INDUCTION OF TUMOR NECROSIS FACTOR ALPHA IN MURINE MACROPHAGES WITH VARIOUS STRAINS OF *COXIELLA BURNETII* AND THEIR LIPOPOLYSACCHARIDES

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Summary. – The ability of various strains of *Coxiella burnetii* (*C.b.*) and their phase I and II lipopolysaccharides (LPSs) to induce tumor necrosis factor alpha (TNF-α) in peritoneal Balb/c mouse macrophages *in vitro* was investigated. Considerable differences in the induction ability were observed in dependence on the strain applied. In a TNF-α bioassay, the most effective inducers were both corpuscles and LPSs of the strains Priscilla and Scurry, followed by Nine Mile, Luga, and Henzerling I. In contrast, in ELISA, the most effective inducers were LPSs of the strains Luga and Henzerling, followed by Nine Mile, Priscilla, and Scurry. The role of toll-like receptor 4 (TLR4) in the induction was confirmed by the use of C3H/HeJ mouse macrophages. Thus, the induction of TNF-α was much higher in Balb/c mouse macrophages than that in TLR4-deficient C3H/HeJ mouse macrophages. Differences in the results of the bioassay and those of ELISA suggest a role of another secreted factor(s) induced with *C.b.* in murine macrophages that could act synergically with TNF-α in L929 cells in the bioassay. The observed differences in TNF-α induction might play a role in the pathobiology of Q fever.

Key words: TNF-α; macrophage; bioassay; ELISA; Coxiella burnetii; lipopolysaccharides

Introduction

C.b., an obligate intracellular bacterium, is the etiologic agent of Q fever that occurs in two major forms: acute and chronic. The acute form can be manifested by a flue-like illness, pneumonia, acute febrile illness, hepatitis, and meningoencephalitis. The chronic form usually occurs as chronic hepatitis, vascular or bone marrow infection and endocarditis (Maurin and Raoult, 1999). Different Q fever forms may correspond to different immunomodulatory properties of individual *C.b.* strains and/or different patient immunogenetic predispositions. Genetic variability among different *C.b.* strains has been tentatively ascribed to their virulence (Hendrix *et al.*, 1991; Samuel *et al.*, 1985). A comparison of various *C.b.* isolates regarding their LPSs by immunoblot analysis resulted in their grouping similar to that of genomic grouping (Hackstadt, 1986). It has been recently found that the monocytes from patients with developing chronic Q fever exhibit defective phagosome maturation and impaired *C.b.* killing (Ghigo *et al.*, 2004).

The establishment of persistent *C.b.* infection probably corresponds not only to the subversion of microbicidal function of macrophages (Capo *et al.*, 1999a), but also to the impairment of the T-cell immune response (Mege *et al.*, 1997). Q fever endocarditis is associated with the suppression of antigen-specific T-cell responses mediated partially by prostaglandin E2 production (Koster *et al.*, 1985) or interleukin-10 release by monocytes (Capo *et al.*, 1996a). Besides their immunosuppressive role, the monocytes from patients with Q fever endocarditis overproduce TNF-α (Capo *et al.*, 1996b). Such a TNF-α overproduction is involved in

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Abbreviations: *C.b.* = *Coxiella burnetii*; NM I = Nine Mile phase I; NM II = Nine Mile phase II; H I = Henzerling phase I; H II = Henzerling phase II; P = Priscilla; L = Luga; S = Scurry; LPS = lipopolysaccharide; TLR = toll-like receptor

the survival of *C.b.* inside the patient monocytes (Dellacasagrande *et al.*, 2000a) and may be related to specific inflammatory syndrome of Q fever endocarditis consisting of an increase in circulating TNF-α without variations in cytokine antagonists (Capo *et al.*, 1999a). Intracellular bacteria have developed specific strategies to prevent production of TNF-α by macrophages. In contrast, *C.b.* stimulates TNF-α production in human and murine macrophages (Tujulin *et al.*, 1999; Dellacasagrande *et al.*, 2000b).

The virulence of *C.b.* is mainly related to the structure of its LPS (Hackstadt *et al.,* 1985; Škultéty *et al.*, 1998). Whereas the bacteria in phase I (virulent) express a smooth-type LPS (phase I LPS) (Amano *et al.*, 1987; Toman, 1999), the bacteria in phase II (avirulent) exhibit a rough-type LPS (phase II LPS) (Toman and Škultéty, 1996; Ftáček *et al.*, 2000). These LPSs were considered poorly endotoxic (Schramek and Brezina, 1976; Toman *et al.*, 2004); however, their ability to induce TNF-α has been reported (Tokarevich *et al.*, 1994; Kubeš *et al.*, 2000; Toman *et al.*, 2004). The TNF-α induction does not directly reflect the virulence of *C.b.*, since avirulent *C.b.* was also shown to induce TNF-α (Tujulin *et al.*, 1999), and avirulent *C.b.* in comparison to virulent *C.b.* was a more potent inducer of TNF-α. It is likely that the induction of TNF-α with avirulent *C.b.* depends on its phase II LPS (Dellacasagrande *et al.*, 2000b). The increase in binding of avirulent *C.b.* to monocytes could explain the overproduction of TNF-α, since the interaction of avirulent *C.b.* with monocytes in comparison to virulent *C.b.* was much stronger (Capo *et al.*, 1999b). In the cell response to LPS integrins are probably engaged. The interaction of *C.b.* with monocyte $\alpha_v\beta_3$ integrin is not sufficient to induce TNF-α, but it probably enables the *C.b.* LPS to interact with TLR4 or TLR2 of monocytes and, in this way, to trigger the TNF-α induction (Honstettre *et al.*, 2004; Zamboni *et al.*, 2004).

As cytokines activate a cascade of secondary inflammatory mediators that eventually leads to endothelial damage (Bone, 1991), we decided to investigate the ability of different *C.b.* strains and their LPSs to induce TNF-α in peritoneal macrophages from Balb/c and C3H/HeJ mice. TNF-α was measured by a bioassay on L929 cells and ELISA. We conclude that the differences in the ability to induce TNF-α observed among different *C.b.* strains might play a role in their pathogenicity.

Materials and Methods

Cells. Mouse L 929 fibroblasts were cultivated in RPMI 1640 medium with L-glutamine (PAA Labs GmbH, Austria) supplemented with 10% of heat-inactivated fetal calf serum (Life Technologies, Germany) and 40 µg/ml gentamicin (Lek A.S., Slovenia) (complete medium). Unless otherwise stated, complete medium was used in all manipulations with the cells.

Table 1. The strains of *C. burnetii* used in the study

C.b. strains	Year and place of isolation	Source
Nine Mile	1937, USA	Tick (*Dermacentor andersoni*)
Priscilla Q 177	1980, USA	Goat placenta
Scurry Q 217	1981, USA	Human liver
Luga	1958, Russia	Tick (*Apodemus flavicollis*)
Henzerling	1945, Italy	Human blood

C.b. strains Nine Mile in phase I (NM I), Nine Mile in phase II (NM II), Henzerling in phase I (H I), Henzerling in phase II (H II), Priscilla in phase I (P), Luga in phase I (L) and Scurry in phase I (S) were used (Table 1). They were cultivated in chick embryo yolk sacs.

Animals. Inbred C3H/HeJ and Balb/c mice of either sex were obtained from MAT Consulting, Czech Republic, and used at 2 to 4 months of age.

Preparation, culturing and induction of macrophages. To collect peritoneal macrophages, mice were injected intraperitoneally with 2 ml of sterile 5% thioglycolate broth (Difco Laboratories) (Park and Rikihisa, 1991). Five days later, the mice were sacrificed by cervical dislocation and macrophages were removed by peritoneal lavage using Hank's balanced salt solution (HBSS). The cells were washed by centrifugation and 5 x 10^6 cells in 0.5 ml of medium was added to each well of 24-well tissue culture microplates (Sarstedt) for 2 hrs at 37°C in 5% CO_2. After removal of non-adherent cells, at least 96% of the adherent cells, which were used in further experiments, were represented by macrophages according to morphological and phagocytic criteria (Akagawa and Tokunaga, 1985). The medium was replaced with the fresh one (0.5 ml) containing the appropriate TNF-α inducer in duplicate (time 0). At appropriate times of cultivation, the medium was filtered (0.22 µm, Costar) and assayed for TNF-α either immediately or later after freezing at -70°C and thawing.

TNF-α bioassay was done by using TNF-α-sensitive L-929 cells according to the method described by Flick and Gifford (1984). Briefly, the cells were seeded in 96-well flat-bottom microplates (4 x 10^4 cells per 100 µl of medium per well) and incubated overnight at 37°C in 5% CO_2. Approximately 20 hrs later, the medium was replaced with 0.1 ml of medium containing 1 µg/ml actinomycin D (Calbiochem) and the plates were further incubated at 37°C for 2 hrs. The medium was then replaced with serial twofold dilutions of tested samples (0.2 ml) in triplicate. The plates were then incubated for 24 hrs at 37°C in 5% CO_2. The medium was then aspirated from the wells to remove dead cells and the cultures were stained with 0.5% crystal violet in 50% ethanol and 5% formalin. The plates were thoroughly washed with water to remove excessive dye, dried and washed with 50% ethanol to elute the dye. A_{590} was determined in a Multiscan MS (Labsystems) by using a lysis control as a blank. The TNF-α activity of a tested sample was calculated by linear regression and expressed in units per ml (U/ml). One unit of TNF-α activity was defined as that causing the lysis of 50% of L cells in the standard assay. A standard with 300–400 U/ml was included in each assay. The SE of the mean was generally less than ±12%.

TNF-α ELISA was performed by using the Duo Set, Development System mouse TNF-α kit from R&D Systems, UK.

C.b. – growth and purification. C.b. cells, serologically in phase I and II (yolk sack passages 3 and 163, respectively) were propagated in chicken embryo yolk sacs. The cells were killed with formalin and purified by differential centrifugation and enzymatic (only phase II cells) and ether treatments (Toman and Škultéty, 1996; Škultéty *et al.*, 1998).

LPS preparation. Highly purified *C.b.* cells were treated with chloroform-methanol (2:1; v/v) at room temperature overnight to remove phospholipids, and then extracted with 45% aqueous phenol three times at 68°C for 20 mins (Schramek and Brezina, 1976).

Results

Induction of TNF-α with various C.b. strains

The induction of TNF-α in Balb/c mouse peritoneal macrophages with various *C.b.* strains in concentrations of 20 and 50 μg/ml was followed by the bioassay (Fig. 1). The highest TNF-α values were obtained with the strain S (365 U/ml), followed by P (287 U/ml), L, and H I (both 242 U/ml). Most strains reached a peak at 3–6 hrs, but in several cases, particularly with NM I and NM II, the peak occurred at 9–15 hrs. Significant levels of TNF-α (30–100 U/ml) were detectable even at 18 hrs.

In contrast to Balb/c mouse macrophages, C3H/HeJ mouse macrophages stimulated with various *C.b.* strains did not show any detectable TNF-α values in the bioassay.

Induction of TNF-α with LPSs of various C.b. strains

In the experiments, in which TNF-α was determined by the bioassay, TNF-α in Balb/c mouse macrophages induced with LPSs of various *C.b.* strains reached a peak already at 3 hrs (Fig. 2). Whereas the highest TNF-α levels were obtained with LPSs from the strains S and P at a concentration of 20 μg/ml (503 and 363 U/ml, respectively), with the strains NM I, H I and H II, the highest TNF-α levels (190, 244 and 58 U/ml, respectively) were obtained with LPSs at a concentration of 50 μg/ml. In the case of C3H/HeJ mouse macrophages, when *C.b.* corpuscles were replaced by their LPSs as potential inducers, TNF-α was again undetectable. An LPS from *Salmonella typhimurium* (20 μg/ml) used as a positive control, induced TNF-α to the level of 660 U/ml.

In the experiments, in which TNF-α was assayed by ELISA, Balb/c macrophages produced the highest values

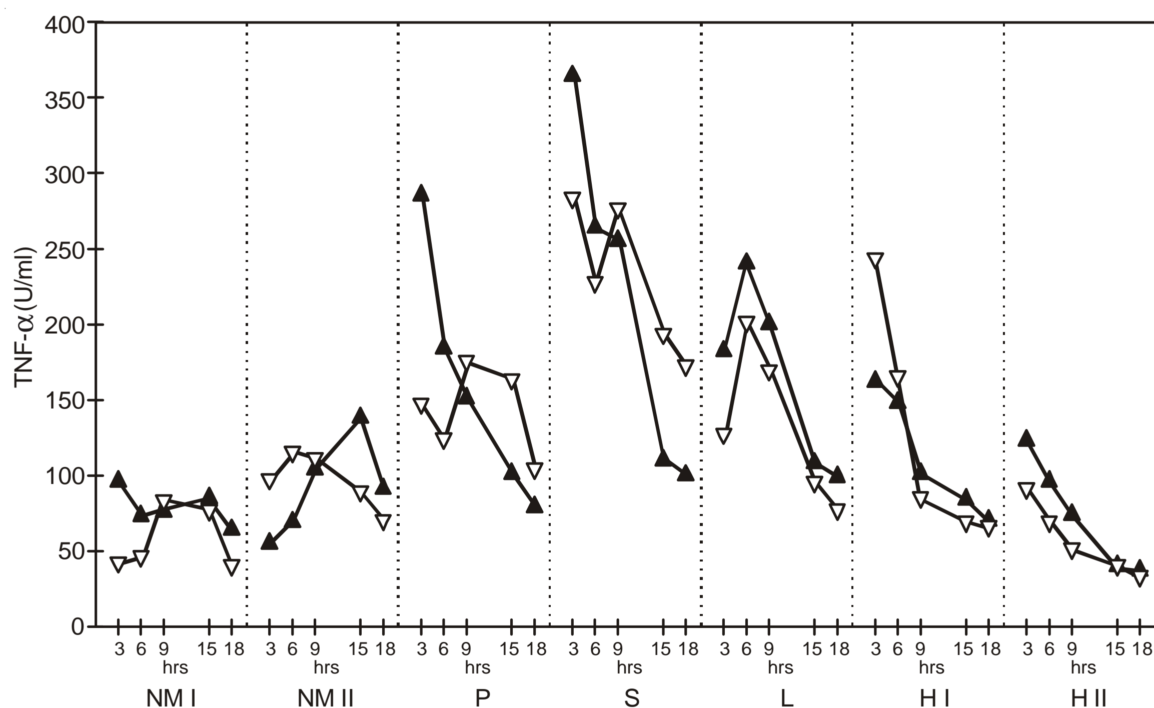

Fig. 1

TNF-α induction in Balb/c mouse macrophages with various *C.b.* strains as revealed by the bioassay

Concentration of *C.b.* corpuscles: 50 μg/ml (full triangles) and 20 μg/ml (open triangles).

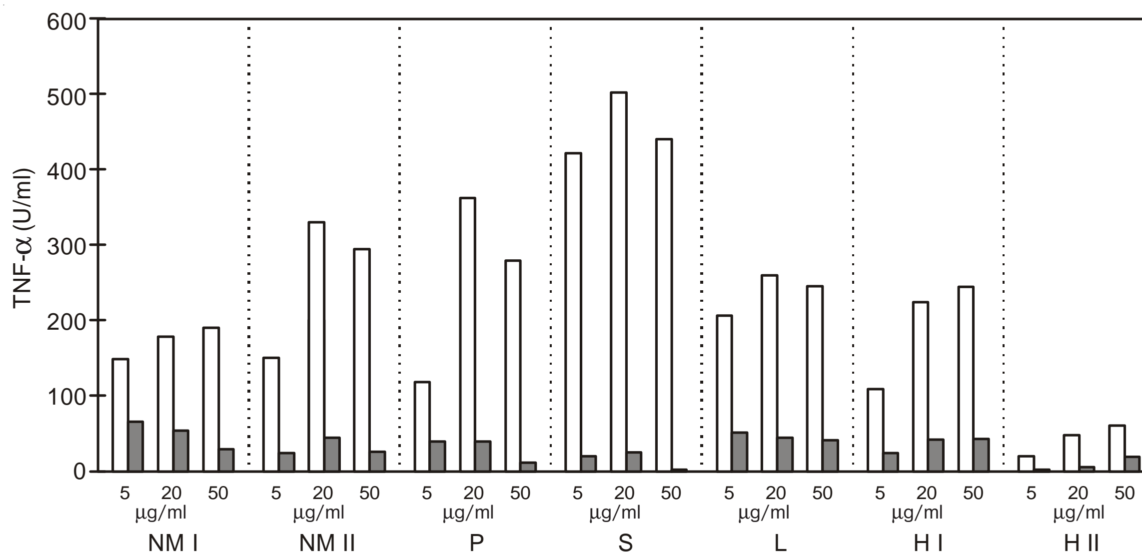

Fig. 2

TNF-α induction in Balb/c mouse macrophages with LPSs of various *C.b.* strains as revealed by the bioassay

TNF-α detected at 3 hrs (open columns) and 6 hrs (stippled columns).

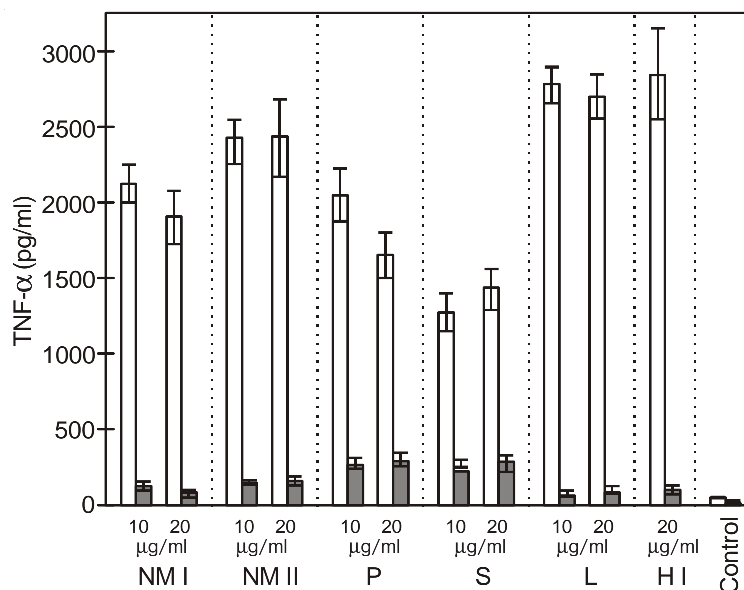

Fig. 3

TNF-α induction in mouse macrophages with LPSs of various *C.b.* strains as revealed by ELISA

Balb/c (open columns) and C3H/HeJ (stippled columns) macrophages. Control: the supernatant from a culture of non-induced macrophages. The data represent the means ± SD from two separate experiments.

of TNF-α at 3 hrs after induction with LPSs from the *C.b.* strains L (2700 pg/ml at 10 μg/ml) and H I (2839 pg/ml at 20 μg/ml) (Fig. 3). These LPSs were followed by NM II (2425 pg/ml and 2437 pg/ml at 10 μg/ml and 20 μg/ml, respectively), NM I (2123 pg/ml and 1906 pg/ml at 10 μg/ml and 20 μg/ml, respectively), P (2052 pg/ml and 1648 pg/ml at 10 μg/ml and 20 μg/ml, respectively), and S (1435 pg/ml and 1275 pg/ml at 20 μg/ml and 10 μg/ml, respectively). At

6 hrs and later, a sharp decrease in TNF-α levels was observed (data not shown). The LPS from *S. typhimurium* (20 μg/ml) used as positive control, induced TNF-α to the level of 2200 pg/ml at 3 hrs. Similarly to the results with C3H/HeJ mouse macrophages given above, these macrophages showed very low levels of TNF-α after stimulation with LPSs from various *C.b.* strains; a maximum of 292 pg/ml was observed with the strain P.

Discussion

In this report, we show that peritoneal macrophages from Balb/c and C3H/HeJ mice (low endotoxin responder) produce TNF-α after contact with corpuscles from different strains of *C.b.* or with their LPSs in a different manner. In the recent years, the ability of *C.b.* to induce TNF-α has been reported not only by us (Kubeš *et al*., 2000), but also by other authors (Dellacasagrande *et al*., 2000b; Tujulin *et al*., 1999; Tokarevich *et al*., 1999), working mostly with the prototype strains of *C.b.*- NM and P. However, the *in vitro* induction with corpuscles or LPSs of various strains of *C.b.* has not yet been demonstrated.

In our experiments, TNF-α was quantified by its cytotoxic activity on L929 cells by the bioassay and by its exact amount by ELISA. The bioassay system seems to mimic better the complexity of the infected organism and offers probably a better possibility to evaluate the putative role of TNF-α in the pathophysiological complications associated with the chronic form of Q-fever. In fact, the highest TNF-α induction was observed with both corpuscles and LPSs of the strains S and P. The S strain is considered to be associated with the chronic form of Q fever. Glazunova *et al.* (2005) have reported that the strains P and S belong to different monophyletic groups, and thus, are not genetically closely related. However, the LPS patterns of both strains appear to be similar on both SDS-PAGE and immunoblot analyses (Hackstadt, 1986; Toman *et al*., 1996). The latter data could be supported by the recent MLVA analysis using the unweighted pair-group method, in which both strains were shown to be genetically very close (Svraka *et al*., 2006).

In contrast, the highest induction of TNF-α in macrophages with LPSs were observed in ELISA with the strains L, H I, NM, P, and S, ordered in descending manner. This finding suggests the role of non-LPS structures of *C.b.* in TNF-α induction in murine macrophages. Recently, Dellacasagrande *et al.* (2000b) have shown that the adherence of *C.b.* to THP-1 monocyte cell line via $\alpha_v\beta_3$ integrin was necessary to trigger the TNF-α production, but that engagement of $\alpha_v\beta_3$ integrin was not sufficient to elicit such a response. An additional signal was provided by the *C.b.* LPS. Futhermore, Zamboni *et al.* (2004) have shown an important role of TLR2 in the induction of cytokines in macrophages with *C.b.* There is a possibility that *C.b.* corpuscles can also bind TLR2 through the lipid A from LPS or other structures, which can synergize with TLR4 in the TNF-α production.

Interestingly, the amount of TNF-α induced with both corpuscles and LPSs of *C.b.* was approximately the same when measured by the bioassay. One might expect higher amounts of TNF-α induced with LPSs compared to those of corpuscles, as LPSs prepared by phenol/water extraction, represent usually up to 10% of the weight of corpuscles (Amano *et al.*, 1987; Ftáček *et al*., 2000). Thus, it appears that the ability of *C.b.* to induce cytokines other than TNF-α, which may synergize in its cytotoxic effect measured by the bioassay, should also be considered. In fact, interferons can sensitize cells to the cytotoxic effect of TNF-α (Tsujimoto and Vilcek, 1986) and *C.b.* corpuscles can induce interferons in mice (Kazár and Schramek, 1984). Thus, also these facts might support the above hypothesis.

In addition, Capo *et al.* (1996b) have evaluated production of proinflammatory cytokines TNF-α, IL-1, and IL-6 in patients with Q fever. Transcription and secretion of TNF-α and IL-1 were significantly higher in the monocytes of the patients with Q fever endocarditis than in those of healthy controls. Moreover, monocytes released significantly higher levels of TNF-α and IL-1 in the patients with recent than with stabilized endocarditis (Capo *et al*., 1996b). Hence, the overproduction of inflammatory cytokines might be involved in the pathophysiology of Q fever.

In conclusion, our attempt to elucidate the pathogenetic behavior of various strains of *C.b.* and their LPSs by comparison of their ability to induce TNF-α showed that there are strain-specific differences in the induction of TNF-α, which could play an important role in the pathogenicity of the strains.

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