

***Yersinia enterocolitica* leads to transient induction of TNF- α and activates NF- κ B in synovial fibroblasts**

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Abstract

Objective

The importance of the presence of bacterial antigen or even living bacteria for the pathogenesis of reactive arthritis has been discussed increasingly ever since bacterial antigen was found in inflamed joints. Bacteria may persist in the body and drive the local immune response, maintaining arthritis. Cytokines, in particular tumor necrosis factor- α (TNF- α), are essential for bacterial elimination. In reactive arthritis, the course of the disease is influenced by several cytokines, including TNF- α . TNF- α expression can be mediated by transcription factor nuclear factor-kappa B (NF- κ B). Moreover, TNF- α is also one of the strongest activators of NF- κ B.

Methods

*In vitro expression of TNF- α and activation of NF- κ B in synovial fibroblasts after infection with *Yersinia enterocolitica* or *Salmonella enteritidis* was analysed by electrophoretic mobility shift assay, Western blot assay and real-time PCR.*

Results

We found that infection of synovial fibroblasts with yersiniae and salmonellae lead to the transient expression of TNF- α mRNA and induction of NF- κ B.

Conclusion

Induction of TNF- α in synovial fibroblasts after infection with yersiniae or salmonellae might be insufficient to eliminate bacteria, and this could allow the intracellular persistence of these bacteria. Our results therefore support the hypothesis that a permissive cytokine pattern might contribute to the pathogenesis of reactive arthritis.

Key words

Reactive arthritis, TNF- α , NF- κ B, *Yersinia enterocolitica*, *Salmonella enteritidis*, pathogenesis.

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Introduction

Reactive arthritis following gastrointestinal or urogenital tract infection with yersinia, salmonella, shigella or campylobacter has been regarded as a human model of spondyloarthropathies. Originally described as aseptic inflammation of the joints, the importance of bacterial antigen or even intact bacteria for the pathogenesis of reactive arthritis has been accepted increasingly, since bacterial antigen and DNA were found in synovial tissue and fluid of reactive arthritis patients (1-4). These data suggest that bacteria may invade joint tissue and that, after eradication of living or culturable bacteria, pathogenic bacterial components, such as LPS, may persist in the joint and lead to the induction and perpetuation of arthritis. To further investigate this hypothesis we established an *in vitro* model of synovial fibroblasts which were infected with different bacteria.

Using this model, we could show that yersinia and salmonella can persist in synovial fibroblasts for several weeks *in vitro* (5-7). However, it is unclear, why elimination of the microorganisms fails subsequently leading to bacterial persistence.

Cytokines are essential for bacterial elimination. In animal models, it has been shown that cytokines such as IL-12, interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α), are crucial for the elimination of bacteria including yersinia and chlamydia (8,9). *In vitro*, intracellular persistence of salmonellae and yersiniae in synovial fibroblasts was influenced by INF- γ and TNF- α (10). Both cytokines led to an accelerated killing of intracellular bacteria.

In reactive arthritis, it has been shown that the course of disease is influenced by several cytokines (11). In particular, low secretion of TNF- α by peripheral blood mononuclear cells seems to be correlated with a chronic course of reactive arthritis (12).

The transcription factor nuclear factor- κ B (NF- κ B) plays a crucial role in the expression of multiple genes involved in inflammatory responses, including TNF- α (13,14). The most common form of NF- κ B is a heterodimer composed of 65 kDa (NF- κ B p65) and 50

kDa (NF- κ B p50) proteins. In unstimulated cells, NF- κ B is present in the cytoplasm in association with an inhibitory protein of the I κ B family, mainly I κ B α (15,16). Stimulation leads to phosphorylation and subsequent degradation of I κ B. The now unmasked subunits of NF- κ B, p65 and p50, are translocated to the nucleus and activate transcription of numerous genes. TNF- α expression is not only mediated by NF- κ B, but TNF- α is also one of the strongest activators of NF- κ B.

Activation of NF- κ B has been found in several chronic inflammatory diseases including ulcerative colitis (17, 18), rheumatoid arthritis (19-21), lung diseases (22) and several acute inflammatory diseases (23).

In reactive arthritis, the role of NF- κ B has so far not been investigated in detail. Therefore, with respect to the pathogenesis of reactive arthritis, using our *in vitro* model, synovial fibroblasts were infected with yersiniae or salmonellae and activation of NF- κ B and induction of TNF- α was analysed.

We found that infection of synovial fibroblasts with *Yersinia enterocolitica* or *Salmonella enteritidis* leads to induction of TNF- α expression and activation of NF- κ B. However, TNF- α expression is only transient and might be therefore insufficient to prevent bacterial persistence in reactive arthritis.

Materials and methods

Cell culture, bacteria and infection of synovial fibroblasts:

Synovial fibroblast-like cells (SFC) were derived from cadaver knee joints from several donors free from rheumatic or infectious diseases (24). All cells were found to be HLA-B27 negative as determined by PCR and immunofluorescence (6). Cells were grown in RPMI containing 10% fetal calf serum (FCS). Cells were used during passages 5 to 12. Twenty-four hours before infection, the FCS concentration was reduced to 0.5% to avoid uncontrolled activation of NF- κ B. Confluent monolayers of synovial fibroblast-like cells were infected with *Yersinia enterocolitica*, *Salmonella enteritidis* or *Escherichia coli* at a multiplicity of infection (MOI) of 100 for 1 hr (5, 6). All bacterial strains were

patient isolates. Infected cells were washed and gentamicin 25 μ g/ml was added to the culture medium to kill extracellular bacteria. After 2 hr, gentamicin concentration was reduced to 4 μ g/ml, which did not affect intracellular growth of bacteria. RNA isolation and protein extracts were prepared after the indicated period of time after infection. Synovial fibroblast-like cells were also stimulated with inactivated bacteria or bacterial supernatants, containing no bacteria. For inactivation, suspensions of bacteria were irradiated at a wavelength of 302 nm (Ultraviolet Transilluminator TM36, UVP, Cambridge, England) by UV-light for 30 minutes. Bacterial supernatant was obtained by centrifugation of the bacterial suspension at 4000 g.

Stimulation of fibroblasts with TNF- α

For positive control, non-infected synovial fibroblast-like cells were stimulated with 20ng/ml recombinant TNF- α (Promega, Madison, USA) for 1 hr (25) and treated in the same way as infected cells.

Enzyme immunoassay (EIA) using monoclonal antibodies to TNF- α and IL-10 was performed according to the manufacturer's instructions using an ELISA of Endogen (Biozol) with a detection limit of 5 pg/ml.

Isolation of RNA and RT PCR

RNA isolation of infected or stimulated synovial fibroblast-like cells was done using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA was reverse transcribed into complementary DNA. Quantification of cytokine mRNA of TNF- α and IL10 was performed using real-time quantitative reverse transcriptase polymerase chain reaction (RT PCR) as described (26, 27).

Preparation of nuclear and cytoplasmic protein extracts

Protein extracts of synovial cells were prepared as described (28) with slight modifications. Briefly, cell monolayers (4×10^6 cells) were harvested with a cell scraper, washed twice with PBS, and incubated with buffer A (10 mM HEPES

pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) for 15 min on ice. The swollen cells were passed 10 times through an injection needle (26G3/8) and centrifuged for 1 minute at 8000 g. Supernatant containing the cytoplasm was stored at -70°C . Pelleted nuclei were resuspended in buffer C (20 mM HEPES pH 7.9, 0.4 M KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF) and mixed vigorously for 40 min at 4°C . Nuclear extracts were centrifuged for 5 min at 12000 g and the supernatant was frozen at -70°C . Protein concentration was measured according to the method of Bradford (29).

Western blot assay

For Western blot assays, 5-10 μ g of cytoplasmic or nuclear proteins were fractionated on 10% polyacrylamide gels containing SDS. Proteins were transferred electrophoretically to a 0.20 μ m pore nitrocellulose membrane (Schleicher and Schuell). Transfer was performed for 3 hr at 200 mA on ice. To ensure equal loading of proteins in all lanes, membranes were stained with Ponceau S dye (Sigma, St. Louis, MO, USA). Membranes were incubated in TBE containing 6% skimmed milk powder to block non-specific binding. Detection was performed using the ECL system (Amersham, GB) according to the manufacturer's instructions. As primary antibody, rabbit polyclonal antibodies to IkBa or NF- κ B p65 were used (Santa Cruz, Biotechnology, sc-371 and sc-109). As secondary antibody, a horseradish peroxidase conjugated polyclonal swine anti rabbit immunoglobulin antibody (DAKO, Denmark) was used. All experiments were repeated at least twice.

Electrophoretic mobility shift assay (EMSA)

In EMSA, 3 or 4 μ g nuclear proteins were incubated with 5000-8000 cpm (equivalent to 0.2 ng) of a ^{32}P -labelled oligonucleotide probe and 2 μ g poly-(di-dC) as non-specific competitor for 30 min on ice. Samples were analysed on non-denaturing 5% polyacrylamide gels in 0.4 TBE at 220V at room temperature. Gels were dried onto filter

paper and visualized by autoradiography at -70°C . For supershift assays, 1 μ l of p65 or p50 antibody (Santa Cruz, sc-109 and sc-298) was added to the EMSA incubation mixture. The sequence used for the NF- κ B-probe was: TCE_{dA>C} 5'-AGCTTGACCAAGAGG GATTTCCCCTAAATC-3' (30; the underlined base pairs correspond to the κ B binding motif).

Results

Induction of TNF- α in synovial fibroblasts

TNF- α and IL-10 could not be found in the supernatant of yersiniae infected cells by EIA (data not shown). Quantitative real time PCR was used to analyse cytokine mRNA production in synovial fibroblast-like cells after infection with yersiniae. Using this method, it could be shown that infection of synovial fibroblast-like cells with yersiniae led to induction of TNF- α mRNA. A first maximum of TNF- α mRNA production was observed 4 hr after infection. A second, but lower peak of expression was seen 24 hr after infection (Fig.1). At later time points, TNF- α mRNA expression was negligibly low (data not shown).

As positive control, synovial fibroblast-like cells were stimulated with TNF- α . This treatment led to a high expression of TNF- α mRNA. In comparison to stimulation with TNF- α , the expression of TNF- α mRNA in synovial fibroblast-like cells after infection with yersiniae was of low magnitude (ratio of approximately 6:1).

Instead of living bacteria, synovial fibroblast-like cells were also stimulated with UV-irradiated bacteria or bacterial supernatant. To confirm inactivation of UV-irradiated bacteria, they were recultivated, and no living bacteria were detected. TNF- α mRNA induction in SFC after stimulation with inactivated bacteria or with bacterial supernatant was increased compared with unstimulated cells. However, it appeared to be weaker than after stimulation with living yersiniae (data not shown).

These results showed that stimulation of synovial fibroblast-like cells with yersiniae led to a transient expression of TNF- α mRNA. TNF- α mRNA ex-

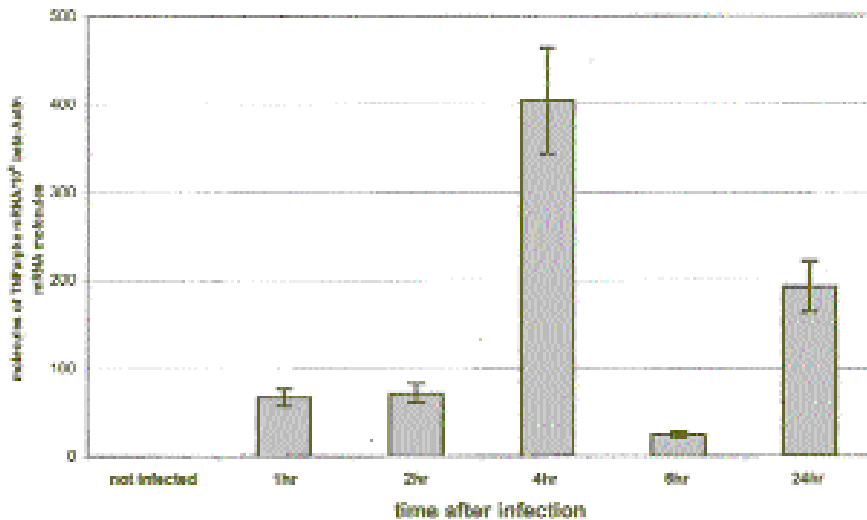


Fig. 1. Expression of TNF- α RNA in synovial fibroblast-like cells after infection with yersiniae. A maximum production of TNF- α RNA molecules was found 4 hours after infection. Non-infected synovial fibroblast-like cells did not produce TNF- α RNA.

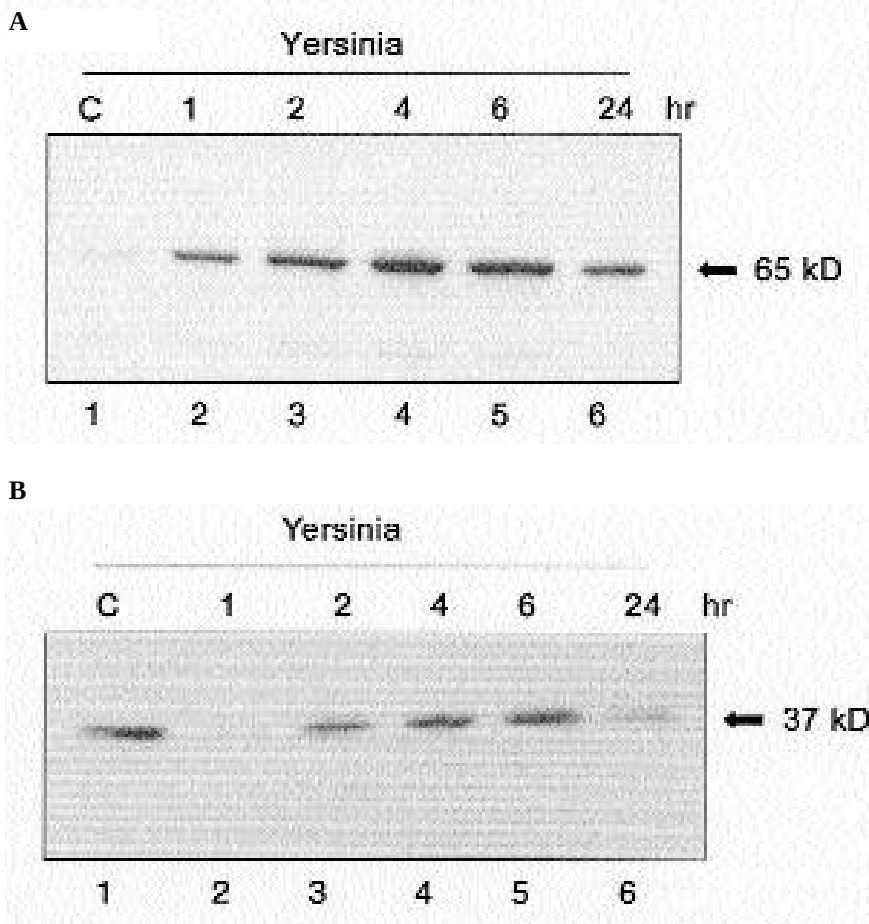


Fig. 2. Western blot analysis of (A) nuclear extracts and (B) cytoplasmic extracts of synovial fibroblast-like cells 1 to 24 hrs after infection with *Yersinia enterocolitica*.

(A) Activation of NF- κ B is demonstrated at a maximum 4 to 6 hrs after infection. Lane 1: control (C); lanes 2 to 6: infection of synovial fibroblast-like cells after 1, 2, 4, 6 and 24 hours (1, 2, 4, 6, 24 hrs). (B) Degradation of Ikba in the cytoplasm of infected synovial fibroblast-like cells found 1 hr after infection. Lane 1: control (C); lanes 2 to 6: infection of synovial fibroblast-like cells after 1, 2, 4, 6 and 24 hours (1, 2, 4, 6, 24 hrs).

pression might be influenced by other cytokines, such as IL-10, which is a possible suppressor of TNF- α expression. For this reason, IL-10 expression was analysed by quantitative real time PCR. However, there was no measurable IL-10 mRNA expression in synovial fibroblast-like cells after infection with yersiniae (data not shown).

Activation of NF- κ B in synovial fibroblasts by infection with yersiniae

Activation of NF- κ B in synovial fibroblast-like cells by infection with yersiniae was analysed by western blot assay and EMSA at different time points after infection. Each experiment was done at least twice. No significant differences in activation of NF- κ B was seen in SFC from different donors.

Western blot of cytoplasmic extracts with antibodies to IkBa showed degradation of the inhibitor protein IkBa with a maximum 1 hr after infection (Fig. 2B). Translocation of NF- κ B into the nucleus was determined by Western blot of nuclear extracts using antibodies to p65. Translocation of NF- κ B was seen within a period from 1 hr to 24 hr and a maximum was observed 4 to 6 hr after infection (Fig. 2A). Results were confirmed in EMSA, which showed activation of NF- κ B with a maximum 4 hr after infection (Fig. 3). Addition of antibody to p65 and p50 caused a supershift, demonstrating that the NF- κ B complex consisted of the subunits p65 and p50. Shifted bands were specifically eliminated by an excess of unlabelled probe.

For a positive control, synovial fibroblast-like cells were incubated with 20 ng/ml TNF- α , a strong inducer of NF- κ B. Strong activation was seen both in western blot and in EMSA at a maximum 30 to 60 min after stimulation. Negative control consisted of NF- κ B activation in uninfected synovial fibroblast-like cells and showed no activation.

Activation of NF- κ B might be induced by surface proteins of yersiniae. To analyse the influence of intracellular bacterial survival and replication on activation of NF- κ B, synovial fibroblast-like cells were stimulated with yer-

siniae which had been inactivated by UV-light irradiation. Activation of NF- κ B was analysed by western blot assay of cytoplasmic and nuclear extracts. Whereas degradation of I κ B α was not detectable a weak activation signal of NF- κ B was detected 4 to 6 hr after infection by translocation of p65 (Fig. 4). Synovial fibroblast-like cells were also incubated with bacterial supernatant after sterile filtration. The activation signal of NF- κ B was even weaker compared to UV-light irradiated-bacteria, but still demonstrable (Fig. 4).

Activation of NF- κ B by salmonellae

NF- κ B activation in synovial fibroblast-like cells was also analysed after infection with *Salmonella enteritidis*, another bacteria that is associated with reactive arthritis, (Figs. 5 and 6). In western blot analysis, stimulation with salmonellae led to degradation of I κ B α 1 hr after infection. Translocation of p65 into the nucleus was seen about 4 hr after infection. This was confirmed by EMSA, which showed a maximum activation of NF- κ B 4 to 6 hr after infection.

NF- κ B complexes could be supershifted in EMSA after addition of antibodies specific for p65 and p50.

Discussion

Although the pathogenesis of reactive arthritis remains still unclear, the triggering agents of reactive arthritis are known. After gastrointestinal infection, bacteria probably persist in the body and induce arthritis. Elimination and persistence of bacteria can be influenced by several cytokines. In animal models it has been shown that Th1 cytokines, such as IL-12, INF- γ and TNF- α , are crucial for the elimination of the reactive arthritis-associated bacteria yersiniae (9). In contrast, a lack of these cytokines or elevated Th2 cytokines can lead to ineffective clearance and persistence of pathogens. *In vitro*, the Th1 cytokines INF- γ and TNF- α decreased intracellular survival of bacteria in synovial fibroblasts after infection with yersiniae (10). This effect was antagonized by the Th2 cytokine IL-4 which led to prolonged intracellular bacterial survival.

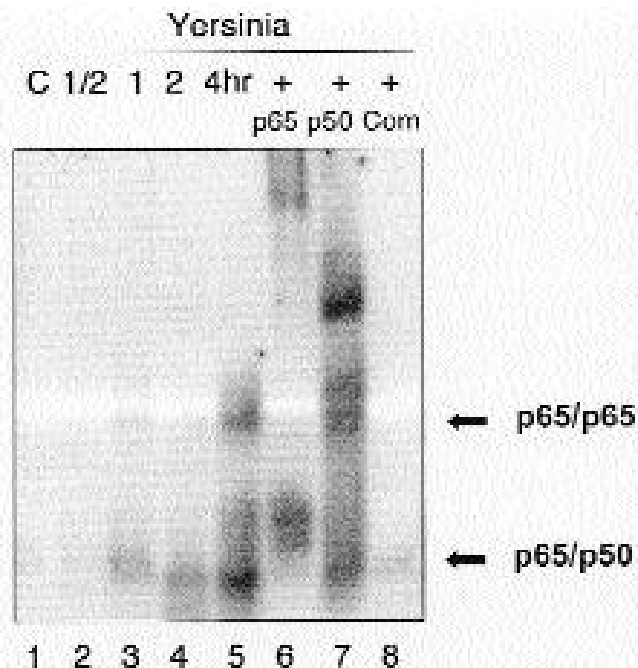


Fig. 3. EMSA of nuclear extracts of synovial fibroblast-like cells 30 min to 4 hrs after infection with *Yersinia* showing a maximum activation of NF- κ B about 4 hrs after infection.

Lane 1: control (C), not infected cells; lanes 2 to 5: stimulation 30 min to 4 hours after infection with yersinia (30 min, 1, 2, 4 hrs), lane 6: addition of antibody to p65 (+p65); lane 7: addition of antibody to p50 (+p50); lane 8: addition of specific competitor (+com).

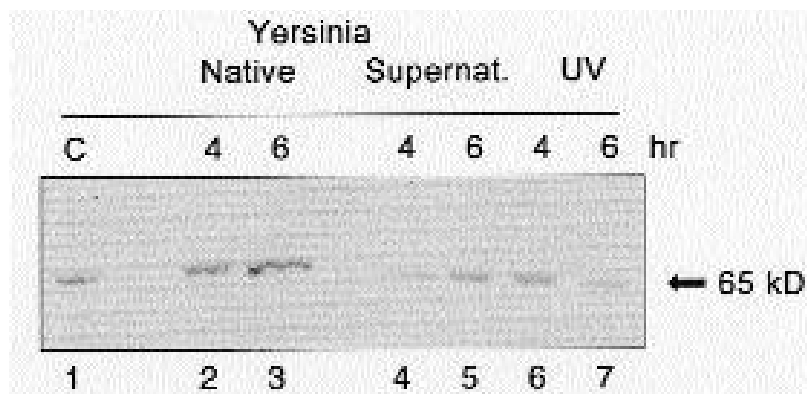


Fig. 4. Western blot analysis of nuclear extract to compare activation of NF- κ B in synovial fibroblast-like cells 4 to 6 hrs after stimulation with yersiniae, bacterial supernatant or UV-light irradiated inactivated yersiniae. Activation of NF- κ B is weaker after stimulation with bacterial supernatant or inactivated bacteria than with living yersiniae.

Lane 1: control (C); lanes 2 and 3: stimulation with yersiniae 4 and 6 hours (4, 6 hrs) after infection; lane 4 and 5: stimulation with bacterial supernatant after 4 and 6 hours; lane 6 and 7: stimulation with UV-light inactivated bacteria after 4 and 6 hours.

In the present study, using an *in vitro* model, we have shown that synovial fibroblasts, as non-professional cells of the immune system, can be induced to produce TNF- α mRNA after infection with yersiniae. Since a correlation between cytokine mRNA and protein expression has been shown (27), we conclude that infection with *Yersinia*

leads to TNF- α expression. However, production of TNF- α was low and could not be detected by ELISA (<5 μ g/ml). Using quantitative real time PCR which is much more sensitive than ELISA, a transient induction of TNF- α mRNA was demonstrable. The maximum was observed 4 to 6 hours after infection, and a second but lower maximum was

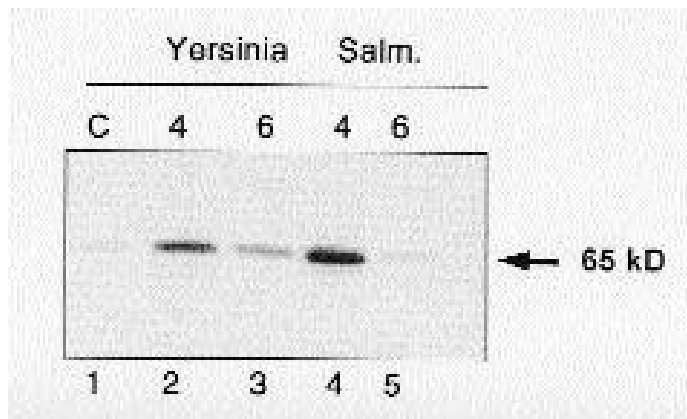


Fig. 5. Western blot analysis of nuclear extracts showing nuclear translocation of NF κ B in synovial fibroblast-like cells 4 to 6 hrs after infection with *Yersinia* or *Salmonella*.

Lane 1: control (C), not infected cells; lanes 2 and 3: stimulation with yersinia 4 and 6 hours after infection (4, 6 hrs); lane 4 and 6: stimulation with salmonellae 4 and 6 hours after infection (4, 6 hrs).

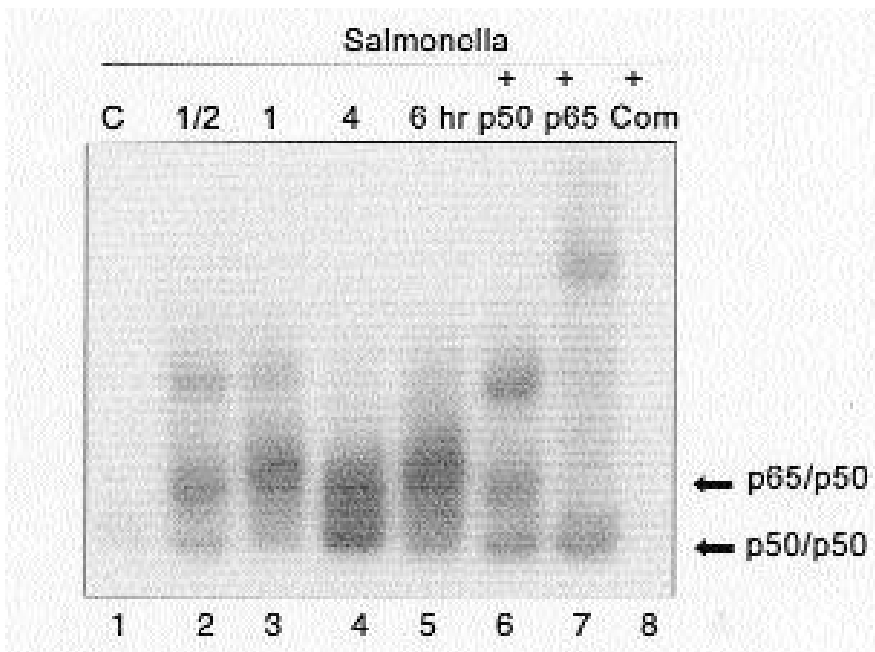


Fig. 6. EMSA of nuclear extracts of synovial fibroblast-like cells 30 min to 6 hrs after infection with *Salmonella* showing a maximum activation of NF κ B about 4 hrs after infection.

Lane 1: control (C), not infected cells; lanes 2 to 5: stimulation 30 min to 6 hours after infection with salmonellae (1/2, 1, 4, 6 hrs), lane 6: addition of antibody to p50 (+p50); lane 7: addition of antibody to p65 (+p65); lane 8: addition of specific competitor (+com).

seen 24 hours after infection. Afterwards, at a time of bacterial persistence and replication (7), TNF- α mRNA expression was negligibly low. Therefore, in this *in vitro* model, induction of TNF- α might be insufficient to eliminate bacteria, and this might permit intracellular persistence of these bacteria for several weeks, as it has been demonstrated previously (5).

Recently, it has been reported by Braun *et al.* that low secretion of TNF- α cor-

relates with morbidity in the course of reactive arthritis in humans (12). TNF- α secretion by peripheral blood mononuclear cells and T cells was significantly lower in patients with a disease duration of ≥ 6 months than in patients with a disease duration of <6 months and low secretion of TNF- α correlated with a chronic course of reactive arthritis. Moreover, cells from HLA-B27 positive patients secreted less TNF- α than those from those patients who

were HLA-B27 negative. These findings led to the conclusion that diminished TNF- α production might reflect a phase of relative immunodeficiency contributing to bacterial persistence in reactive arthritis. This supports our hypothesis that a permissive cytokine pattern might contribute to the pathogenesis of reactive arthritis.

In contrast, Granfors and colleagues found that activation of NF- κ B and secretion of TNF- α are enhanced upon LPS stimulation in U937 human monocytic cells transfected with HLA-B27 (31). These results suggest that increased inflammatory responses contribute to the development of reactive arthritis. All SFC used in our study were HLA-B27 negative and therefore the influence of HLA-B27 was not analysed. Future studies may clarify these controversies. However, previous results from our laboratory in human fibroblasts suggested no influence of endogenous HLA B27 on invasion and persistence of gram-negative bacteria (6).

Having shown that synovial fibroblast-like cells produce TNF- α after infection with *Yersinia enterocolitica*, we were interested to elucidate, which signal transduction pathways may lead to TNF- α expression. NF- κ B is not only one of the most important transcription factors for TNF- α expression, but is also itself activated by TNF- α (32). Therefore, activation of NF- κ B in synovial fibroblast-like cells after infection with yersinia or stimulation with TNF- α was analysed by western blot and EMSA.

In the present study, NF- κ B activation in synovial fibroblast-like cells after infection with yersinia was analysed by western blot analysis and EMSA. Peak activation was seen 4 to 6 hours after infection. Similar results were obtained by infection with salmonellae. To analyse the importance of living bacteria for the activation of NF- κ B, yersinia were inactivated by UV-light irradiation. Activation of NF- κ B was much weaker compared to stimulation with living bacteria. Activation might be induced by components of the bacterial wall. To further exclude these influences, bacterial supernatant was sterile filtrated. Stimulation of synovial fi-

broblasts with this supernatant lead to an even weaker but still demonstrable activation of NF- κ B. The most plausible explanation is that the supernatant still contains bacterial products, which lead to activation of NF- κ B. We presume that this agent might be LPS since LPS is an important inducer of NF- κ B and difficult to eliminate (33).

Activation of NF- κ B with respect to the pathogenesis of arthritis of different aetiologies has been analysed by several authors. Activation of NF- κ B in response to TNF- α has been shown in human synovial cells with subsequent transcription of several genes, including TNF- α and IL-6 (25), and in mouse fibroblast-like synoviocytes (34). Activation of NF- κ B has also been detected in human inflamed synovial tissue of patients with rheumatoid arthritis or osteoarthritis (21). In a mouse model, the role of NF- κ B in the pathogenesis of inflammatory arthritis was demonstrated (20).

In conclusion, our results show that infection of synovial fibroblast-like cells with yersiniae leads to expression of TNF- α and induction of NF- κ B. Expression of TNF- α was probably mediated by NF- κ B. However, both TNF- α expression and NF- κ B activation were weak and only transient. Thus, TNF- α production might be insufficient to kill invading bacteria. Therefore, with respect to the pathogenesis of reactive arthritis, yersiniae or salmonellae might be able to persist in fibroblasts and produce the critical amount of bacterial antigen, necessary for the induction of arthritis in susceptible individuals.

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