Anthrax lethal factor cleaves MKK3 in macrophages and inhibits the LPS/IFNγ-induced release of NO and TNFα

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Abstract The lethal toxin of *Bacillus anthracis* consists of two proteins, PA and LF, which together induce lethal effects in animals and cause macrophage lysis. LF is a zinc-endopeptidase which cleaves two mitogen-activated proten kinase kinases (MAPKKs), Mek1 and Mek2, within the cytosol. Here, we show that also MKK3, another dual-specificity kinase that phosphorylates and activates p38 MAP kinase, is cleaved by LF in macrophages. No direct correlation between LF-induced cell death and cleavage of these MAPKKs was found in macrophage cell lines and primary peritoneal cells exhibiting different sensitivity to LF. However, we present the first evidence that sublytic doses of LF cleave Meks and cause a substantial reduction in the production of NO and tumour necrosis factor-α induced by lipopolysaccharide/interferonγ. We suggest that this effect of LF is relevant during the first stages of *B. anthracis* infection, when a reduction of the inflammatory response would permit growth and diffusion of the bacterium.

Key words: Anthrax; Lethal factor; Metalloprotease; Mitogen-activated protein kinase kinase; Cytokine; Inflammation

1. Introduction

Anthrax is an acute disease of animals and humans caused by the ingestion or inhalation of spores of pathogenic strains of *Bacillus anthracis*. This Gram-positive bacterium produces two primary virulence factors: poly-D-glutamic acid capsule and two toxic complexes composed of three distinct proteins: protective antigen (PA, 83 kDa), oedema factor (EF, 88 kDa) and lethal factor (LF, 89 kDa) [1]. In laboratory animals, intradermal injection of a PA-EF mixture induces oedema which is associated with the adenylate cyclase activity of EF [2,3]. The intravenous injection of PA and LF (Letx) produces rapid lethal effects. In vitro, Letx causes death of macrophages and macrophage cell lines with a few hours, whereas all other types of cells tested appear essentially unaffected, even though they internalise the toxin [4]. There is a large variation in the development of anthrax among laboratory animals, basically due to a different ability to interfere with the mechanism of initial germination of spores and the ensuing bacterial multiplication with production of large amounts of Letx [5–7].

PA binds to an unknown cellular receptor and it is then cleaved by a furin-like surface protease leading to the exposure of a binding site for EF and LF. Their binding to PA triggers a receptor-mediated endocytosis which drives LF or EF inside endosomes [8,9]. There is evidence that LF escapes from the lumen of endosomal compartments into the cytosol [10], where it displays its zinc-endoprotease activity and cytotoxicity, which is inhibited by some membrane-permeant metalloprotease inhibitors [11].

Recently, the mitogen-activated protein kinase kinase (MAPKK) isoforms Mek1 and Mek2 were identified as cytosolic targets of LF activity using two entirely different experimental approaches [12,13]. Mek1 and Mek2 are cleaved by LF at peptide bonds very close to their N-termini. Mek1 and 2 are dual-specificity kinases with a key role in cell growth, proliferation, differentiation and apoptosis [14]. They are key mediators of signal transduction from the cell surface to the nucleus. The signalling pathways involving MAPKK1/2 (Mek1 and Mek2) and MAPKK3 (MKK3) play a crucial role in the activation of macrophages and are directly involved in the production of cytokines, i.e. in the biosynthesis of tumour necrosis factor-α (TNFα), interleukin (IL)-1 and IL-6 [15–17]. Cleavage of MAPKKs by Letx in the N-terminal region is associated with a transient phosphorylation of their substrates ERK1 and ERK2 [13]. These results established LF as a novel tool for the study of cell biology processes and as such it has already been used in probing the role of Meks in Golgi transport [18]. These findings also opened the possibility of linking a defined intracellular biochemical lesion to the pathogenesis of anthrax, as previously done for many bacterial toxins with intracellular targets [19].

Here, we attempted to correlate the LF cleavage of MAPKKs to LF toxicity on macrophages by using macrophage cell lines sensitive or resistant to LF as well as peritoneal macrophages isolated from mouse strains with different sensitivity to anthrax. We examined a number of macrophage activities and we found that LF is capable of cleaving MKK3, an additional MAP kinase kinase isoform. However, no evidence of a direct connection between this N-terminal proteolysis of the MAPKK isoforms 2 and 3 and macrophage cell death was found. We report here that the release of TNFα and NO from macrophages is inhibited by LF and suggest that such inhibition is advantageous to the growth of the bacterium in the initial phase of infection.

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Abbreviations: PLA_2, phospholipase A_2; EF, oedema factor; FCS, foetal calf serum; IFNγ, interferon-γ; IL, interleukin; Letx, lethal toxin; LF, lethal factor; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; PA, protective antigen; PMA, phorbol myristate acetate; TNFα, tumour necrosis factor

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2. Materials and methods

2.1. Reagents

Interferon-γ (IFNγ), phorbol myristate acetate (PMA) and *Escherichia coli* 026:B6 endotoxin (LPS) were purchased from Sigma Chemical Co.

2.2. Cell cultures

Cells were incubated at 37°C, 5% CO_2 in a humidified atmosphere. The murine macrophage cell lines J774.A1 (ATCC TIB 67) and Raw 264.7 (ATCC TIB 71) were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium supplemented with heat-inactivated foetal calf serum (FCS) 10%, 10 μg/ml streptomycin, 10 U/ml penicillin. The MT2 cell line, a kind gift from Prof. P. Ricciardi-Castagnoli (University of Milan), is a murine macrophage cell line generated from the thymus of BALB/cJ strain mice by transformation with a recombinant retrovirus carrying an avian v-*myc* oncogene [20]. MT2 cells exhibit responses typical of primary macrophages when maintained in Iscove medium 5% FCS, 10 μg/ml streptomycin, 10 U/ml penicillin.

Primary macrophages were isolated from the peritoneum of BALB/cJ or DBA/2J or A/J mice. Cells were recovered with 5 ml of RPMI medium, containing FCS 10%, 10 μg/ml streptomycin, 10 U/ml penicillin. 8×10^5 peritoneal cells were seeded in four-well Lab-Tek chamber slides (Nunc) 1 day before the experiment. The slides were washed three times with medium to remove non-adherent cells before treatment with Letx.

2.3. Anthrax lethal factor and cytotoxicity assay

LF was expressed in *E. coli* strain BL21 and purified as described [13]. PA was obtained from the culture supernatant fluids of *Bacillus anthracis* strain RP42 (PA^+ LF^- EF^-) [21] and was purified as previously described [22].

Macrophage cytotoxicity of LF was determined by the method of Hansen et al. [23] with minor modifications. Briefly, cells were harvested by scraping and plated at a density of 8×10^4 in 96-well plates (Falcon, Becton Dickinson), 3.5×10^5 in four-well plates (Nalgene Nunc International) or 8×10^5 in six-well plates (Falcon, Becton Dickinson) 1 day before the experiments. After treatment with Letx and/or other reagents and inhibitors, cells were washed with phosphate-buffered saline (PBS), and tetrazolium salt (MTT, 1 mg/ml in PBS) was added. After 1.5 h incubation, the reaction was stopped by adding the same volume of 20% sodium dodecyl sulphate (SDS), 50% dimethylformamide, pH 4.7. Plates were shaken overnight at room temperature and absorbance was read at 530 nm in a multiwell plate reader.

2.4. Assay of reactive oxygen indermediates and NO production and TNFα secretion

Supernatants of Raw 264.7 and MT2 cell cultures, seeded in 96-well plates and incubated for 14 h with medium or with medium supplemented with LPS 10 ng/ml and IFNγ 0.2 U/ml, were assayed for the presence of TNFα by a specific ELISA test (Genzyme). NO production was determined after conversion into nitrites with the Griess reaction [24].

2.5. MAPKK cleavage

To determine the LF-induced cleavage of Mek2 and MKK3, 3.5×10^5 MT2 or Raw 264.7 cells were plated in four-well microdishes (Nalgene Nunc International). The next day, cells were treated with LF and PA in 0.5 ml of medium. Cells were lysed at different times with 100 μl of Laemmli sample buffer containing 5% (v/v) 2-mercaptoethanol, 10 mM orthophenanthroline and a tablet of inhibitors of proteases (Complete Mini, Roche Molecular Biochemicals) per 10 ml. Peritoneal macrophages were treated with the same amount of toxin for different times and samples were prepared similarly. All samples were boiled for 10 min and 10 μl of each of them was loaded on SDS 10% (w/v) polyacrylamide gels, transferred to Protran nitrocellulose transfer membranes (Schleicher and Schuell) and probed with a rabbit polyclonal antibody against Mek2 (N-20, Santa Cruz Biotechnology) or against MKK3 (C-19, Santa Cruz Biotechnology), both of them diluted 1:1000. Bound primary antibody was revealed with a horseradish peroxidase-conjugated horse anti-rabbit IgG (Calbiochem, 1:2000) and detected by enhanced chemiluminescent substrate Super Signal West Pico (Pierce). Autoradiographs were analysed by Multi analyst Gel Doc 2000 (Bio-Rad). At least 10^5 macrophages have to be loaded on one lane to detect MAPKK cleavage with variations among different experiments within 10%.

3. Results

3.1. MT2 and Raw264.7: two cell lines with different sensitivity to Letx as a model to understand the mechanism of action of LF

Macrophage cell lines differ greatly with respect to the cytotoxic effect of Letx. No differences in receptor binding, proteolytic activation of PA or LF internalisation were detected between resistant and sensitive macrophages [9,25]. In preliminary experiments we found that the MT2 cell line, which exhibits a well defined macrophage phenotype [20], is resistant to the cytotoxic effect of Letx (Fig. 1). MT2 cells were then compared to the Raw 264.7 cell line, which is Letx-sensitive and which is the one most commonly used to study LF cytotoxicity [26,27]. Fig. 1 shows that these two cell lines have a clearly different response to LF, with MT2 being unaffected by 8 h exposure to the toxin, while Raw cells are no longer

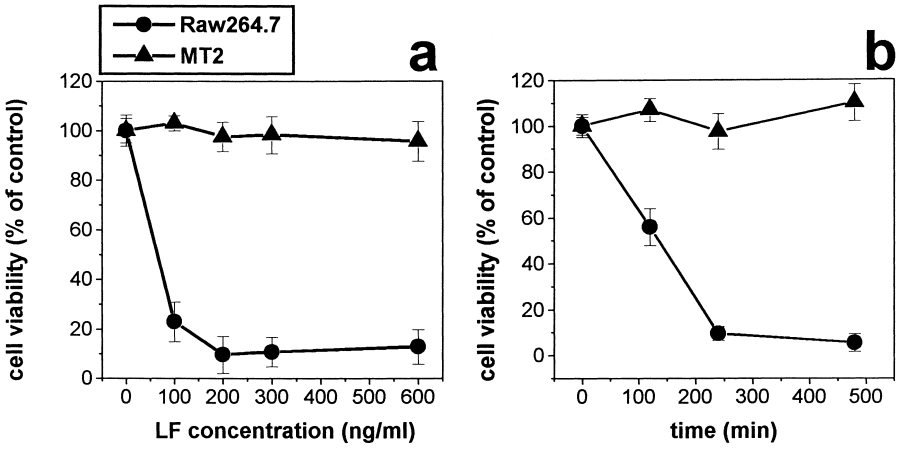

Fig. 1. MT2 and Raw 264.7 macrophage cell lines are differentially sensitive to Letx. a: Cells were plated in 96-well plates as described, and were treated with PA 200 ng/ml at the indicated LF concentrations. After 4 h of incubation, cell death was assayed as described in Section 2. b: Cells were treated with 200 ng/ml of LF and 200 ng/ml of PA for different periods of time up to 4 h and then cell death was determined as in a. Values are expressed as percentage of the control value taken as 100%. The average of three independent experiments performed in duplicate is reported, bars represent S.D.

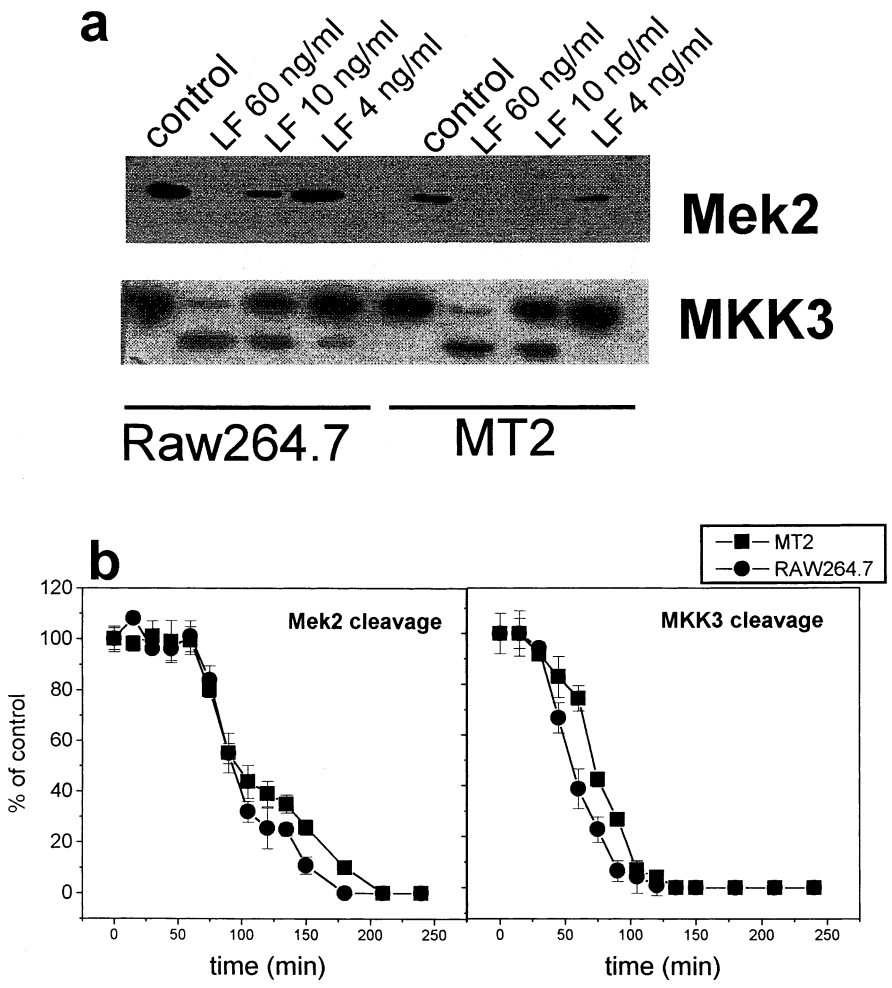

Fig. 2. LF cleaves Mek2 and MKK3 with similar rates in sensitive and non-sensitive cells. a: Western blot showing the cleavage of Mek2 and MKK3 achieved 5 h after the addition of PA (200 ng/ml) plus LF, at the indicated concentrations, to Raw 264.7 and MT2 cells. Cell viability was identical to controls in samples treated with 4 ng/ml or 10 ng/ml of LF, while it was 50% reduced in the presence of 60 ng/ml of LF. b: Extent of cleavage as a function of time of Mek2 and MKK3 in macrophage cell lines, determined by densitometric scanning of western blots, prepared as described in Section 2. Values are expressed as percentage of the control value taken as 100%. Data are the average of three independent experiments performed in duplicate and bars represent S.D.

viable after 2 h, similar to J774.1 cells, another LF-sensitive macrophage cell line [10,11,28,29].

3.2. LF cleaves MAPKK isoform 3

It was recently found that Mek1 and Mek2 are cleaved by LF within their N-termini. Here, using a MKK3-specific antibody, we show that MKK3 is also a good substrate of the LF proteolytic activity (Fig. 2a). Such proteolytic cleavage generates a MKK3 fragment easily distinguishable from the uncleaved form, thus permitting the accurate determination of the kinetics of cleavage.

Unexpectedly, Mek2 and MKK3 are cleaved with similar rates in the two cell lines (Fig. 2b). These results do not allow us to establish a correlation between the isoform of MAPKK cleaved or the kinetics of the proteolytic reaction and the different toxin effect on the two macrophage cell lines.

The opposite fate of Raw264.7 and MT2 exposed to Letx could be related to a difference in the cascade of events following MAPKK activation. In macrophage cells, MAPKKs are involved in the cell signalling pathway leading to the release of cytokines such as TNFα and to other fundamental activities related to the host defence against bacterial invasions. This possibility was evaluated by comparing Raw264.7 and MT2 cells for the production of TNFα, suggested to play a central role in the lethal effects of the toxin in animals [26], and for the production of NO, a central mediator of the vascular and inflammatory response [30] as well as a microbicidal agent [31]. However, the amount of TNFα and NO released upon LPS/IFNγ stimulation is comparable in the two cell lines (Fig. 3).

3.3. Primary cell cultures of peritoneal macrophages with different Letx sensitivity show a similar cleavage rate of Mek2 and MKK3

In order to overcome possible problems related to the use of macrophage cell lines, which may have altered sets of biochemical activities compared to cells in vivo, peritoneal macrophages were isolated from strains of mice characterised by different sensitivities to Letx [5,32]. In particular, A/J and DBA/2J macrophages are Letx-resistant, whereas BALB/cJ macrophages are sensitive to LF. Here, the possible correlation between the LF-induced cleavage of MAPKK proteins

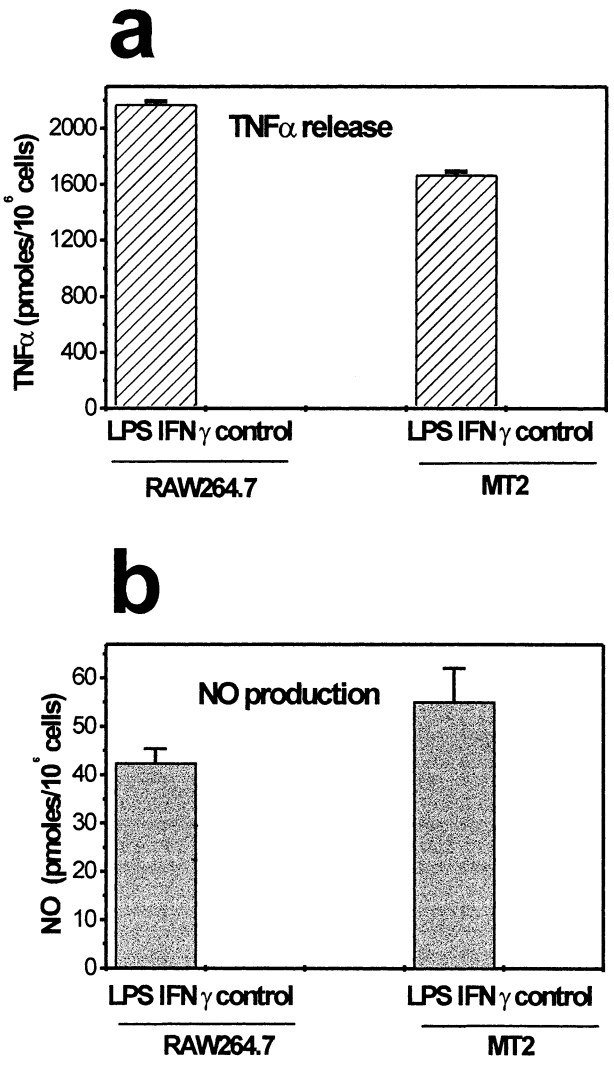

Fig. 3. LPS/IFNγ-induced production of TNFα and NO by macrophage cell lines. Cells were stimulated for 14 h with LPS (10 ng/ml) and IFNγ (0.2 U/ml). Cell culture media were collected and assayed for TNFα (a) and NO (b).

and cell cytotoxicity should become apparent. The result of cell viability tests, reported in the inset of Fig. 4a, confirms the different sensitivity of primary macrophages of A/J, DBA/2J and BALB/cJ mice, exposed for 4 h to Letx. However, both Mek2 and MKK3 are cleaved to the same extent and with very similar kinetics in the macrophages isolated from the three different strains (Fig. 4). These data extend the findings obtained with macrophage cell lines and reinforce the conclusion that there is no evident direct correlation between LF-induced cleavage of the MAPKK isoforms considered here and LF-induced cell cytotoxicity.

3.4. Treatment with sublytic concentration of Letx impairs activation of macrophages induced by LPS/IFNγ

Impairment of MAPK pathways appears to be an excellent strategy for pathogenic microbes because it affects the host inflammation and immune defence thus creating advantageous conditions for the survival and multiplication of bacteria [33]. Letx concentration during the pathogenesis of anthrax is low in the beginning, but increases in parallel with the growth of the toxin-producing organisms. Therefore, we tested the effects of sublytic Letx concentrations, such as those present at the beginning of *B. anthracis* multiplication, on the cleavage of MAPKK proteins. We had found (Fig. 2a) that a consistent cleavage of Mek2 and MKK3 takes place at concentrations of LF (4 and 10 ng/ml) which cause no reduction of cell viability. For comparative reasons, some samples were treated with 60 ng/ml of LF, a concentration which leads to complete proteolysis of the MAPKK isoforms tested here (Fig. 2a) with a concomitant 50% reduction of cell viability (not shown).

Fig. 5 shows that the stimulation of macrophages with LPS/IFNγ has different effects on cells pre-treated with Letx and untreated cells. The production of TNFα and NO is lowered in cells whose Mek proteins are cleaved, suggesting a role for LF in the suppression of the release of TNFα and other proinflammatory cytokines. It is then possible that *B. anthracis* can achieve the enormous bacterial proliferation characteristic of anthrax helped by a preliminary impairment of the host inflammatory response.

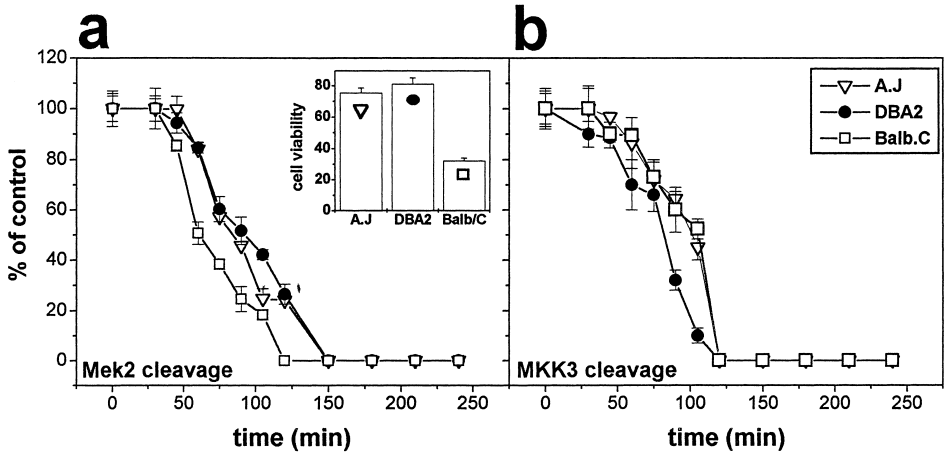

Fig. 4. LF-induced cleavage of Meks in peritoneal macrophages. Peritoneal primary macrophages were plated as described in Section 2 and were treated for the indicated times with 200 ng/ml of LF and 200 ng/ml of PA. The extent of proteolytic cleavage rate of Mek2 (a) and MKK3 (b) and cell viability (inset), after 4 h, were determined as described in Section 2. Values are the average of two experiments run in duplicate and bars represent S.D. values.

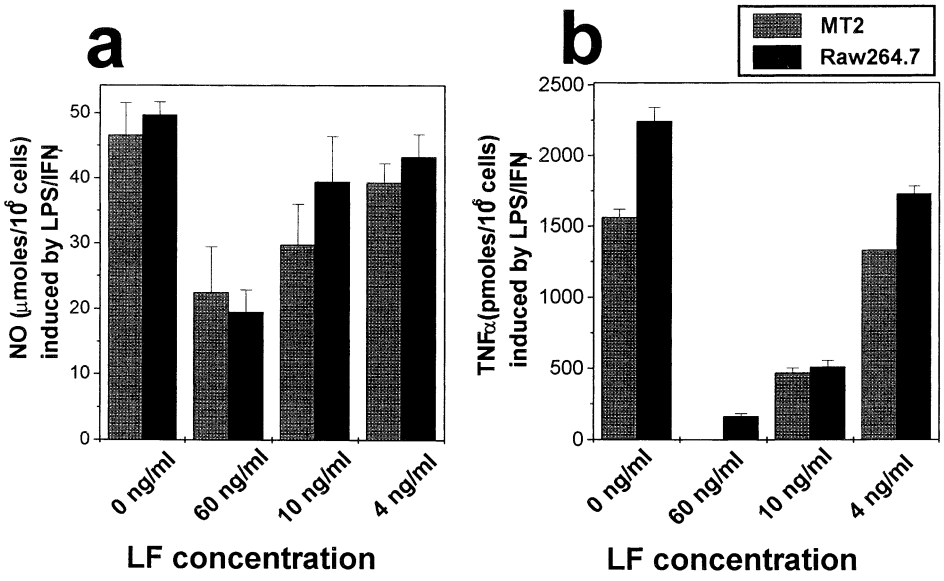

Fig. 5. Effect of sublytic doses of anthrax toxic complex on the LPS/IFNγ-induced production of NO and TNFα. Raw 264.7 and MT2 cells were treated with the indicated amounts of LF together with 200 ng/ml of PA, and then with LPS (10 ng/ml) and IFNγ (0.2 U/ml), and incubated for 14 h before determination of NO (a) and TNFα (b). Undetectable amounts of NO and TNFα were released by untreated cells (not shown). Values are the average of three different experiments run in duplicate and bars represent S.D. values.

4. Discussion

The MAPK cell activation pathway is involved in a variety of cellular processes, which include activation of nuclear genes and of cytosolic activities [14]. Different isoforms of MAPKK play a central role in the cascade of phosphorylation events. LF is a zinc-dependent endopeptidase specific for two MAPKKs, Mek1 and 2, which are cleaved within their N-terminus [12,13]. The intravenous injection of PA plus LF causes the rapid death of animals via haemorrhagic oedema and macrophages play a key role in the process [26]. The challenge is now to correlate the biochemical lesion caused by LF inside cells with the physio-pathological mechanism leading to animal death. It was shown that PA plus LF causes the cytolysis of macrophages in culture [4,25–27]. This death with liberation of pro-inflammatory cytokines, lysosomal hydrolases and other cell necrotic compounds would sustain an acute inflammatory reaction. However, we found that MT2 macrophages are resistant to Letx, and that this resistance cannot be attributed to a lack of cleavage of isoforms 2 and 3 of MAPKKs. In fact, no difference in extent and rate of LF proteolysis of these MAPKKs could be observed between Raw264.7 and MT2 macrophages. Thus, an immediate direct link between the proteolytic cleavage induced by LF in the cytosol and macrophage cell death is not apparent from these experiments. Cell lines may deviate from the original phenotype and macrophage cell lines are no exception in this respect. However, the lack of correlation is reinforced by the present findings that MAPKKs isoforms 2 and 3 are similarly cleaved by Letx in peritoneal macrophages isolated from the anthrax-resistant A/J and DBA/2J mice.

The biochemical link between the MAPKK-specific proteolytic activity of LF and macrophage cell death remains to be established. It was previously found that the LF-induced cytolysis of macrophages requires protein synthesis [34]. Nuclear transcription and MAPKK-induced activation of cytosolic phospholipase A_2 (PLA_2) are not influenced by LF, since LF cytotoxicity in Raw264.7 cells is not affected by the specific inhibitors α-amanitin and $AACOCF_3$ (our unpublished results), which block transcription and cytosolic PLA_2, respectively.

The major finding described here is of potential relevance in the first phase of the development of anthrax, when the number of bacteria is relatively small and, in parallel, the concentration of circulating Letx is sublytic. In macrophage cell lines exposed to sublytic concentrations of Letx, MAPKK isoforms 2 and 3 are cleaved with concomitant reduction in the amount of pro-inflammatory mediators produced in response to stimulation with LPS/IFNγ. One is tempted to speculate that an important role of the Letx toxic anthrax complex is that of reducing the release of pro-inflammatory mediators from macrophages in the very first phases of bacterial proliferation, those which are critical for the determination of the infection outcome. Among them, the germination of spores inside macrophages appears to be the most important one [7]. If, at this stage, the amount of Letx produced is sufficient to prevent release of inflammatory mediators from the infected macrophage, this would greatly increase the chances of an ensuing bacterial proliferation. Later on, the extent of bacterial growth would reach such dimensions that it can no longer be controlled by the inflammatory response, also because the large amount of released LF would extend its inhibitory effect to neutrophils [35–36].

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