Effect of erythromycin on biological activities induced by
*Clostridium perfringens* alpha-toxin

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Inhibitory effect of erythromycin on inflammation

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Nonstandard abbreviations:

ERM: erythromycin
KTM: kitasamycin
TNF: tumor necrosis factor
IL: interleukin
IFN: interferon
MAPK: mitogen-activated protein kinase
ERK: extracellular regulated kinase
TrkA: toropomyosin-related kinase receptor A
ELISA: enzyme-linked immunosorbent assay
PDK1: phosphoinositide-dependent kinase 1
DG: diacylglycerol
PT: pertussis toxin
Abstract

Clostridium perfringens alpha-toxin, an important agent of gas gangrene with inflammatory myopathies, possesses lethal, hemolytic and necrotic activities. Here we show that alpha-toxin-induced lethality in mice was inhibited by intravenous preadministration of erythromycin (ERM). Administration of ERM resulted in a drastic reduction in the release of tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) and systemic hemolysis induced by alpha-toxin, whereas the administration of kitasamycin (KTM) did not. Furthermore, the lethality and systemic hemolysis caused by alpha-toxin were blocked by the preinjection of anti-TNF-α, but not anti-IL-1β- or anti-IL-6-antibody. In addition, TNF-α-deficient mice were resistant to alpha-toxin, indicating that TNF-α plays an important role in the lethality. ERM inhibited the toxin-induced release of TNF-α from neutrophils and phosphorylation of TrkA and ERK1/2. Furthermore, K252a, a TrkA inhibitor, and PD98059, an ERK1/2 inhibitor, inhibited the toxin-induced release of TNF-α from neutrophils. The observation shows that the toxin-induced release of TNF-α is dependent on the activation of ERK/MAPK signal transduction via TrkA in neutrophils, and that ERM specifically blocks the toxin-induced events through the activation of neutrophils.
**Introduction**

*C. perfringens* alpha-toxin has been shown to be a virulence factor required for the development of gas gangrene with inflammatory myopathies (Titball, 1997; Stevens, 2000; Sakurai et al., 2004). Stevens et al. reported that alpha-toxin may cause shock indirectly by stimulating the release of endogenous mediators such as IL-8 and platelet-activating factor (PAF) (Bryant and Stevens, 1996; Stevens, 2000). Cytokines are thought to be a key element in the inflammatory response that characterizes sepsis and septic shock. Cytokines are immunoregulatory peptides with a potent inflammatory action, mediating the immune/metabolic response to an external noxious stimulus and fueling the transition from sepsis to septic shock, multiple organ dysfunction syndromes, and/or multiple organ failure (Tracey et al., 1987; Riedemann et al., 2003; Dinarello, 2004). It is thought that synergistic interactions between cytokines can cause or attenuate tissue injury (Calandra et al., 2002). TNF-α, which is released early from neutrophils and macrophages, is one of the important cytokines involved in the pathophysiology of sepsis (Tracey et al., 1987; Lum et al., 1999). TNF-α-induced tissue injury is largely mediated through neutrophils, that respond by producing elastase, superoxide ion, hydrogen peroxide, sPLA2, PAF, leukotriene B1, and thromboxane A2 (Aldridge, 2002). IL-1 stimulates the synthesis and release of prostaglandins, elastases,
and collagenases and transendothelial microvascular cells, which respond by releasing the powerful neutrophil-stimulating agents, PAF and IL-8 (Leirisalo-Repo, 1994). IL-1 and TNF-α are synergistic and share many biological effects in sepsis (Herbertson et al., 1995). We reported that alpha-toxin triggered events such as superoxide generation and adhesion to the matrix in rabbit neutrophils. Furthermore, we reported that it did so by activating the formation of DG by endogenous PLC via pertussis toxin-sensitive GTP-binding protein and the PI3K-PDK1 pathway via the TrkA receptor (Ochi et al., 2002; Oda et al., 2006). It is therefore possible that the toxin’s actions in vivo are related to the release of cytokines from neutrophils and macrophages.

Macrolide antibiotics including ERM, clarithromycin and azithromycin, are recognized as potent antibiotics for the treatment of various microbial infections (Schmid, 1971). Considerable attention has been paid to the multiple biologic actions, inhibition of neutrophil chemotaxis and oxidative burst and the release of proinflammatory cytokines from monocytes, of macrolide antibiotics (Sato et al., 1998; Khan et al., 1999; Rubin and Tamaoki, 2000). In view of their unique pharmacological actions, macrolide antibiotics may function as an immunomodulator. In fact, low-dose and long-term treatment with these antibiotics has been reported to be effective against diffuse panbronchiolitis, proposed as a clinicopathologic disease entity characterized by
chronic inflammation with inflammatory-cell infiltration (Kadota et al., 1993; Fujii et al., 1995). However, whether macrolide antibiotics can modulate alpha-toxin-induced inflammation has not been investigated. Furthermore, little is known about the mechanism behind the inhibitory effect of macrolide antibiotics on the activation of neutrophils.

In the present study, we investigated the effect of macrolide antibiotics on the toxin-induced activation of neutrophils and death of mice, and the mechanism involved in the toxicity.
Methods

Mice.

BALB/c mice of approximately 6 weeks of age were supplied by Nihon-SLC (Hamamatsu, Japan). TNF-α-deficient mice were from K Sekikawa (National Institute of Animal Health) and K Saito (Gifu University). All animal experiments were approved by the Animal Care Committee and carried out under the guide for care and use of laboratory animals (Tokushima Bunri University and Aichi Medical University).

Macrolide antibiotics.

The macrolide antibiotics (injectable agents) were purchased commercially: ERM from Abbott (Tokyo, Japan) and KTM from Asahi Kasei (Tokyo, Japan). Both agents were dissolved in distilled water.

Other drugs

TrkA inhibitor (K252a) and 2’-amino-3’-methoxyflavone (PD98059) were purchased from Merck-Calbiochem, Japan. Purtussis toxin (PT) were from Seikagaku Bio Business Co., Japan. Anti-phospho-TrkA antibody and anti-phospho-ERK1/2 antibody were obtained from Cell Signal Technology. All other drugs were analytical grade.

Purification of wild-type alpha-toxin.
A recombinant form of the plasmid pHY300PLK harboring the structural gene of wild-type alpha-toxin was introduced into *Bacillus subtilis* ISW1214 by transformation. Transformants were grown in Luria-Bertani broth containing 50 μg of ampicillin/ml to an OD$_{600}$ of 0.8-0.85. Purification of wild-type toxin was performed as described previously (Nagahama et al., 1995). The toxin preparation contained no detectable endotoxin (less than 0.03 endotoxin units per ml) as determined by a Limulus amebocyte lysate assay (Sato et al., 1998).

**Preparation of mouse neutrophils.**

Mouse neutrophils were prepared from peripheral blood of BALB/c mice by using Lymphoprep (New England Nuclear) gradient centrifugation (280 x g for 20 min) (Ochi et al., 2002). The neutrophils were of high purity (>90%) and viability (>95%).

**Alpha-toxin challenge experiments in macrolide antibiotic-treated mice**

BALB/c mice aged 6 weeks old were used. They were purchased from Nihon-SLC, Co. (Hamamatsu, Japan) and fed a standard diet and water. The animals were randomly divided into 5 groups (10 mice each); four macrolide antibiotic-injected groups and one saline-injected group. The mice were injected intravenously with ERM and KTM (0.1 and 0.5 mg/mouse, respectively) every 24 h for 5 days. They were then injected with 20 ng of alpha-toxin/mouse. The dose of alpha-toxin was chosen based on a mortality of
>80% at 10 to 12 h post-injection. Approximately 0.1 ml of ERM or KTM solution was administered intravenously into the tail to achieve a dose of 0.1 and 0.5 mg/mouse. Untreated mice received approximately 0.1 ml of sterile saline. The survival of the mice was monitored every hour after alpha-toxin was injected.

**Determination of cytokines**

The concentrations of TNF-α, IL-1β, IL-6, IFN-γ, IL-4 and IL-10 were determined with enzyme-linked absorbent assay (ELISA) kits for these cytokines (R&D systems, Minneapolis, MN, USA).

**Measurement of superoxide in mouse neutrophils.**

The generation of superoxide was evaluated by the 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo(1,2-a)pyrazin-3-one (MCLA)-dependent chemiluminescence method. Neutrophils (1 x 10⁶ cells/ml) were prepared from mice treated with ERM or KTM every 24 h for 5 days. They were incubated with alpha-toxin at 37°C in a final volume of 0.2 ml of HBSS containing 0.3 mM CaCl₂ and 1.25 µM MCLA. The chemiluminescence was measured with a chemiluminescence reader (Ochi et al., 2002).

**Determination of hemolytic activity**

The serum from mice treated with alpha-toxin was diluted 10-fold-diluted with
HBS. The level of hemoglobin in the solution was analyzed spectrophotometrically at A550.

**Detection of phosphorylation of various proteins induced by alpha-toxin**

Mouse neutrophils were incubated with alpha-toxin at 37°C for 10 min in HBS containing 0.3 mM CaCl2, 1 mM MgCl2 and 0.1 mL Na3VO4. The reaction was terminated by the addition of 0.5 mL of ice-cold 7.5% trichloroacetic acid and kept on ice for 30 min. The precipitate was collected by centrifugation at 10,000 rpm for 20 min. Phosphorylated proteins were electrophoresed by SDS-PAGE and then transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Inc). The membranes were incubated with 5% [w/v] nonfat milk in TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% [v/v] Tween 20) and probed with specific rabbit monoclonal antibodies against various phosphorylated proteins and unphosphorylated proteins. Detection was conducted using an enhanced chemiluminescence (ECL) kit (GE healthcare). A quantitative analysis of bands was performed using densitometry (LAS-4000, Fujifilm).

**Statistical analysis.**

All values are expressed as means ± SEM. Student’s unpaired t-test and a one-way ANOVA were used for statistical analyses. P < 0.05 was considered
statistically significant.
Results

Effect of macrolide antibiotics on lethality induced by alpha-toxin in mice.

The effect of macrolide antibiotics on alpha-toxin-induced lethality was examined in mice. The animals were injected with various concentrations of ERM or KTM every 24 h for 5 days and then intravenously with 20 ng of alpha-toxin/mouse (Fig. 1). Alpha-toxin-injected mice began to die after about 8 h, and all mice died within 11 h of the administration (Fig. 1). ERM-preadministered mice survived for about 10 h after the injection of alpha-toxin. The survival rate 24 h after the administration (0.1 or 0.5 mg of ERM/mouse) of the toxin was about 30 and 80%, respectively, showing that ERM inhibited the toxin-induced lethality in a dose-dependent manner. The preadministration of KTM had little effect on the toxin-induced lethality.

Effect of macrolide antibiotics on release of cytokines induced by alpha-toxin in mice.

The overproduction of inflammatory cytokines induced by shiga toxin (van Setten et al., 1996; Yoshimura et al., 1997) and lipopolysaccharide (Palsson-McDermott and O'Neill, 2004) leads to a cytokine storm, damaging various cells and tissues. To investigate the relationship between alpha-toxin-induced production of cytokines and lethality, the toxin-induced release of cytokines in the serum of mouse was measured.
We measured the release of these cytokines induced by the toxin in mice preinjected with various concentrations of ERM or KTM every 24 h for 5 days. Alpha-toxin induced the release of TNF-α, IL-1β, IL-6, IL-10, IFN-γ and IL-2, as shown in Fig. 2. In mice preinjected with ERM, levels of proinflammatory cytokines, TNF-α, IL-1β and IL-6, in serum were markedly decreased, compared with control values (Fig. 2), whereas the toxin-induced release of the T-helper type 1 cytokines, IFN-γ and IL-2, and the T-helper type 2 cytokine, IL-10, increased about two fold compared to that in mice preinjected with ERM (Fig. 2). In mice preinjected with KTM, the release of these cytokines was the same as that in the control mice. In addition, the administration of ERM or KTM alone had no effect on the release of TNF-α, IL-1β, IL-6, IL-2, IFN-γ and IL-10 (data not shown).

**Effect of macrolide antibiotics on hemolysis induced by alpha-toxin in mice.**

Intravenous injection of alpha-toxin into mice resulted in massive intravascular hemolysis (Sugahara and Osaka, 1970; Kreidl et al., 2002). To investigate the effect of ERM and KTM on the intravascular hemolysis induced by alpha-toxin, mice preinjected with ERM and KTM were intravenously administered with 20 ng of alpha-toxin per mouse. The alpha-toxin-induced hemolysis in vivo markedly decreased in ERM-injected mice, compared with controls, but not in KTM-injected mice (Fig. 3A).
Next, erythrocytes of mice in vitro were treated with a sub-hemolytic dose of alpha-toxin (20 ng/ml) at 37°C for 30 min, and then incubated with various concentrations of TNF-α, IL-1β or IL-6 for 60 min. TNF-α enhanced the toxin-induced hemolysis of mouse erythrocytes in a dose-dependent manner, but IL-1β and IL-6 did not (Fig. 3B).

The effect of proinflammatory cytokines on alpha-toxin-induced lethality

Mice were intravenously injected with 20 ng of alpha-toxin/mouse 2 h after the administration of 50 μg of anti-TNF-α, IL-1β or IL-6 antiserum/mouse (Fig. 4). Untreated mice began to die within 10 h after the administration of the toxin and all mice died within 15 h. The survival rate of anti-TNF-α antibody-preinjected mice was about 80% within 15 h post-administration of the toxin (Fig. 4). The anti-IL-1β and anti-IL-6 antibodies had little effect on the lethality.

The participation of TNF-α in the toxin-induced lethality was examined in TNF-α-deficient mice (Fig. 5). The administration of alpha-toxin killed all of the wild-type mice (B10.D2) within 12 h. The survival rate of the TNF-α-deficient mice was 100% and 75% within 12 and 72 h, respectively, post-administration (Fig. 5). The result was consistent with that obtained by injection of anti-TNF-α antibody in mice.

The effect of ERM and KTM on alpha-toxin-induced release of TNF-α from
neutrophils.

To examine whether alpha-toxin stimulates the release of TNF-α from neutrophils, the toxin was incubated with the cells at 37°C for 3 h. Alpha-toxin induced the release of TNF-α in a dose-dependent manner (data not shown). We investigated the effect of ERM and KTM on the release. Neutrophils were isolated from mice preinjected with ERM or KTM every 24 hr for 5 days. The release induced by the toxin from neutrophils of ERM-treated mice was about 20% of that from neutrophils of untreated mice (Fig. 6A). With KTM, little significant inhibition was observed (Fig. 6A). It therefore appears that ERM inhibits the toxin-induced release of TNF-α from neutrophils. Furthermore, the toxin-induced release of TNF-α from macrophages of ERM-treated mice was significantly low, compared with that from macrophages of untreated mice (data not shown).

Effect of macrolide antibiotics on superoxide generation induced by alpha-toxin.

We reported that alpha-toxin stimulates the generation of superoxide in rabbit neutrophils (Ochi et al., 2002; Oda et al., 2006). The effect of ERM on the toxin-induced superoxide generation was examined. Preinjection of mice with ERM (0.5 mg/mouse) significantly attenuated the alpha-toxin-stimulated generation of superoxide in neutrophils, compared with that in the toxin-treated neutrophils of
untreated mice (Fig. 6B), but that with KTM did not (Fig. 6B).

We reported that alpha-toxin induced the generation of superoxide through an ERK1/2-associated signaling event stimulated by PDK1’s activation via Toropomyosin-related kinase receptor A (TrkA) and the production of DG via pertussis toxin (PT) sensitive GTP-binding protein (Oda et al., 2006). The effect of ERM and KTM on the alpha-toxin-induced phosphorylation of TrkA and ERK1/2 and formation of diacylglycerol (DG) was investigated. The phosphorylation of both TrkA and ERK1/2 was markedly reduced in the neutrophils prepared from mice treated with ERM (0.5 mg/mouse), but not with KTM (Fig. 6C). On the other hand, ERM and KTM had no effect on the production of DG induced by alpha-toxin in neutrophils (Fig. 6D), whereas PT inhibited it.

The effect of TrkA and ERK1/2 inhibitors and anti-TrkA antibody on release of TNF-α from neutrophils induced by alpha-toxin.

To assess the involvement of TrkA and ERK1/2 in the release of TNF-α induced by alpha-toxin, we investigated if K252a, a TrkA inhibitor, and PD98059, an ERK1/2 inhibitor, affect the release. As shown in Fig. 7, treatment of the neutrophils with K252a or PD98059 significantly inhibited the release of TNF-α induced by alpha-toxin. PT also inhibited the release (Fig. 7). Moreover, the alpha-toxin-induced release of TNF-α
was attenuated by pretreatment with the anti-TrkA antibody, but not control IgG (Fig. 8).

Therefore, it appears that the release of TNF-α from neutrophils treated with the toxin is closely related to the activation of TrkA and PT-sensitive GTP-binding protein.
Discussion

The present study demonstrated that ERM, a 14-ring macrolide antibiotic, has inhibitory effects on events triggered by alpha-toxin, death and in vivo hemolysis, in mice. Preadministration of ERM to mice resulted in prevention of the release of inflammatory cytokines (TNF-α and IL-1β) and lethality caused by the toxin. Anti-TNF-α antibody inhibited the death of mice induced by alpha-toxin. Furthermore, TNF-α-deficient mice were resistant to alpha-toxin. These observations suggest that the lethal effect of alpha-toxin is closely related to the release of TNF-α into the bloodstream. Stevens et al. and Bunting et al. suggested that alpha-toxin contributes indirectly to shock by stimulating production of endogenous mediators such as TNF-α and platelet-activating factor (Bunting et al., 1997; Stevens and Bryant, 1997). It therefore appears that TNF-α released by alpha-toxin is important in enhancing the toxic actions of alpha-toxin in vivo. However, TNF-α in the range of concentrations found in mice treated with alpha-toxin did not induce lethality. Therefore, it appears that TNF-α alone does not participate in lethality induced by alpha-toxin under our experimental condition. Inhibitors for release and expression of TNF-α may be worth pursuing as a novel therapeutic approach to the treatment of gas gangrene and sepsis caused by C. perfringens. On the other hand, preadministration of ERM to mice resulted in increases
in T-helper type 1 (IFN-γ and IL-2) and T-helper type 2 (IL-4) cytokines. Increases in IFN-γ, IL-2 (Tzianabos et al., 1999) and IL-4 (D'Andrea et al., 1995) have been reported to have an inhibitory effect on sepsis. Therefore, it is possible that the change in the release of these cytokines induced by ERM contributes to improved survival.

We have reported the mechanism of hemolysis of rabbit, horse and sheep erythrocytes induced by alpha-toxin in vitro (Sakurai et al., 1994; Ochi et al., 1996; Ochi et al., 2004; Oda et al., 2008). However, little is known about the mechanism of massive hemolysis induced by the toxin in vivo. ERM simultaneously inhibited the release of TNF-α and systemic hemolysis induced by alpha-toxin. Moreover, TNF-α dose-dependently enhanced the hemolysis induced by the sub-hemolytic dose of alpha-toxin in vitro. The result shows that TNF-α released by alpha-toxin plays an important role in systemic hemolysis, perhaps inflammation in gas gangrene caused by C. perfringens.

Several papers have reported that 14- and 15-member-macrolide antibiotics attenuated the activation of neutrophils induced by various inflammatory stimuli (Kanai et al., 2004; Tsai et al., 2004; Simpson et al., 2008). We found that alpha-toxin induced the generation of superoxide through activation of an ERK/MAPK signaling cascade by phosphorylation of PKCθ via the TrkA receptor and formation of DG via PT-sensitive
Gi in rabbit neutrophils (Oda et al., 2006). ERM simultaneously inhibited the toxin-induced production of superoxide from neutrophils, release of TNF-α, and phosphorylation of TrkA and ERK1/2, but KTM did not. On the other hand, treatment with ERM had no effect on the formation of DG via PT-sensitive Gi in rabbit neutrophils treated with alpha-toxin. Moreover, K252a and PD98059 inhibited the release of TNF-α from neutrophils induced by alpha-toxin. These observations provided evidence that inhibition of the toxin-induced phosphorylation of TrkA receptor by ERM results in suppression of activation of neutrophils, superoxide formation and TNF-α release. This is the first report of the mechanism for inhibition of neutrophils by ERM.

It has been reported that ERM, clarythromycin and roxithromycin inhibit the generation of oxidants by stimulus-activated neutrophils (Anderson, 1989; Hand et al., 1990). A 15-member-macrolide antibiotic also inhibited the generation of superoxide induced by alpha-toxin in rabbit neutrophils (data not shown). It therefore appears that 14- and 15-member-macrolide antibiotics are effective in the treatment of infectious diseases caused by *C. perfringens*.

In conclusion, preadministration of ERM leads to inhibitory effects on the alpha-toxin-induced release of TNF-α, and death, as a consequence of inhibition of the
phosphorylation of the TrkA receptor. The phosphorylation of ERK1/2 via the TrkA receptor activated by alpha-toxin in neutrophils is linked to the release of TNF-α. Furthermore, the cytokine storm induced by alpha-toxin, mainly the release of TNF-α, plays an important role in the death and massive hemolysis of mice treated with the toxin.
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References


Riedemann NC, Guo RF and Ward PA (2003) Novel strategies for the treatment of


Footnotes

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Legends for Figures

Fig. 1. Effect of macrolide antibiotics on lethality induced by alpha-toxin.

Mice received various concentrations of ERM or KTM for 5 days every 24 h, and were then injected intravenously with 20 ng of alpha-toxin. Survival of the mice was monitored every 30 min after the injection of the toxin. A typical result from one of five experiments is shown \((n = 10-12)\). *, \(P < 0.05\); **, \(P < 0.01\), compared with the lethality induced by alpha-toxin alone. Symbols: control, ●; Saline + alpha-toxin, ■; ERM (0.1 mg/mouse) + alpha-toxin, ▲; ERM (0.5 mg/mouse) + alpha-toxin, ◆; KTM (0.1 mg/mouse) + alpha-toxin, □; KTM (0.5 mg/mouse) + alpha-toxin, ○.

Fig. 2. Effect of macrolide antibiotics on release of various cytokines induced by alpha-toxin in the serum of mice.

Mice received ERM or KTM for 5 days every 24 h and were injected intravenously with 20 ng of alpha-toxin. The release of cytokines (TNF-\(\alpha\), IL-1\(\beta\), IL-6, IL-10, IFN-\(\gamma\) and IL-2) in the serum was assayed with ELISA kits. Values are mean \(±\) s.e.m. \((n = 5)\). *, \(P < 0.05\); **, \(P < 0.01\), compared with alpha-toxin alone. Symbols: control, ○; Saline + alpha-toxin, ■; ERM (0.5 mg/mouse) + alpha-toxin, ▲; KTM (0.5 mg/mouse) + alpha-toxin, □.

Fig. 3. Intravascular hemolysis induced by alpha-toxin in mice pretreated with
macrolide antibiotics

A) Mice received various concentrations of ERM or KTM for 5 days every 24 h, and were then injected intravenously with 20 ng of alpha-toxin. The amount of hemoglobin in the serum of peripheral blood was measured spectrophotometrically at A550. Values are mean ± s.e.m. (n = 5). Symbols: control, ○; Saline + alpha-toxin, ■; ERM (0.5 mg/mouse) + alpha-toxin; ▲; KTM (0.5 mg/mouse) + alpha-toxin, □. B) The washed erythrocytes (1.0 x 10^10cells/ml) of mice were incubated with 20 ng/mL of alpha-toxin at 37°C for 30 min, and then treated with various concentrations of cytokines at 37°C for 60 min. Values are mean ± s.e.m. (n = 5). *, P < 0.01, compared with the hemolysis induced by alpha-toxin alone. Symbols: TNF-α, ●; IL-1β, △; IL-6; ▲.

Fig. 4. Effect of anti-TNF-α, -IL-1β and -IL-6 antibodies on lethality induced by alpha-toxin

Mice received 50 μg of anti-TNF-α, anti-IL-1β or anti-IL-6 antibodies, and after 2 h, the treated mice were injected intravenously with 20 ng of alpha-toxin. Survival of the mice was monitored every 30 min after the injection of the toxin. A typical result from one of five experiments is shown (n = 3). *, P < 0.01, compared with the lethality induced by alpha-toxin alone. Symbols: control, ●; Saline + alpha-toxin, ■; anti-TNF-α antibody + alpha-toxin, △; anti-IL-1β antibody + alpha-toxin, ◇;
anti-IL-6 antibody + alpha-toxin, □.

**Fig. 5. Comparison of lethality induced by alpha-toxin in wild-type mice with that in TNF-α knockout mice.**

Mice were injected intravenously with 20 ng of alpha-toxin. Survival of the mice was monitored at indicated times after the injection of the toxin. Values are mean ± s.e.m. (n = 6). *, P < 0.01, compared with the lethality induced by alpha-toxin alone.

Symbols: control mouse (B10D2), ●; TNF-α knock out mouse, ○.

**Fig. 6. Involvement of TrkA-mediated signal transduction in mouse neutrophils treated with alpha-toxin**

Neutrophils (1 x 10^7 cells) from the mice treated with ERM or KTM were incubated with 100 ng/ml of alpha-toxin. A) The neutrophils (10^7 cells/ml) were incubated with 100 ng of alpha-toxin at 37°C for 3 h. The release of TNF-α was assayed with an ELISA kit. Values are mean ± s.e.m. (n = 5). *, p < 0.01. B) Production of superoxide was monitored for 15 min based on MCLA-chemiluminescence. The integrated data (area under the curve) are plotted. Superoxide production induced by alpha-toxin alone was set as the maximal response (100%). Values are mean ± s.e.m. (n = 5). ***, p < 0.05. C) The neutrophils were incubated with 100 ng/ml of alpha-toxin at 37°C for 10 min, and subjected to SDS-PAGE and Western blotting using specific antibody. Values
are mean ± s.e.m. (n = 5). D) The neutrophils were incubated with 100 ng/ml of alpha-toxin at 37°C for 10 min, and the intracellular DG was measured. Values are mean ± s.e.m. (n = 5). ***, p < 0.01.

**Fig. 7. Effect of various inhibitors on release of TNF-α induced by alpha-toxin in mouse neutrophils**

Mouse neutrophils (10⁷ cells/ml) were pretreated with 30 nM K252a (60 min), 10 μM PD98059 (60 min) or 10 μg/ml PT (120 min) for the periods indicated, and incubated with 100 ng/ml of alpha-toxin at 37°C for 3 h. The release of TNF-α was assayed with an ELISA kit. Values are mean ± s.e.m. (n = 5). *P < 0.01

**Fig. 8. Effect of anti-TrkA antibody on alpha-toxin-induced production of TNF-α in mouse neutrophils**

Mouse neutrophils (10⁶ cells/ml) were pretreated with anti-TrkA anti-body (1 μg/ml) and control rabbit IgG (1 μg/ml) for 60 min, and incubated with or without 100 ng/ml of alpha-toxin at 37°C for 3 h. The release of TNF- α was measured with an ELISA kit. Values are mean ± s.e.m. (n = 5). *P < 0.01.
Figure 1

Graph showing survival (%) over time after injection of toxin (h) for different groups with various symbols and annotations.
Figure 2

(a) TNF-α (pg/ml) vs. Time after injection of toxin (h)
(b) IL-1β (pg/ml) vs. Time after injection of toxin (h)
(c) IL-6 (pg/ml) vs. Time after injection of toxin (h)
(d) IL-10 (pg/ml) vs. Time after injection of toxin (h)
(e) IL-2 (pg/ml) vs. Time after injection of toxin (h)
(f) IFN-γ (pg/ml) vs. Time after injection of toxin (h)
Figure 8

The graph shows the levels of TNF-α (pg/ml) in response to different treatments. The y-axis represents the concentration of TNF-α, ranging from 0 to 1200 pg/ml. The x-axis indicates the type of treatment: Control rabbit IgG and Anti-TrkA.

- The control group treated with rabbit IgG shows a low level of TNF-α, close to 0 pg/ml.
- The group treated with Anti-TrkA has a significantly higher level of TNF-α, reaching up to 1200 pg/ml.

An asterisk (*) indicates a statistically significant difference compared to the control group.