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Full Length Research Paper

NFκB is differentially activated in macrophages from J774A.1 cell line infected with vaccine or virulent strains of *Brucella abortus*

Maribel Cervantes-Flores[1], Aurora Martínez-Romero[2], Eda Guadalupe Ramírez-Valles[1], Leticia Saucedo Mendiola[1], Martha C. Moreno-Lafont[3], Rubén López-Moreno[3], Iris Estrada-García[3] and Virgilio Mojica-Marín[1]*

[1]Facultad de Ciencias Químicas. Universidad Juárez Del Estado de Durango (UJED). Av. Veterinária s/n, Circuito Universitário, Col. Valle del Sur. CP. 34200. Durango, Dgo., México.
[2]Facultad de Ciencias Químicas unidad Gómez-Palacio. Universidad Juárez Del Estado de Durango (UJED). Av. Artículo 123 s/n Colonia Filadelfia CP 35010; AP. No. 51 Gómez Palacio, Dgo., México.
[3]Escuela Nacional de Ciencias Biológicas-IPN. Prol. de Carpio y Plan de Ayala s/n. Col. Santo Tomás. 11340 México, D.F., México.

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The activation and nuclear translocation of NF-κB and the expression of the pro-inflammatory cytokine genes by macrophages infected with the attenuated *Brucella abortus* RB51 and virulent 2308 strains were evaluated. pIκBα and NF-κB were determined by immunoblot, and cytokines IFN-γ and IL12 mRNA were determined by reverse transcriptase polymerase chain reaction (qPCR) and translocation of NF-κB protein to the nucleus was determined by electrophoretic mobility shift assay (EMSA). We demonstrate that the attenuated *B. abortus* RB51 strain stimulates cells resulting in NF-κB activation and nuclear translocation, during experimental infection in macrophages J774A.1 which induced a pro-inflammatory response producing IL-6, 12 TNF-s INF-g and iNOS. The virulent strain *B. abortus* 2308 also stimulated the cells but induced a p50 homodimer of NF-κB which is inactive. The p50 homodimer of NF-κB binds to DNA, and thus blocked the activation of pro-inflammatory cytokines genes. Therefore, an evasion mechanism of the strain 2308 is to produce an inactive homodimer of NF-κB which does not give rise to pro-inflammatory response to eliminate the bacteria.

Key words: *Brucella abortus*, RB51, 2308, NF-κB, transduction signals.

INTRODUCTION

Toll-like receptors (TLR) are membrane molecules that, upon engagement of pathogen associated molecular patterns (PAMP) induce signal transduction pathway through adaptor molecules, such as the myeloid differentiation protein MyD88, IRAK, and TRAF6. This signaling leads to activation of the pro-inflammatory response. Activation of the MyD88-dependent pathways release NF-κB from its inhibitor (IκBs), promoting its translocation to the nucleus (Dueñas et al., 2004; Huang et al., 2003; Liew et al., 2005). NF-κB comprises a family of ubiquitously expressed, eukaryotic transcription factors that participate in the regulation of multiple immediate genes that are expressed at the onset of many vital biological processes such as cell growth, immunoregulation, apoptosis, and inflammation (Tak and Firestein, 2001; Yamamoto and Gaynor, 2001). One of the major functions of NF-κB is its key involvement in inducing an effective immune/inflammatory response against viral and bacterial infections. The importance of NF-κB role in initiating a potent inflammatory response

*Corresponding author. E-mail: vmojica@citologica.com.

cannot be better signified than recognizing that the κB consensus sequence is found in the promoter/enhancer regions of more than 50 diverse genes whose expression is known to be crucial in driving an inflammatory response (Majdalawieh and Hyo-Sung, 2010). Inducible genes that are known to be transactivated by NF-κB include, but are not limited to, IL-1β, IL-6, IL-8, TNFα, IFNγ, MCP-1, iNOS, COX-2, intracellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) (Baeuerle and Henkel, 1994; Baldwin, 1996; Kopp and Ghosh, 1995). These molecules play critical roles in key biological events involving cell recruitment, attachment, differentiation, proliferation, and activation constituting an active inflammatory response (Majdalawieh and hyo-Sung, 2010). In this report we studied the cytokine gene expression and demonstrated that infection by the virulent *Brucella abortus* 2308 strain releases an inactive transcriptional homodimer of NF-κB and did not induce activation of pro-inflammatory cytokine genes; neither did it induce expression of IFN-γ, TNF-κ nor of the iNOS enzyme. In contrast, *B. abortus* RB51 vaccine strain releases an active transcriptional heterodimer of NF-κB and stimulates the pro-inflammatory response by the activation of IL-12 and IFN-γ as well as iNOS genes.

MATERIALS AND METHODS

Infection and mice vaccination

Five- to 7-week old BALB/c female mice (CINVESTAV-IPN, Mexico, D.F.) were divided in three experimental groups consisting of 18 animals each. The first group of mice was intraperitoneal (i.p.) vaccinated with 0.4 ml PBS containing 4×10^8 CFU of *B. abortus* RB51. This high dose was applied because lower doses do not consistently colonize mice, as established by Schurig et al. (1991). A second group of mice was infected under the above conditions with 5×10^4 CFU of *B. abortus* 2308 in 0.4 ml PBS, since this dose provides the most rigorous challenge (Montaraz and Winter, 1986), and higher doses are 100% lethal (unpublished data of our group). Unvaccinated negative control animals were injected with 0.4 ml PBS. Animals were kept under standard hygiene conditions and food and water were given *ad libitum*.

Peritoneal exudate cells and macrophage cell line culture

Three mice per group were euthanized at 3 h, and 1, 2, 3, and 7 days after infection. Peritoneal exudate cells (PEC) were obtained by washing the peritoneal cavity with 3 ml of minimal essential medium (MEM) containing 2 U/ml heparin and 1% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY). PEC's were placed in siliconized tubes, washed and resuspended in 1 ml RPMI 1640 (Gibco BRL) supplemented with 10% FBS and 2 mM glutamine (Sigma Chemical, St Louis, MO). PEC were counted by Trypan blue dye exclusion and the cell suspension was adjusted to 1×10^6 PEC/ml.

Macrophages from the J774A.1 cell line were maintained in RPMI 1640 medium (Gibco BRL) supplemented with 2 mM L-glutamine, 7% horse serum (Gibco BRL) and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin, and 250 µg/ml amphotericin B).

Infection of macrophages with *B. abortus*

The vaccine strain RB51 and the virulent 2308 strains of *B. abortus* were kindly donated by G. Schurig (Virginia Tech, USA.) and maintained in trypticase soy agar. Since the analysis of nuclear and cytoplasmic proteins needed a greater amount of material, this part of the study was performed onto the macrophage cell line J774A.1. 2.4×10^7 cells were seeded in each 144 x 21 mm Petri dish for cell culture (Nunc, Denmark); they were infected with 2.4×10^9 CFU of *B. abortus* RB51 or of *B. abortus* 2308, and incubated for different time periods at 37°C in 5% CO_2 atmosphere. Cells were washed three times with PBS; then, the nuclear and cytoplasmic protein extracts were obtained.

Stimulation of macrophages with heat-inactivated *B. abortus* RB51 or 2308

Macrophages J774A.1 (2.4×10^7) were seeded in cell culture Petri dishes adding 2.4×10^9 CFU of heat inactivated *B. abortus* RB51 or 2308 strain (70°C for 30 min), and incubated for different times at 37°C in CO_2 atmosphere. For times longer than 30 min, macrophages were incubated with dead bacteria during 30 min, washed, and supplemented with antibiotic-free RPMI, and then incubated for the remainder time. Cells were washed three times with PBS and then the nuclear and cytoplasmic protein extracts were obtained.

Analysis of cytokine genes expression

Total RNA was isolated from 1×10^6 PEC at different post-infection times as indicated in each experiment, with Trizol (Life Technologies, Gaithersburg, MD.) and chloroform according to manufacturer's guidelines. cDNA was prepared from total RNA using 0.5 µg oligo-dT primers (Life Technologies) and 200 U Moloney murine leukemia virus reverse transcriptase (MMLVRT) enzyme (Life Technologies), following vendor's recommendations. The primers used and amplified products predicted sizes are shown in Table 1. To ensure that all RT reaction samples contained RNA, control RT-PCR with primers for mRNA of the housekeeping gene glyceraldehyde 3-phospate dehydrogenase (G3PDH) were screened in parallel. PCR products were analyzed by electrophoresis of 5 µl of the reaction mix at 95 V for 1.5 h in 1.5% agarose in TBE buffer (90 mM Tris, 90 mM boric acid, and 2 mM EDTA) containing 0.05 µg/ml of ethidium bromide; 1Kb DNA ladder (Gibco BRL) was used as size marker. The difference in cytokine mRNA levels in PEC was estimated with an internal standard derived from PCR amplifications of G3PDH mRNA's. These calculations, using a ChemiImager 4000 Low Light Imaging System (San Leandro, CA) and the Alpha Ease software, allowed us to obtain arbitrary units expressing the value of cDNA in each band. The density of each cytokine band was normalized according to the corresponding G3PDH band from the same RT-PCR reaction mix.

Collection of cytoplasmic and nuclear proteins from macrophages

Macrophages were washed with cold PBS, released with a cell scraper, and centrifuged at 1500 × g, at 8°C during 5 min. Cells were resuspended in buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1mM DTT), and then frozen in a dry ice-acetone bath. Afterwards, they were thawed in ice bath and centrifuged at 1200 × g at 4°C during 10 min. The supernatants were collected as cytoplasmic extracts and the pellet containing the nuclei was resuspended in buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1.5 mM $MgCl_2$, 25% glycerol, 0.2 mM EDTA, 1mM DTT), supplemented

Table 1. Sequence of primers used to amplify housekeeping gen, cytokines and iNOS mRNAs and predicted sizes of the amplified products.

Primer	Sequence	Predicted Size (bp)
β-actin	5´sense: 5´-GTG GGC CGC TCT AGG CAC CAA-3´ 3´antisense: 5´-CTC TTT GAT GTC ACG CAC GAT TCC-3´	540
G3PDH	5´sense: 5´-ACC ACA GTC CAT GCC ATC AC-3´ 3´antisense: 5´-TCC ACC ACC CTG TTG CTG T-3´	452
IL-6	5´sense: 5´-ATG AAG TTC CTC TCT GCA AGA GAC T-3´ 3´antisense: 5´-CAC TAG GTT TGC CGA GTA GAT CT C-3´	638
IL-12p40	5´sense: 5´-CAG AAG CTA ACC ATC TCC TGG TTT G-3´ 3´antisense: 5´-TCC GGA GTA ATT TGG TGC TCC ACA C-3´	394
TNF-α	5´sense: 5´-ATG AGC ACA GAA AGC ATG ATC CGC-3´ 3´antisense: 5´-CC AAA GTA GAC CTG CCC GGA CTC-3´	692
IFN-☐	5´sense: 5´-TGA ACG CTA CAC ACT GCA TCT TGG-3´ 3´antisense: 5´-CGA CTC CTT TTC CGC TTC CTG AG-3´	460
iNOS	5´sense: 5´-CAG CTC CAC AAG CTG GCT CG-3´ 3´antisense: 5´-CAG GAT GCT CTG AAC GTA GAC CTT G-3´	700

with 0.5 mM PMSF proteases inhibitor. This mixture was incubated at 4°C during 30 min under gentle stirring in an angular platform, and then centrifuged at 20,000 × g at 4°C for 20 min. The supernatant was collected as the nuclear extract. Buffer D (20 mM HEPES, pH 7.9, 50 mM KCl, 25% glycerol, 0.2 mM EDTA, 1 mM DTT), supplemented with 0.5 mM PMSF proteases inhibitor, was added to the cytoplasmic and nuclear extracts, which were stored and maintained at -70°C until needed. Protein concentration was measured by means of the reaction with bicinconinic acid (BCA, Pierce, Rockford, IL), using bovine serum albumin as standard.

Immunoblot analysis of pIκBα and NF-κB proteins

The cytoplasmic and nuclear protein extracts were subjected to 10% SDS-PAGE, transferred to nitrocellulose membranes and blocked with 3% skim milk in TBS buffer (20 mM Tris, pH 7.6, 0.14 M NaCl) supplemented with 0.05% Tween 20 (TBS-T), during 1 h at room temperature. Membranes were washed with TBS-T and incubated overnight at 4°C with the polyclonal pIκBα antiserum, and polyclonal NF-κB antiserum (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:1000 dilution. After washing with TBS-T, membranes were incubated with the horseradish peroxidase-conjugated goat anti-rabbit antibodies (Santa Cruz Biotechnology) during 1 h more at room temperature. The ECL Plus kit (Amersham, Biosciences, Buckinghamshire, England) was used to develop the chemiluminescence for visualization on Kodak BioMax-MR film (Sigma-Aldrich, St Louis, MO).

Electrophoretic mobility shift assay (EMSA)

The cytoplasmic or nuclear proteins were incubated with a double chain oligonucleotide labeled with [γ32P] ATP. To label the oligonucleotide, 2 µl (1 µg) of the double chain oligonucleotide, 1 µl of the enzyme T4 polynucleotidil kinase buffer (Gibco BRL), 1 µl of enzyme T4 (Gibco BRL), 1 µl of (γ 32P) ATP, and 6 µl of sterile water were added, the mixture was incubated for 1 h at room temperature, led to a final volume of 100 µl, and stored at -20°C. The labeled oligonucleotide was incubated with either the cytoplasmic or the nuclear extract, adding 5 µg of protein with 0.05 µg/µl poly poly(dI)-poly(dC), incubated for 15 min on ice and adding 2 µl of the [γ32P] ATP-labeled oligonucleotide.

The mixture was incubated 30 min at room temperature. The oligonucleotide sequence for NF-κB binding (Biosynthesis) was 5´-AGT TGA GGG GAC TTT CCC AGG C-3´. DNA-protein complexes were electrophoretically run in a 6% polyacrylamide gel (29:1) with a 0.5x TBE buffer (40 mM Tris, 40 mM boric acid, 1 mM EDTA).

RESULTS

Vaccination with *B. abortus* RB51 produced a decrease in the expression of the IL-6 gene starting at 3 h post-vaccination. Expression of this gene was reestablished at 1 day, increased on day 3 post-infection, and disappeared on day 7 post-infection. In PEC from *B. abortus* 2308 infected mice, the mRNA for IL-6 was not detected at any of the studied times (Figure 1). In PEC from *B. abortus* RB51, mRNA for IL-12 was also detected at 3 h, and at least until 7 days after vaccination. In PBS-inoculated mice, no mRNA for IL-12p40 was detected, whereas PEC from mice infected with *B. abortus* 2308 showed a low expression of the gene (Figure 1).

As shown in Figure 1, *B. abortus* RB51 induced biphasic activation in TNF-α gene expression at 3 h and at 3 days post-vaccination, reaching the highest expression at Day 1. In contrast, PEC from *B. abortus* 2308-infected mice revealed no TNF-α expression at any of the studied times. On the other hand, the IFN-γ gene

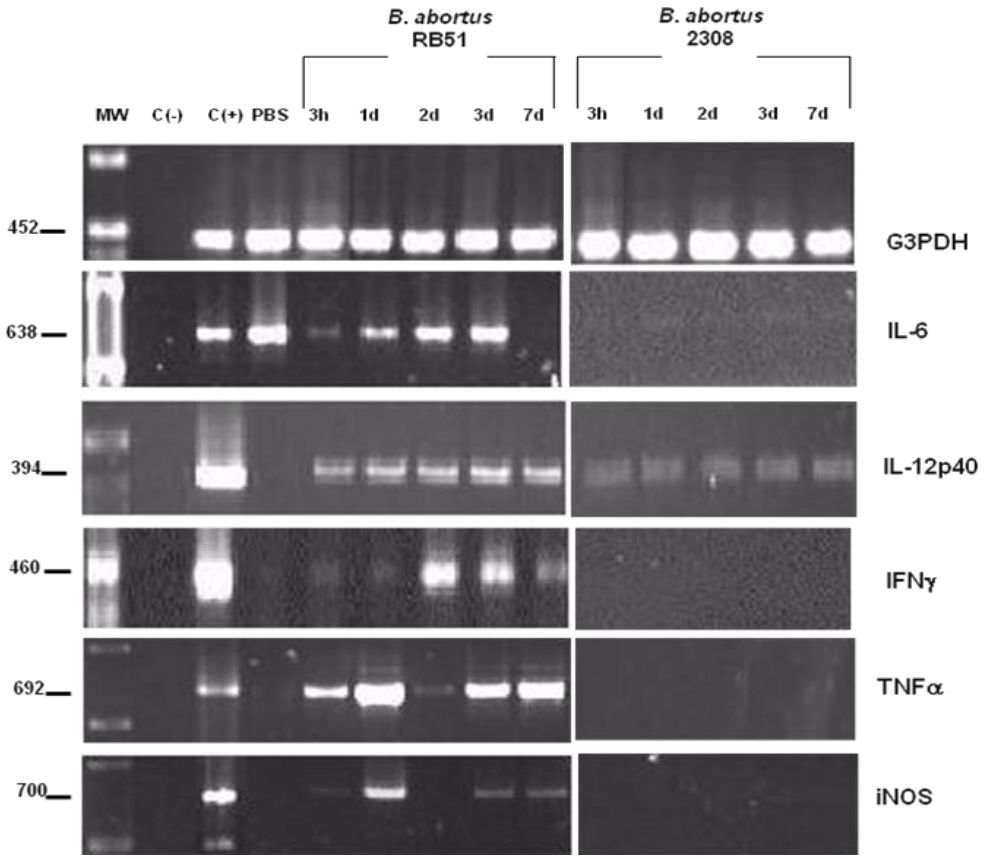

Figure 1. Expression of IL-6, IL-12p40, TNF-α, IFN-γ, and iNOS cytokines. Cytokines and iNOS mRNA by BALB/c peritoneal exudate cells (PEC) were determined at 3 h and 1, 2, 3 and 7 days post vaccination with *B. abortus* RB51 or post infection with *B. abortus* 2308 analyzed by RT-PCR. Control group was inoculated with PBS. Total RNA was prepared from 106 PEC. Negative [lane C(-)] and positive controls [lane C(+)] were included. MW, molecular weight markers. The base-pair numbers of cDNA are indicated on the left.

was detected at 3 h post-vaccination with *B. abortus* RB51, peaking at 2 days, and was still detectable at day 7. It is worthwhile mentioning that IFN-γ expression occurred after IL-12p40 expression (from 3 h on). IFN-γgene expression was not observed either in strain 2308-infected mice, at least not during the first 7 days p.i., or in PBS-inoculated mice (Figure 1). The absence of mRNA for IFN-γ correlates with the fact that strain 2308 does not induce protective immunity.

The gene for iNOS was expressed at 24 h post-vaccination with *B. abortus* RB51 (Figure 1). However, its expression ceased on day 2 post-vaccination, being re-expressed at Days 3 and 7 at very low levels. The expression of this gene was not detected in either control or *B. abortus* 2308-infected mice.

To assess the effect of infection with different *B. abortus* strains on the signaling that leads to activation of NF-κB, J774A.1 cell line macrophages were infected with either *B. abortus* RB51 or *B. abortus* 2308 and activation IκBα was determined, as well as the nuclear translocation of NF-κB. Infection with either bacterial strain induced activation of pIκBα, although each at different times (Figure 2A). In *B. abortus* RB51-infected cells, the activated proteins were detected at 5 min post-infection, whereas cells infected with *B. abortus* 2308 showed a delay in the activation of these proteins; the protein pIκBα at 15 min. Because NF-κB must be translocated to the nucleus once released by its inhibitor in order to activate gene transcription, we investigated the effect of *B. abortus* infection on NF-κB translocation. As shown in Figure 2A, NF-κB was detected in the nucleus of cells infected with *B. abortus* RB51; translocation started at 5 min and was detectable until 30 min [NF-κB (NE)]. A second phase of NF-κB release and translocation was observed at 90 min, disappearing afterwards. A third response phase was noticeable at 6 h. In *B. abortus* 2308-infected cells, nuclear translocation of NF-κB was observed from 5 min on, maintained until 90 min, observing a second response phase from 2 to 6 h p.i. These results indicate that both *B. abortus* strains induce

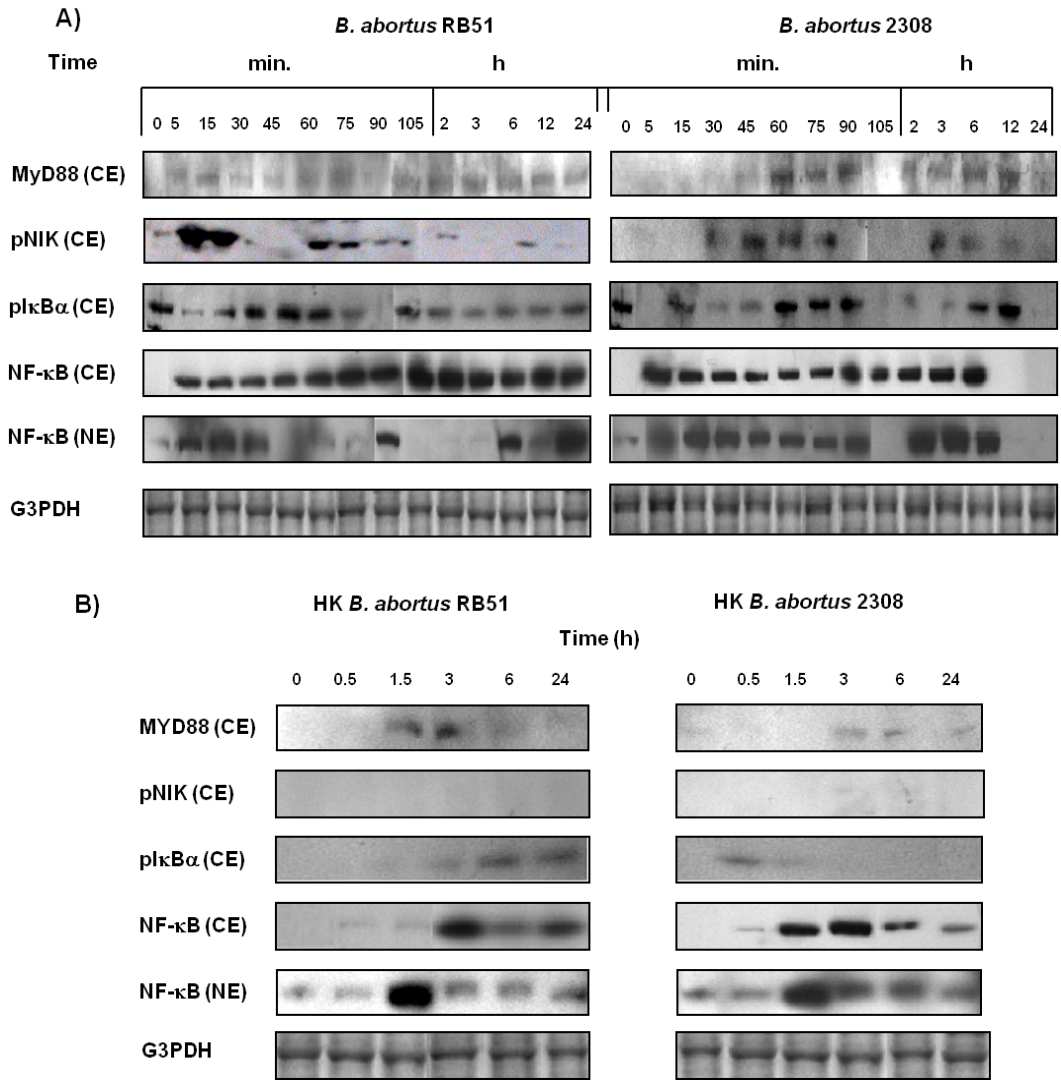

Figure 2. Activation of IκBκ and NF-κB, and translocation of NF-κB. Infection of macrophages with either strain (RB51 or 2308) of live *B. abortus* induced activation IκBκ and the translocation of factor NF-κB towards the nucleus (a). Stimulation of macrophages with heat- inactivated *B. abortus* RB51 or 2308 induced activation of IκBκ leading to nuclear translocation of factor NF-κB (b); 60 µg of each extract (cytoplasmic and nuclear) were SDS-PAGE separated and transferred to nitrocellulose membranes. Membranes were incubated with the antibody for pIκBα, and NF-κB, and the bound antibody was visualized by means of chemiluminescence. Results are from one of three different experiments with same results. CE= cytoplasmic extract, NE= nuclear extract.

nuclear release and translocation of NF-κB.

Stimulation with heat-inactivated *B. abortus* RB51 induced nuclear release and translocation of NF-κB (Fig. 2B). The same behavior was observed in cells stimulated with heat-inactivated *B. abortus* 2308.

Because the NF-κB dimers released from IκB translocate towards the nucleus to activate transcription, we searched for the effect of infection with *B. abortus* RB51 or 2308 on the nuclear translocation of NF-κB. As shown in Figure 3A, free NF-κB was present in the cytoplasm and nucleus of macrophages infected with *B. abortus* RB51, indicating that this strain induces activation and translocation of NF-κB towards the nucleus. These results reveal that infection with *B. abortus* RB51 does not affect the ability of NF-κB to translocate to the nucleus and bind to specific DNA sequences. With the virulent strain *B. abortus* 2308, two complexes were observed in the cytoplasmic extract samples (Figure 3A), indicating the presence of two NF-κB protein complexes (termed I and II) that, according to their molecular weight, could correspond to dimers p-65-p50 and p50-p50, respectively. It must be noted that in

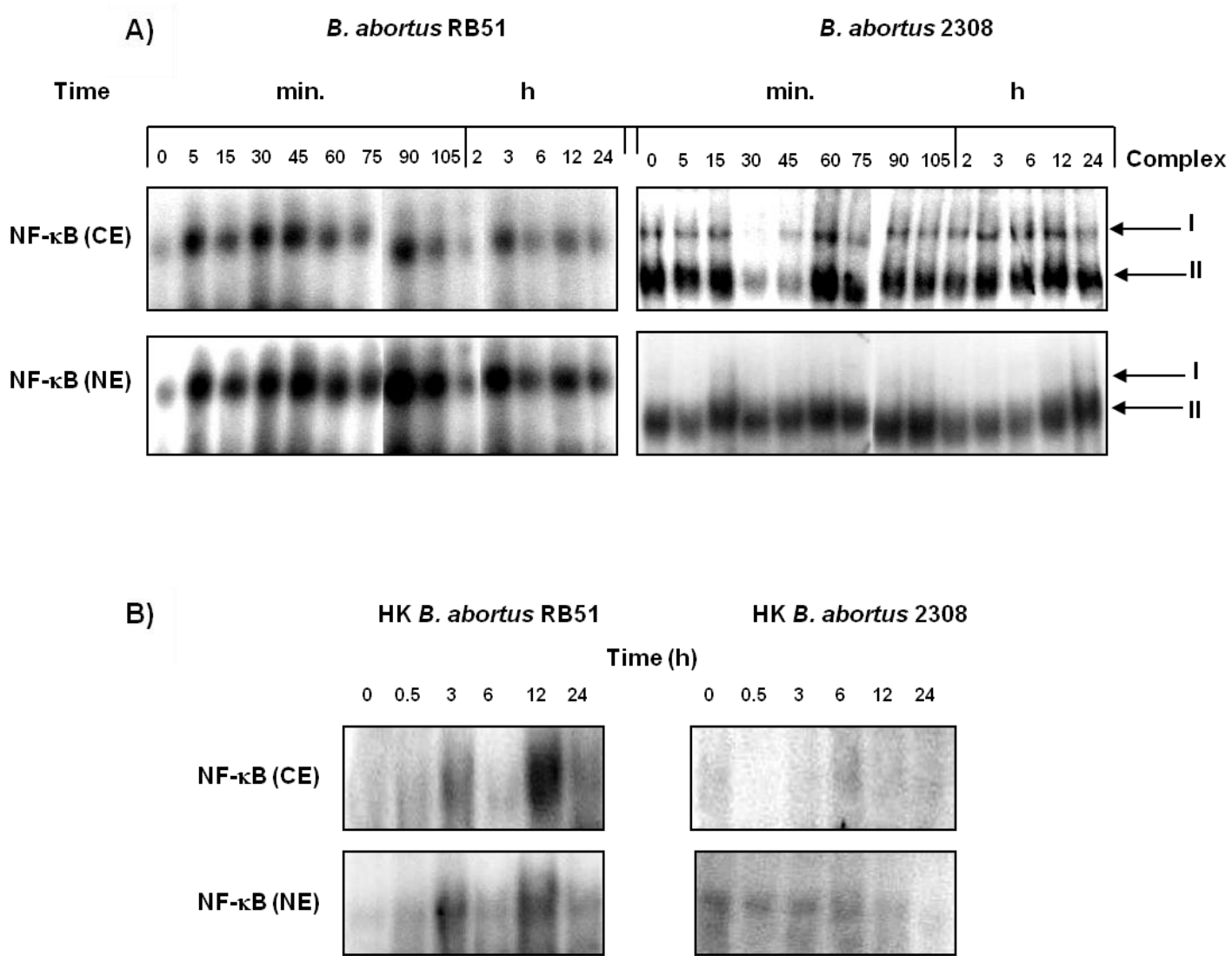

Figure 3. EMSA analysis of release and nuclear translocation of NF-κB. EMSA revealed the nuclear translocation of factor NF-κB and apparently this does not affect the capacity of NF-κB to bind to the specific DNA sequence in macrophages infected with either strain RB51 or 2308 (a) or heat killed strains (b). After each infection time, the cytoplasmic (CE) and nuclear (NE) proteins were prepared and analyzed by means of EMSA using extracts with the same number of cells and a [γ 32P] ATP-labeled oligonucleotide.

the nuclear extract samples only one band was observed corresponding to Complex II (probably the heterodimer p50-p50). The one band observed in the cytoplasmic and nuclear samples of strain RB51-infected macrophages corresponds to complex I (probably the homodimer p65-p50).

Stimulation of macrophages with heat-inactivated *B. abortus* RB51 induced formation of NF-κB complexes in nuclear and cytoplasmic extracts (Figure 3B). These results reveal that stimulation with the heat-inactivated strain RB51 induces NF-κB activation, translocation, and DNA binding. In cells stimulated with the heat inactivated strain 2308, the NF-κB translocated to the nucleus despite not being detected in the cytoplasm (Figure 3B).

DISCUSSION AND CONCLUSION

Macrophage activation is an essential element required to resolve an infection caused by *Brucella* spp. A key mechanism in cell-mediated immunity is the antimicrobial activity of macrophages stimulated by IFN-γ(Divekar et al., 2006; Huang et al., 2003; Thale and Kiderlen, 2005). Murphy et al., 2001b, reported that IFN-γ knockout C57BL/6 mice decreased their capacity to control the infection caused by *B. abortus*, in contrast to the wild strain, which show resistance to the infection. In humans, it has been demonstrated that T cells from patients with brucellosis produce IFN-γ when stimulated *in vitro* with *Brucella* antigens. This production by CD8+T cells is

higher during the acute stage of the disease (Moreno et al., 2002). In the present study we observed that PEC from *B. abortus* RB51-vaccinated mice expressed the genes that encode cytokines IL-6, IL-12, TNF-α, IFN-γ, and iNOS. However, no mRNA for cytokines TNF-α, IFN-γ, and iNOS were detected in mice infected with *B. abortus* 2308 or in non-infected control mice.

Expression of IL-6 and IL-12 genes decreased in mice infected with *B. abortus* 2308 in comparison with mice inoculated with *B. abortus* RB51. The lower or lack of expression of these genes could have been produced directly by the virulent bacterium, either by a deficient signaling in the plasmatic membrane or by the inhibition or blockage of signal transduction during intracellular transit in the macrophage. Studies on the effect of bacterial surface on the organization of lipids in the lipid rafts suggest that there is an alteration in the organization of receptors and transduction molecules (Kim et al., 2004; Lapaque et al., 2006). The present results allow us to suggest that the protective immunity induced by *B. abortus* RB51 is related with the production of IL-12 by the macrophage during the first hours post-inoculation which, in turn, induces early expression of the IFN-γ gene. Although *B. abortus* 2308 seems to induce IL-12 expression, this is at a very low level, and could, therefore, not reach the threshold to activate the IFN-γ gene and other genes. Our results confirm that there is a very tight relation between IL-12 gene expression and the resistance to infection by *B. abortus*, mediated by IFN-γ, as reported by other authors (Huang et al., 2003; Paranavitana et al., 2005; Zhan and Cheers, 1995b), and for other infections with intracellular microorganisms, such as *Mycobacterium tuberculosis, Salmonella* or *Listeria* (Kawakami et al., 2004; Puertollano et al., 2005; Stoycheva and Murdjeva, 2005). This phenomenon that involves IL-12 and IFN-γ responses could be relevant, since the differences in the expression of these two cytokines occurred at the moment in which T cells were compromised in their activation. The presence of IL-12 and IFN-γ at the moment of antigenic presentation favors differentiation to Th1. Most papers analyze the late response, in which there is also an association between the presence of IL-12 and IFN-γ and protection. The second stage of production of these cytokines completes a response model in which both cytokines form a positive feedback loop (Campos et al., 2004; Huang et al., 2003; Paranavitana et al., 2005).

In turn, the role of TNF-α depends on the presence of IFN-γ during the first infection stages, as shown by neutralizing TNF-α in IFN-γ knockout mice that developed an inflammatory process in the spleen but did not reduce the number of CFU (Murphy et al., 2001a). As demonstrated in the present study, *B. abortus* RB51 induced the activation of the TNF-α gene in PEC, which was not achieved in mice infected with the virulent strain 2308. It is possible to assume that *B. abortus* 2308 inhibits TNF-α production during infection, which could be a virulence mechanism. Jubier-Maurin et al. (2001) demonstrated that protein *Omp25* of *Brucella suis* regulates negatively TNF-α production in infected human macrophages, and Ding et al. (2001) demonstrated that TNF-α, IL-10, and IL-12 production was inhibited in macrophages transfected with the gene of the protein HSP-70 from *Brucella melitensis.* Nitric oxide (NO) production, catalyzed by the iNOS enzyme, represents one of the bactericidal mechanisms of macrophages, as demonstrated by the iNOS and NO increased production in macrophages infected with rough *B. abortus* strains (Jimenez de Bagues et al., 2004). The fact that expression of the iNOS gene was not observed in *B. abortus* 2308-infected mice correlated with the absence of IFN-γ gene expression, the latter being required for iNOS production. Although it was not determined, we can infer that there is no NO synthesis during *B. abortus* 2308 infection. In the present study, we demonstrated that infection of macrophages with *B. abortus* RB51 induced synthesis of the mRNA of iNOS, which correlated with mRNA synthesis of TNF-α and IFN-γ in the same macrophages. Induction of NO synthesis by the iNOS enzyme and induction of IL-12 and IFN-γ could explain the anti-*Brucella* immunity conferred by the vaccination with *B. abortus* RB51. The lack of IL-6 mRNA in *B. abortus* 2308-infected mice suggests an important participation of this cytokine in the protective mechanisms, probably promoting an acute response. Giambartolomei et al. (2004) demonstrated that IL-6 production by infected macrophages was not due to the presence of bacterial LPS but due to lipoproteins.

Zhan and Cheers (1995a) showed that the uncontrolled growth of *Listeria* in culture produced low TNF-α and IL-6 responses. These findings are related with our work, since *B. abortus* 2308 replicated much faster within the macrophage than the RB51 strain (data not shown); hence, bacterial growth could inhibited in some way TNF-α and IL-6 production. On the other hand, LPS starts a complex cascade of events, particularly in monocytes and macrophages that lead to the production of pro-inflammatory cytokines (Campos et al., 2004; Harju et al., 2001). Binding of LPS to TLR2 and TLR4, in conjunction with MD2, starts the cytoplasmic signaling cascade that leads to IκB degradation, nuclear translocation of NF-κB, and transcriptional activation of the genes of the pro-inflammatory response (Akira and Takeda, 2004; Liew et al., 2005), such as the cytokines IL-1, IL-6, IL-12, TNF-α, adhesion molecules, acute stage proteins, and inducible enzymes, such as iNOS and COX-2 (Liew et al., 2005; Zhang and Ghosh, 2001). After LPS binding to the TLR4-CD14-MD2 complex, the adaptor MyD88 molecule is recruited, and it interacts with protein kinase IRAK, which in turn recruits the adaptor TRAF-6 towards the receptor complex, that is involved in the activation of IKK that phosphorylates IκB for its ulterior degradation in proteasomes (Anderson, 2000; Liew et al., 2005). We investigated whether IκBα could be affected in *B. abortus*

2308-infected cells as an explanation to the lack of pro-inflammatory cytokine genes expression. Both *B. abortus* RB51 and *B. abortus* 2308 induced activation of protein IκBα. However, strain RB51 achieved an earlier activation of these proteins than strain 2308. Infection of macrophages with any of the *B. abortus* strains used here did not inhibit either NF-κB release or its translocation towards the nucleus. However, we think that although NF-κB was translocated to the nucleus, and since the NF-κB present in the cytoplasm and nucleus of *B. abortus*-infected macrophages did bind to DNA, in the case of cells infected with 2308 strain, NF-κB apparently had no transcriptional activity. In RB51-infected macrophages, a heterodimer p65-p50 (deduced by its molecular weight) forming a complex with its synthetic oligonucleotide was observed, this was named complex I. Interestingly, in the cytoplasmic and nuclear extracts of 2308-infected macrophages, two delayed complexes were observed, the so called complex I and an additional complex II, which was smaller in size, most probably formed by homodimers p50-p50, and apparently present in higher concentration than Complex I. Hence, the explanation for the differential expression of cytokines by 2308 or RB51-infected macrophages could be attributed to the dimmer form of NF-κB induced by each strain. Complex II could be responsible for the lack of gene expression of the analyzed cytokines since it is well documented that the homodimer p50-p50 of NF-κB is not a transcriptional activator (El Gazzar et al., 2007; Pereira et al., 2005; Plaksin et al., 1993). *Brucella*, with its non-classical LPS, leads to a decreased production of cytokines by monocytes and macrophages, including TNF-α, IL-1 and IL-6 (Campos et al., 2004; Jimenez de Bagues et al., 2005; Lapaque et al., 2006), and iNOS (López-Urrutia et al, 2000; Wang et al., 2001). In our hands, characterization of NF-κB proteins revealed a predominance of p50-p50 homodimer in 2308-infected macrophages. Over expression of this molecule inhibits the transactivity of the NF-κB heterodimer, which is given by the specific attachment of the p65 subunit to the promoter site on the TNF gene (El Gazzar et al., 2007; Kastenbauer and Ziegler-Heitbrock, 1999). Based on our results, we consider that *B. abortus* 2308 exerts on the infected cells a process similar to that of LPS tolerance, during which homodimer p50-p50 (Complex II) is induced hindering the transactivation of cytokine genes. To determine whether inhibition of pro-inflammatory cytokines gene expression by NF-κB homodimers induced by *B. abortus* was due to a bacterial component produced after infection or due to a constitutively expressed bacterial component, we stimulated macrophages with heat-inactivated bacteria.

Activation of IκBα and NF-κB was observed as well as translocation of NF-κB towards the nucleus and its DNA binding. By analyzing cytoplasmic and nuclear proteins from cells stimulated with the heat-inactivated strain 2308, only the band corresponding to complex I was observed; hence, inhibition of gene expression is induced by a bacterial component of live bacteria most probably actively secreted after their internalization. Strain 2308 used as a mechanism of virulence of a homodimer induction inactive NF-κB which does not activate pro-inflammatory response that is required to initiate the elimination of bacteria which causes it to replicate. Finally, efforts should be focused to analyze the molecules in *Brucella abortus* 2308 that could be responsible for the results presented in this paper.

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