchased from Santa Cruz Biotechnologies, Inc. Prior to the addition of radiolabelled oligonucleotide probe, 1 μg per gel shift reaction was added and the mixtures were incubated at 37°C for 20 min. Each reaction mixture was analyzed, as for the gel shift assays described above, on a 4% nondenaturing polyacrylamide gel.

Semiquantitative RT-PCR. Total cellular RNA was isolated from EC cultured in 25-cm^2 flasks with Tri-Reagent (Molecular Research Center, Inc., Cincinnati, Ohio) as specified by the manufacturer. Total RNA (0.5 μg) was subjected to reverse transcription (RT) with Superscript RNase H reverse transcriptase (10 U; Bethesda Research Laboratories, Gaithersburg, Md.) with 2.5 μM oligo(dT)$_{16}$ in a 20-μl reaction mixture consisting of 5 mM $MgCl_2$, PCR buffer II (Perkin-Elmer, Madison, Wis.), 1 mM each deoxynucleoside triphosphate, and 1 U of RNase inhibitor (Perkin-Elmer) and amplified with a Gene Amp PCR system 9600 apparatus (Perkin-Elmer). The cycles consisted of an initial incubation at 95°C for 105 s followed by cycling at 95°C for 30 s, 65°C for 30 s, and 72°C for 60 s and by a final incubation at 72°C for 7 min. For detection of IκBα mRNA, 15 μl of the RT reaction mixture was amplified in a 100-μl volume. The primers used were as follows: IκBα forward primer, 5′-GCT CGG AGC CCT GGA AGC-3′; IκBα reverse primer, 5′-GCC CTG GTA GGT AAC TCT-3′ (566-bp product) (15); tissue factor forward primer, 5′-ACT CCC CAG AGT TCA CAC CTT ACC-3′; and tissue factor reverse primer, 5′ TGA CCA CAA ATG CCA CAG CTC C-3′ (398-bp product). To normalize among samples, 2 μl of the RT reaction mixture was amplified in a 100-μl volume with primers for the housekeeping species glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (8): forward primer, 5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′; reverse primer, 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′ (588-bp product). To ensure that the PCR amplification had not reached the plateau phase, the amplification products were compared after completion of 25, 30, and 35 cycles. Aliquots of PCR products were compared with a 1-kb DNA ladder (Bethesda Research Laboratories) separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining.

RESULTS

NF-κB was assayed in nuclear extracts of EC by a gel shift assay with a ^{32}P-labelled, double-stranded consensus NF-κB oligonucleotide corresponding to the κB binding domain of the murine kappa light-chain gene enhancer (25). Exposure of EC to *R. rickettsii* preparations for 3 h resulted in the appearance of two gel-shifted complexes (C1 and C2), both of which were barely detectable in untreated EC (Fig. 1A). Consistent with prior reports (18, 20), a faster-migrating complex (C3) was observed when extracts from both control and infected EC were used; the intensity of this complex was not increased due to infection. Formation of all three gel-shifted complexes was inhibited by the inclusion of a 10-fold molar excess of unlabelled consensus NF-κB oligonucleotide (Fig. 1A) but not by excess oligonucleotides corresponding to the AP-1 or SP-1 binding sequences (data not shown). A mutant NF-κB oligonucleotide containing a 1-bp substitution failed to produce a gel shift (Fig. 1B).

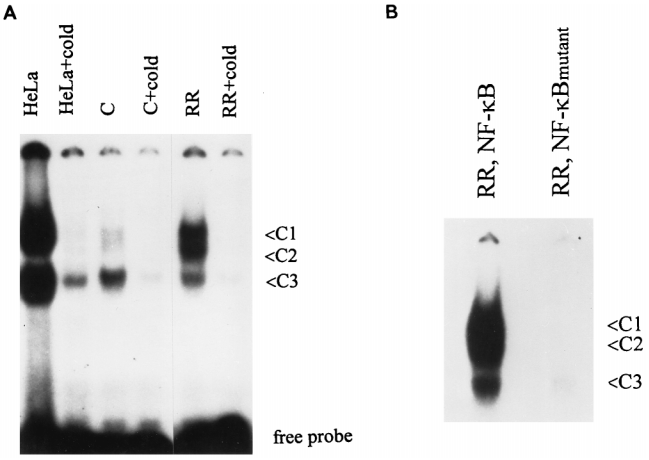


FIG. 1. Gel shift assay of *R. rickettsii*-induced NF-κB activation in EC. (A) Nuclear extracts prepared from HeLa cells and from uninfected (C) and 3-h *R. rickettsii*-infected (RR) EC (2 μg of protein per sample) were incubated with the double-stranded, ^{32}P-labelled NF-κB oligonucleotide probe and then analyzed on a 4% nondenaturing polyacrylamide gel in the absence or presence (+cold) of a 10-fold molar excess of unlabelled oligonucleotide to demonstrate the specificity of complex formation. Autoradiographic exposures are from 15 to 18 h. The positions of the gel-shifted complexes, denoted C1, C2, and C3, are denoted by arrows, and the position of migration of the uncomplexed, ^{32}P-labelled probe is indicated (free probe). (B) A gel shift assay was performed with extracts prepared from 3-h *R. rickettsii*-infected EC (RR) with the NF-κB oligonucleotide probe (NF-κB) or a mutant NF-κB probe (NF-κB$_{mutant}$) containing a 1-bp substitution.

EC uptake of *R. rickettsii* organisms probably participated in the activation response, since the intensity of the gel-shifted complexes produced was diminished an average of 70% (n = 2) when *R. rickettsii* entry was inhibited with 10 μM cytochalasin B (10 μM) (30) (Fig. 2A). As expected, cytochalasin B treatment resulted in rounding of the EC due to depolymerization of the actin cytoskeleton (Fig. 2B). Such treatment nearly completely inhibited the uptake of *R. rickettsii* organisms by the EC at 6 h, as monitored by immunofluorescence staining (Fig. 2B); however, *R. rickettsii* organisms were observed in contact with EC plasma membrane.

Gel supershift analysis was performed to determine the subunit composition of the gel-shifted complexes (Fig. 3). In nuclear extracts prepared from EC infected for 3 h, the addition of anti-p65 and anti-p50 to the reaction mixture prior to the addition of the oligonucleotide probe resulted in the formation of a slower-migrating species (supershifted complexes). As others have observed (20), there was no detectable reactivity of any complex with anti-c-Rel. Uninfected EC contained only a trace of activated complex (complex 1 and 2) and only a trace of supershifted complex which reacted with anti-p50. The use of anti-p50 resulted in a supershifted complex, which probably represented the p50 homodimer. A faint, slower-migrating complex was also seen when anti-p50 was used; this probably represented the p65-p50 heterodimer. The supershifted complex produced when anti-p65 was used was slower migrating, and probably represented the p65-p50 heterodimer.

To explore the kinetics of *R. rickettsii*-induced NF-κB activation, gel shift assays were performed on EC infected for times varying from 1 to 24 h. Infection was monitored in parallel by using EC cultured on coverslips. As shown in Table 1, both the percentage of EC infected and the average number of organisms per EC increased with time during the 24-h in-

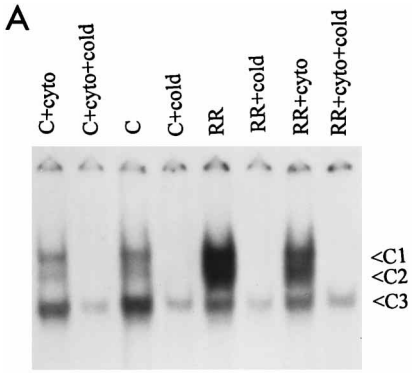
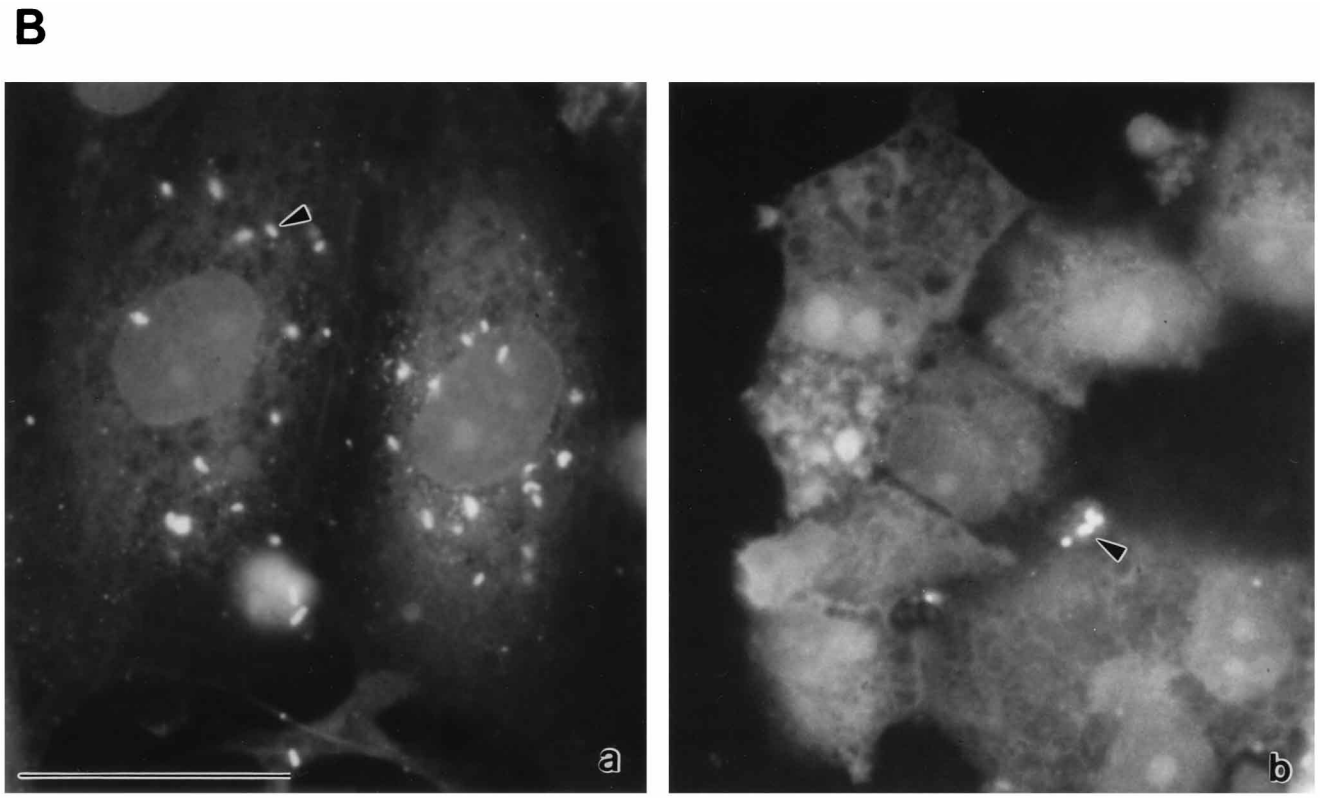


FIG. 2. (A) A gel shift assay was performed with the NF-κB oligonucleotide probe on nuclear extracts prepared from uninfected EC (C), uninfected EC treated with 10 μM cytochalasin B for 3 h (C+cyto), 3-h *R. rickettsii*-infected EC (RR) or EC treated with cytochalasin B (RR+cyto) for 1 h prior to and during incubation with *R. rickettsii*. (B) EC were cultured in parallel on coverslips, infected in the absence (a) or presence (b) of cytochalasin B, and stained by fluorescence with anti-*R. rickettsii* antiserum. *R. rickettsii* organisms are indicated by the arrowheads. Bar, 50 μM.



fection, with 34% of cells infected and 0.9 organism per cell at 3 h and 88% of cells infected and 10.0 organisms per cell at 24 h. Gel shift analysis revealed that activation, which was barely detectable at 1.5 h but not at 1 h, reached an early peak at 3 to 7 h, returned to baseline at 14 h, then reached a more dramatically increased level at 24 h (Fig. 4A). The migration pattern of the gel-shifted species appeared identical at all time points (Fig. 4A). The subunit composition of the NF-κB species activated at 24 h was confirmed, by gel supershift analysis, to be composed of both homodimers of p50 and heterodimers of p65 and p50 (not shown). To determine if the sustained time course truly reflected the kinetics of activation and not simply a lagging infection, total RNA was isolated from EC infected in parallel and subjected to analysis of tissue factor mRNA by RT-PCR (Fig. 4B). As expected (29), tissue factor mRNA levels rose transiently due to infection, with elevated levels observed at 4 h and a return to baseline by 24 h.

NF-κB positively regulates the expression of IκBα, since sequences within the IκBα promoter bind several NF-κB species including p50-p65 (12). Therefore, an increase in the steady-state level of IκBα mRNA is a sensitive indicator of NF-κB activation. IκBα mRNA, as measured by RT-PCR, which was barely detectable in uninfected EC, was clearly increased at 3 h (Fig. 5). No detectable alteration in levels of the housekeeping mRNA species, GAPDH, occurred due to infection (Fig. 5).

DISCUSSION

In this report, we demonstrate that *R. rickettsii* infection of EC induces the activation of host cell transcription factor, NF-κB. Gel shift assays performed with nuclei isolated from *R. rickettsii*-infected EC revealed nuclear translocation of proteins which formed specific complexes with the NF-κB consen-

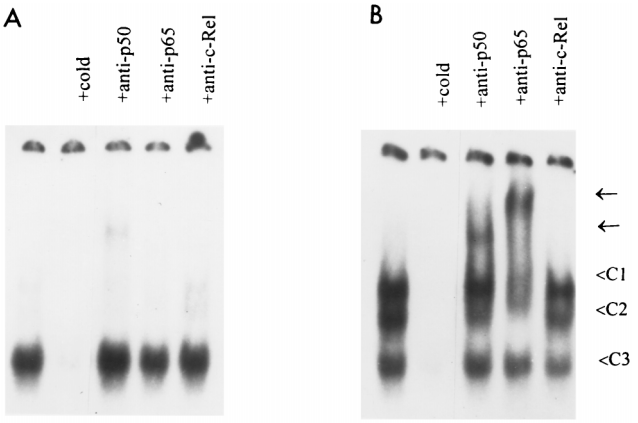


FIG. 3. Gel supershift analysis of activated NF-κB species. EC nuclear extracts from Fig. 1A (control [A] and *R. rickettsii* infected [B]) were subjected to gel supershift analysis with antibodies against p50, p65, and c-Rel. Antibody (2 μg) was incubated with 2 μg of protein from nuclear extracts for 20 min at 37°C prior to the addition of radiolabelled oligonucleotide probe in the gel shift assay. The positions of the gel-shifted complexes are denoted by the arrows, and the original migration position of each gel-shifted complex (C1, C2, and C3) is indicated.

sus oligonucleotide (Fig. 1). This oligonucleotide binds to several NF-κB species in EC, which are composed predominantly of homo- and heterodimers of p50 and p65 subunits, including a homodimer of a 50-kDa subunit (p50) and a heterodimer of p50 and a 65-kDa subunit (p65 or relA) (24). The nuclear complexes activated by *R. rickettsii* infection reacted in gel supershift assays with polyclonal antibodies to both p50 and p65 (Fig. 3) and probably represented the p50 homodimer as well as the p50-p65 heterodimer. Consistent with the activation of these species, *R. rickettsii* infection resulted in an increased steady-state level of IκBα mRNA (Fig. 5), the promoter of which is specifically activated by several NF-κB species including p50-p65, p50–c-Rel, and p65–c-Rel (12, 32). Induction of IκBα transcription by NF-κB probably allows for replenishment of cytoplasmic IκBα following its degradation (16, 32) and serves as a sensitive marker of NF-κB activation.

NF-κB activation in response to *R. rickettsii* infection exhibited sustained, biphasic kinetics, with activation first detected at 1.5 h, peaking at 3 to 7 h, and then reaching a nadir at 14 h (Fig. 4A). The highest levels of activation were observed at 24 h. It has not yet been determined whether activation is sustained until the time of cell death due to infection. The slight delay in activation observed following *R. rickettsii* infection, as compared with the rapidity of activation when cells are stimulated with soluble agonists (5), was probably due to the time required for *R. rickettsii* entry into the EC and is consistent with a requirement for rickettsial uptake in the activation

TABLE 1. Time course of EC infection with *R. rickettsii*[a]

Time (h)	% of EC infected	Mean no. of organisms/cell[b]
3	34	0.9
7	54	2.4
14	73	5.8
24	88	10.0

[a] EC cultured on coverslips were treated in parallel with those used for the gel shift assays described in the legend to Fig. 4 and stained by fluorescence with anti-*R. rickettsii* antiserum.

[b] For each time point, 100 cells were randomly selected and scored for the number of *R. rickettsii* organisms per cell.

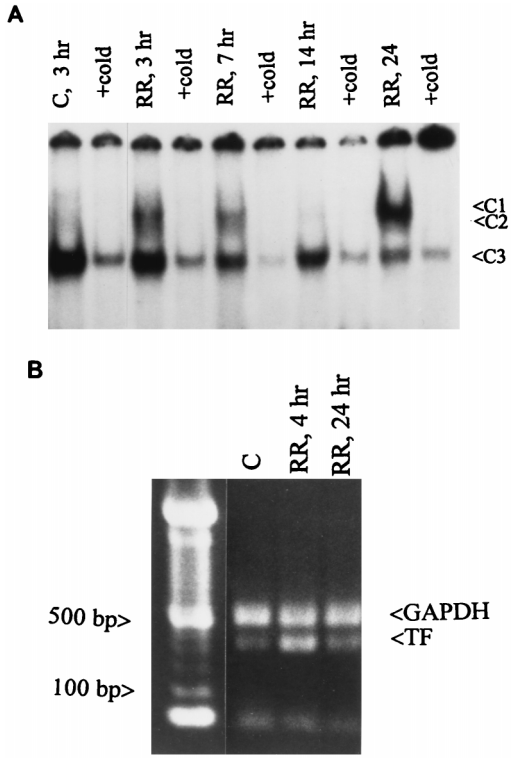


FIG. 4. Kinetics of *R. rickettsii*-induced activation of NF-κB. (A) EC were incubated with culture medium alone for 3 h (C, 3 hr) or with *R. rickettsii* for 3 h (RR, 3 hr), 7 h (RR, 7 hr), 14 (RR, 14 hr), or 24 h (RR, 24 hr). Nuclear extracts were prepared and subjected to gel shift assay with the ^{32}P-labelled NF-κB oligonucleotide probe (2 μg of protein per sample) in the absence or presence of excess unlabelled oligonucleotide (+cold). (B) Uninfected EC (C) and EC infected in parallel with *R. rickettsii* were harvested after 4 and 24 h (RR, 4 hr; RR, 24 hr), total RNA was extracted, and then steady-state levels of tissue factor (398-bp product) and GAPDH (588-bp product) transcripts were measured by RT-PCR analysis with specific primer pairs. Shown are amplification products generated following 30 amplification cycles.

response. The sustained nature of the kinetics observed during *R. rickettsii* infection is similar to that observed following human cytomegalovirus infection, which also displays biphasic kinetics (38), type 5 adenovirus infection (14), and infection

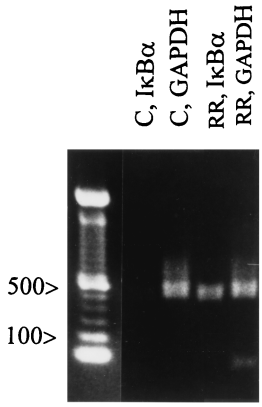


FIG. 5. Measurement of steady-state levels of IκBα mRNA by RT-PCR in EC following *R. rickettsii* infection. Total RNA was isolated from control EC and EC infected for 3 h with *R. rickettsii*, subjected to RT, and PCR amplified with primer pairs specific for IκBα (566-bp product) and GAPDH (588-bp product). Shown are PCR products generated following 30 amplification cycles.

with the intracellular protozoan *Theileria parva* (9), wherein infected cells maintain high levels of activated NF-κB.

The sustained, biphasic kinetics of activation observed during *R. rickettsii* infection could reflect either truly sustained activation on a cellular level or the kinetics of the infection in the population of EC under study. Both the percentage of EC infected and the average number of *R. rickettsii* organisms per cell rose more or less linearly during the 24-h period (Table 1). Tissue factor mRNA levels, measured in parallel, rose and fell transiently during the 24-h infection (Fig. 4B), suggesting that the majority of cells responded to infection early in the time course and arguing in favor of sustained, biphasic kinetics on a cellular level. Tissue factor expression is controlled by NF-κB but is governed by c-Rel–p65 (18), which does not adequately bind the consensus NF-κB oligonucleotide used in this study (17). It is implied from these results that the kinetics of activation of c-Rel–p65 in response to *R. rickettsii* infection may differ from that of p50-p50 and p50-p65. It is possible, therefore, that activation of the NF-κB species measured in this study may result from *R. rickettsii*-induced signalling pathways distinct from or more complex than those resulting in increased transcription of tissue factor mRNA.

R. rickettsii interaction with EC, resulting in entry and subsequent intracellular replication, may stimulate a series of host cell responses beginning with the interaction of *R. rickettsii* with the cell surface. Cytochalasin B, which reportedly allows rickettsial interaction with the host cell surface but prevents entry of the organisms (35, 36), partially blocked NF-κB activation (Fig. 2), implicating *R. rickettsii* entry. *L. monocytogenes* infection of macrophage-like cells results in activation of p50-p65 prior to invasion and escape from the phagolysosome (7, 24). The late phase of activation during *R. rickettsii* infection could perhaps be explained by production of a rickettsial metabolite or replication of the organisms and the early phase could be induced by cell surface interaction, entry, or simple contact of organisms with the host cell cytoplasm. Future studies will use inhibitors of transcription and translation to determine if any phase of activation requires de novo synthesis of NF-κB subunits.

Although the point(s) at which *R. rickettsii* initiates signal transduction leading to NF-κB activation is unknown, it is likely that the pathways involved converge at some point upon those of physiologic agents such as inflammatory cytokines. Since the *R. rickettsii* organisms reside in host cell cytoplasm, as yet unidentified transactivator molecules, which initiate intracellular signalling, may be present on or be produced by *R. rickettsii*. NF-κB activation induced by *R. rickettsii* may underlie important aspects of the host cell response to intracellular infection.

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