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# Effect of Macrolide Antibiotics on Biological Activities Induced by *Clostridium perfringens* Alpha-Toxin

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## 1. Introduction

*Clostridium perfringens* type A (*C. perfringens*) causes gas gangrene with inflammatory myopathies and infrequently septicemia associated with massive intravascular hemolysis. The gas gangrene involves any part of the body; the most common sites being the toes, fingers, feet, and hands. Features include localized pain, swelling and myonecrosis, and finally, shock and death. The septicemia with massive hemolysis and fever leads to death within a few hours.

The microorganism is known to produce a variety of toxins and enzymes that are responsible for severe myonecrotic lesions. Notably, alpha-toxin, which possesses hemolytic, necrotic and lethal activities, and phospholipase C (PLC) and sphingomyelinase (SMase) activities, is an important agent for the diseases (Bryant et al. 2000, Sakurai, Nagahama and Oda 2004). It has been reported that the toxin is required for myonecrosis, hemolysis, inhibition of neutrophil infiltration and thrombosis (Stevens and Bryant 1997, Awad et al. 2001, Ellemor et al. 1999). We also reported that the toxin stimulated  $O_2^-$  production in neutrophils, and firm adhesion of the cells to matrix ligands, fibrinogen, fibronectin, and collagen (Ochi et al. 2002), suggesting that the toxin stimulates the binding of neutrophils to the vascular endothelium and inflammation there. The findings suggest that neutrophils activated by the toxin are unable to migrate across the vascular endothelium in an infectious focus. Actually, gas gangrene caused by *C. perfringens* is reported to be a fulminant necrotizing infection in which inflammatory cells are notably absent from infected tissues, but are often accumulated in vascular wall (Bunting et al. 1997).

Stevens et al. reported that alpha-toxin may cause shock indirectly by stimulating the release of endogenous mediators (Bryant and Stevens 1996, Stevens 2000). We found that the intravenous injection of alpha-toxin in mice resulted in the release of various cytokines (Oda et al. 2008). Cytokines are immunoregulatory peptides with a strong inflammatory action, mediating the immune/metabolic response to an external noxious stimulus and later the transition from septicemia to septic shock, multiple organ dysfunction syndromes, and/or multiple organ failure (Tracey et al. 1987, Riedemann, Guo and Ward 2003, Dinarello 2004). It is thought that synergistic interactions between cytokines can cause or attenuate tissue injury (Calandra, Bochud and Heumann 2002). Tumor necrosis factor (TNF)- $\alpha$ , released from neutrophils, macrophages, and endothelial cells, is an important cytokine involved in

the pathophysiology of septicemia (Tracey et al. 1987, Lum et al. 1999). TNF- $\alpha$ -induced tissue injury is largely mediated through neutrophils which respond by producing elastase, superoxide ion, hydrogen peroxide, phospholipase A, platelet-activating factor, leukotriene B<sub>1</sub>, and thromboxane A<sub>2</sub> (Aldridge 2002). Therefore, it is possible that the exacerbation of gas gangrene with inflammatory symptom and septicemia with massive intravascular hemolysis caused by *C. perfringens* is dependent on cytokines released from neutrophils and macrophages activated by the toxin.

Macrolide antibiotics, including erythromycin (ERM), azithromycin (AZM), clarithromycin and kitasamycin (KTM), are recognized as potent antibiotics for the treatment of various microbial infections (Schmid 1971). Some of these antibiotics have been reported to be effective against diffuse panbronchiolitis characterized by chronic inflammation with inflammatory cell infiltration (Kadota et al. 1993, Fujii et al. 1995). Thereafter, macrolides were shown to exert immunomodulatory effects on a wide range of cells; epithelial cells, macrophages, monocytes, eosinophils, neutrophils, and lymphocytes. The 14- and 15-membered macrolides are known to lead to suppression of neutrophil chemotaxis and oxidative burst, and inhibition of the release of proinflammatory cytokines from monocytes (Sato et al. 1998, Khan et al. 1999, Rubin and Tamaoki 2000). From the point of view of their unique pharmacological actions, recently we revealed that these macrolides inhibited alpha-toxin-induced events in vivo and in vitro. In this review, we show the mechanism for the action of macrolides on the biological activities of the toxin.

## 2. Characterization of alpha-toxin

The genes encoding alpha-toxin (Titball et al. 1989), *Bacillus cereus* PLC (BC-PLC) (Gilmore et al. 1989), and PLCs from *C. bifermentans* (Tso and Siebel 1989) and *Listeria monocytogenes* (Vazquez-Boland et al. 1992) have been isolated and their nucleotide sequences were determined. (Titball 1993). It therefore was found that the deduced amino acid sequences of alpha-toxin and these enzymes exhibit significant homology up to approximately 250 residues from the N-terminus. Therefore, the findings show that alpha-toxin (370 amino acid residues) belongs to the PLC family. BC-PLC has two tightly bound and one loosely bound zinc ions (Hough et al. 1989, Vallee and Auld 1993). Crystallographic and site-directed mutagenesis analysis of alpha-toxin revealed that one zinc ion is tightly coordinated with His-11 and Asp-130, a second is coordinated tightly with His-148 and loosely with Glu-152, and a divalent cation is loosely associated with His-68, -126, -136 and Asp-130 (Nagahama et al. 1995, Nagahama et al. 1996b) and that Asp-56 is essential for catalytic activity (Nagahama et al. 1997), indicating that the catalytic site of the toxin is located in the N-terminal domain. Furthermore, the crystallographic study indicated that the structure is divided into two domains (Naylor et al. 1998): the N-domain (250 residues), consisting of nine tightly packed  $\alpha$ -helices, and the C-domain (120 residues), consisting of an eight-stranded anti-parallel  $\beta$ -sandwich motif. It was confirmed that the N-domain has a structural topology similar to the entire BC-PLC (Hough et al. 1989) and three divalent cations containing zinc ions in the active site, and that amino acid residues involved in zinc-coordination are essential for the enzymatic activities. We reported that mixing the individual N-domain and C-domain restored the hemolytic activity (Nagahama et al. 2002), suggesting that the C-domain affects the activity of the N-domain. Guillouard *et al.* (Guillouard et al. 1997) and Naylor *et al.* (Naylor et al. 1998) reported that the fold of the C-domain is similar to those of the "C2" and "C2-like" domains, present in eukaryotic proteins involved in signal transduction, of

eukaryotic phospholipid-binding proteins such as synaptotagmin. Guillouard *et al.* (Guillouard *et al.* 1997) reported that the C-domain of alpha-toxin mediates interactions with membrane phospholipids in a calcium-dependent manner. Furthermore, Alape-Giron *et al.* (Alape-Giron *et al.* 2000) reported that Tyr-275, -307 and -331 residues are critical for binding of the toxin. We also reported that acrylodan-labeled C-domain variants (S263C and S365C) bound to liposomes and internalized into the hydrophobic environment in liposomes (Nagahama *et al.* 2002, Nagahama, Michiue and Sakurai 1996a). It therefore appears that the C-domain plays an important role in binding to membranes. From these observations, it is apparent that alpha-toxin consists of two distinct modules: the N-domain catalyses phospholipid hydrolysis in membranes and the C-domain binds to and inserts into membranes (Fig. 1)

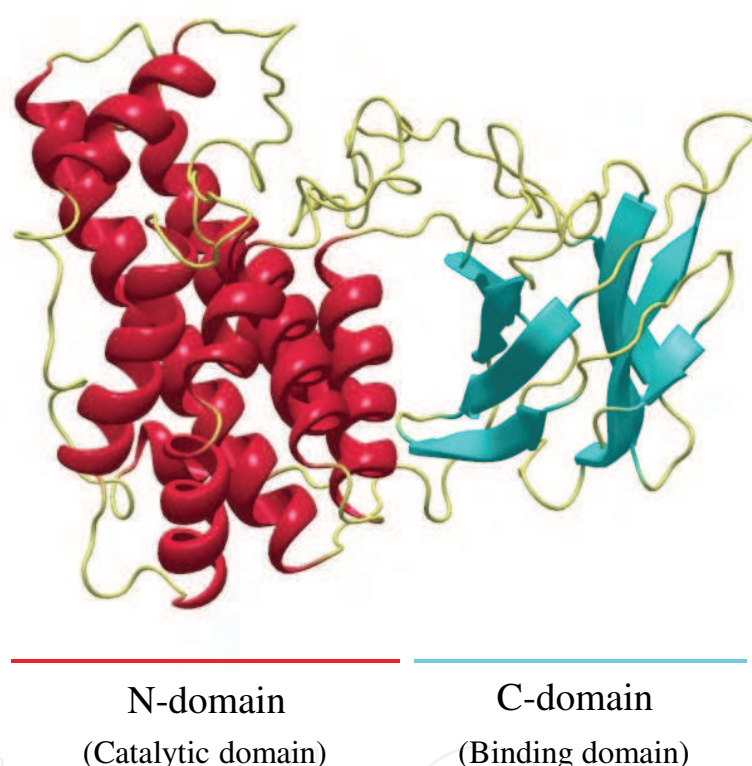


Fig. 1. Structure of *Clostridium perfringens* alpha-toxin

### 3. The action of *Clostridium perfringens* alpha-toxin

#### 3.1 The effect of pro-inflammatory cytokines on alpha-toxin-induced death

Cytokines are small proteins involved in key events of the inflammatory process. Beutler *et al.* reported that neutralization of TNF- $\alpha$  released by the intravenous injection of a lethal dose of lipopolysaccharide (LPS) prevented death in mice (Beutler, Milsark and Cerami 1985). Later, Tracey *et al.* demonstrated that a monoclonal anti-TNF- $\alpha$  antibody protected baboons against sepsis elicited by *Escherichia coli* (Tracey *et al.* 1987). It has been reported that the overproduction of inflammatory cytokines induced by shiga toxin (van Setten *et al.* 1996, Yoshimura *et al.* 1997) and LPS (Palsson-McDermott and O'Neill 2004) led to a cytokine storm, damaging various cells and tissues. Stevens *et al.* and Bunting *et al.* suggested that alpha-toxin contributed to shock by stimulating production of endogenous

mediators such as TNF- $\alpha$  and platelet-activating factor (Bunting et al. 1997, Stevens and Bryant 1997). Fig. 2 shows that intravenous injection of alpha-toxin in mice resulted in the release of TNF- $\alpha$ , interleukin (IL)-1 $\beta$ , IL-6, IL-10, IFN- $\gamma$ , and IL-2 in serum (Fig. 2), and eventually death. TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 are known to be important elements in the inflammatory response. Therefore, it is possible that the lethality of alpha-toxin is associated with the release of these inflammatory cytokines.

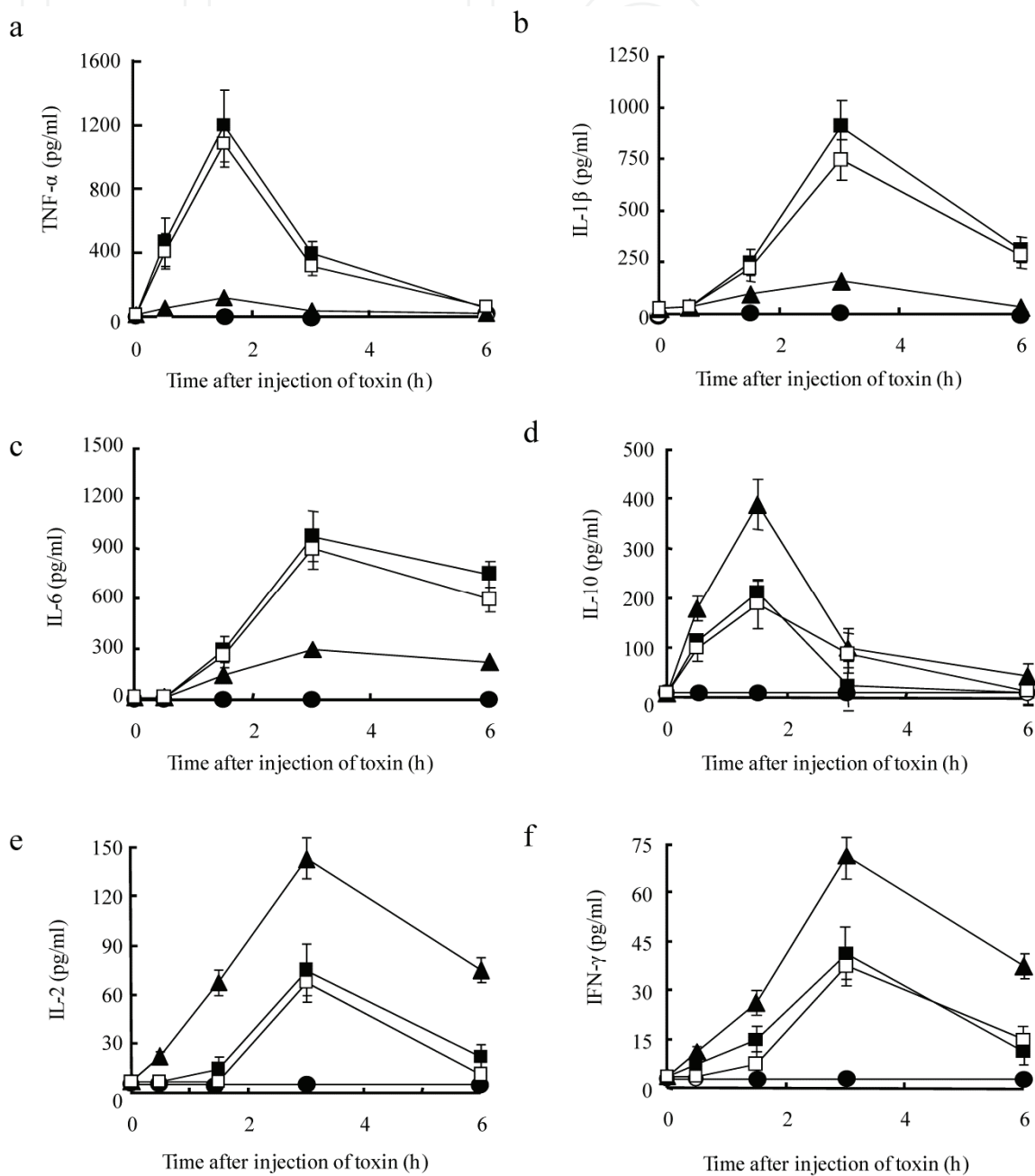


Fig. 2. Effect of macrolide antibiotics on the toxin-induced release of cytokines in blood of mice Mice received ERM or KTM for 5 days every 24 h and were injected intravenously with 20 ng of alpha-toxin. Cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-10, IFN- $\gamma$  and IL-2) in the blood were assayed with ELISA kits. Symbols: control, ●; Saline + alpha-toxin, ■; ERM (0.5 mg/mouse) + alpha-toxin; ▲; KTM (0.5 mg/mouse) + alpha-toxin, □.



To examine the role of inflammatory cytokines in the death caused by the toxin, mice were intravenously injected with the toxin after the administration of an anti-TNF- $\alpha$ , IL-1 $\beta$ , or IL-6 antibody. Untreated mice began to die within 10 hr after the administration of the toxin and all mice died within 12 hr. The survival rate of anti-TNF- $\alpha$  antibody-preinjected mice was 100 and 80% after 8 and 12 hr, respectively, under the conditions (Fig. 3). The anti-IL-1 $\beta$  and anti-IL-6 antibodies had little effect on the lethality (Fig. 3). From the result, it is likely that the lethality of the toxin is related to the release of TNF- $\alpha$ , not of IL-1 $\beta$  or IL-6. To confirm this, the effect of the toxin on TNF- $\alpha$ -deficient mice was tested (Fig. 4). The administration of alpha-toxin killed all of the wild-type mice within 12 h. The survival rate of the TNF- $\alpha$ -knockout mice was 100% and 75% within 12 and 24 h, respectively, consistent with the result obtained by injection of the anti-TNF- $\alpha$  antibody in mice. The observations showed that TNF- $\alpha$  released by the toxin is important in the death caused by the toxin. On the other hand, TNF- $\alpha$  in the range of concentrations found in mice treated with alpha-toxin was not lethal. Therefore, it is apparent that TNF- $\alpha$  alone did not participate in the death from alpha-toxin under our experimental condition. It is likely that TNF- $\alpha$  released by alpha-toxin plays a role in enhancing the actions of the toxin in vivo, but is not a major factor. Therefore, we cannot exclude the possibility that a TNF- $\alpha$  inhibitor is worth pursuing as a novel therapeutic approach to the treatment of gas gangrene and septicemia caused by the microorganism.

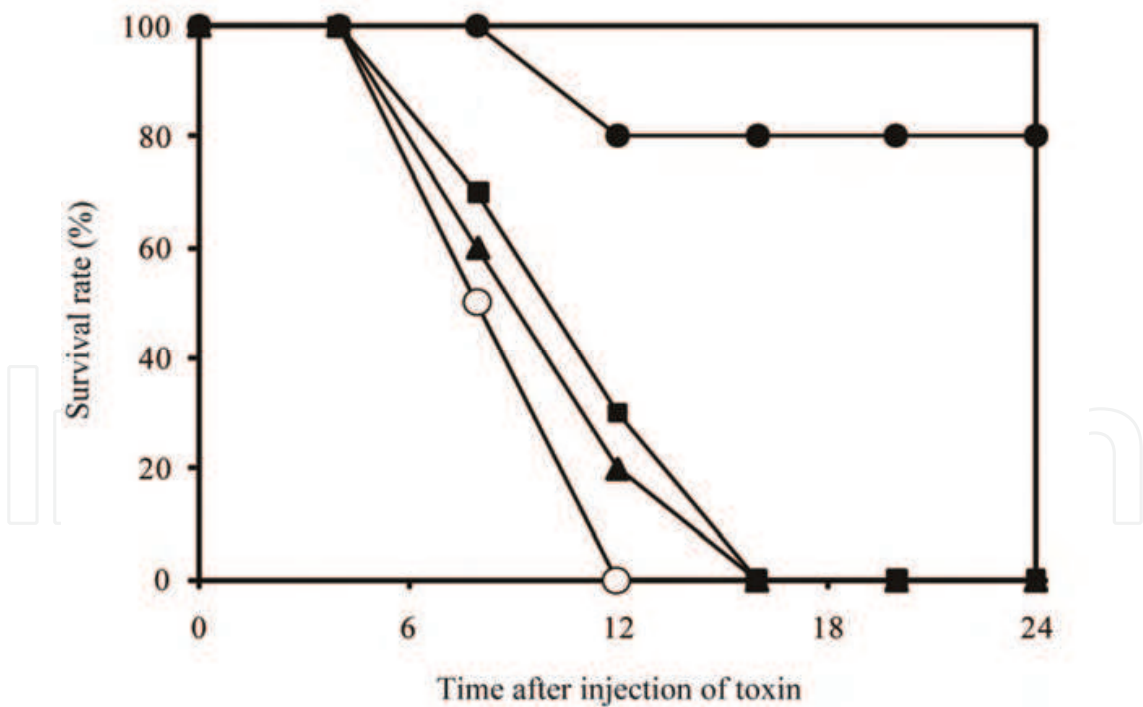


Fig. 3 Effect of anti-TNF- $\alpha$  -IL-1 $\beta$  and -IL-6 antibodies on the lethality of alpha-toxin. Mice received 50  $\mu$ g of anti-TNF- $\alpha$ , anti-IL-1 $\beta$ , or anti-IL-6 antibody, and after 2 h, were injected intravenously with 20 ng of alpha-toxin. Survival of the mice was monitored at the indicated times after the injection of the toxin. Symbols: Saline + alpha-toxin, ○; anti-TNF- $\alpha$  antibody + alpha-toxin, ●; anti-IL-1 $\beta$  antibody + alpha-toxin, ▲; anti-IL-6 antibody + alpha-toxin, ■.

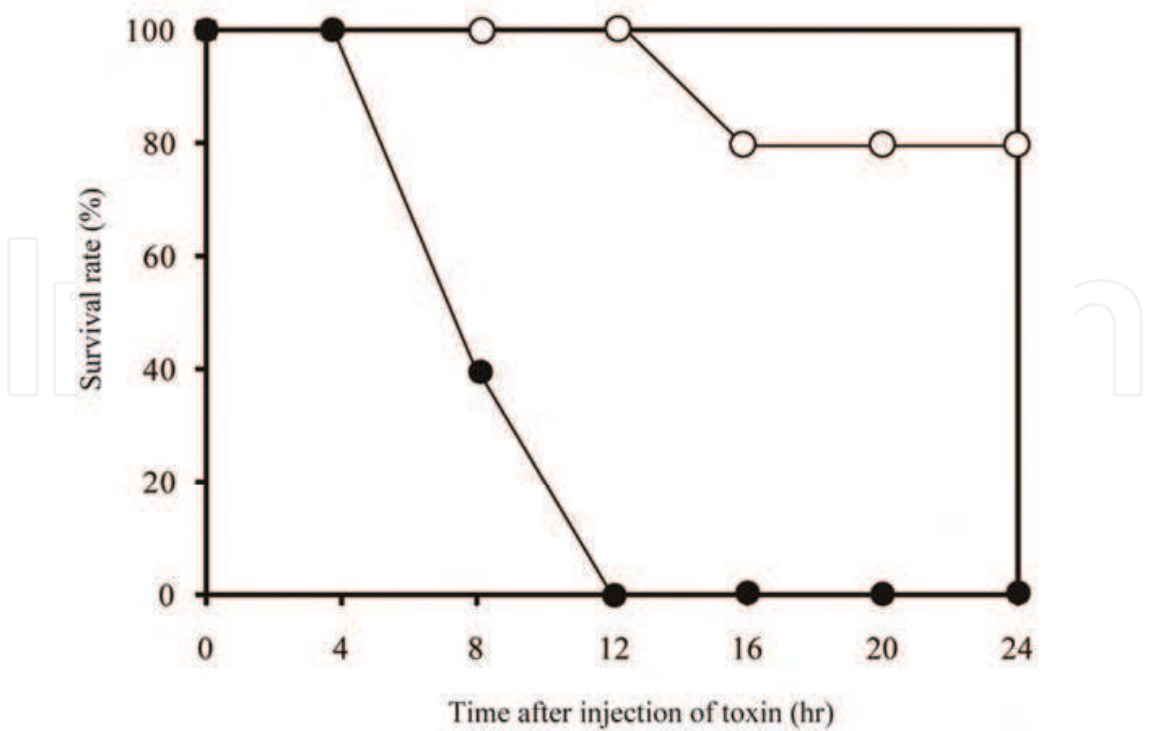


Fig. 4. Comparison of the lethality of alpha-toxin in wild-type mice and TNF- $\alpha$  knockout mice Mice were injected intravenously with 20 ng of alpha-toxin. Survival of the mice was monitored at the indicated times after the injection. Symbols: control mouse (B10D2), ●; TNF- $\alpha$  knockout mouse, ○.

4. The potency of macrolide antibiotics

4.1 The effect on alpha-toxin-induced death

Macrolides, particularly those derived from 14- and 15-membered rings, exert anti-inflammatory effects through a variety of signaling pathways including activator protein-1 (AP-1) and nuclear factor-kappaB (NF- $\kappa$ B) (Sato et al. 1998, Khan et al. 1999, Desaki et al. 2000, Rubin and Tamaoki 2000, Kikuchi et al. 2002). The antibiotics have been reported to impair the production of pro-inflammatory cytokines (Kadota et al. 1993, Fujii et al. 1995). Abe et al. reported that macrolides repressed IL-8 gene expression by suppressing both AP-1 binding sites and NF- $\kappa$ B (Abe et al. 2000). Shchultz et al. postulated that the treatment with macrolides results in suppression of the production of TNF- $\alpha$  and granulocyte-macrophage colony-stimulating factor (Schultz et al. 1998). Simpson et al. have also reported that 14- and 15-member macrolide antibiotics attenuated the activation of neutrophils induced by various inflammatory stimuli (Simpson et al. 2008). We measured the release of these cytokines induced by the toxin in mice preinjected with the 14-membered macrolide, ERM or the 16-membered, KTM. In mice preinjected with ERM, the toxin-induced release of pro-inflammatory cytokines, TNF-  $\alpha$ , IL-1 $\beta$ , and IL-6, in blood was markedly decreased (Fig. 2), whereas the toxin-induced release of the T-helper type 1 (Th1) cytokines, IFN- $\gamma$  and IL-2, and the T-helper type 2 (Th2) cytokine, IL-10, increased approximately 2-fold, compared with that in mice preinjected with ERM (Fig. 2). The action of AZM resembled that of ERM. In mice preinjected with KTM, the toxin-induced release of these cytokines was the same as that in the control mice. The administration of

ERM or KTM alone caused no release of these cytokines. It therefore is apparent that ERM and AZM inhibit the toxin-induced release of pro-inflammatory cytokines, and enhance that of Th1 and Th2 cytokines in vivo. It is interesting that the antibiotics increased levels of Th1 and Th2 cytokines under the conditions. The antibiotics may control a balance of the immune system disrupted by the toxin.

We examined the effect of ERM, AZM, and KTM on the toxin-induced death. Alpha-toxin-injected mice began to die after about 8 hr, and all mice died within 12 hr of the administration (Fig. 5). ERM- or AZM-preinjected mice survived up to 18 hr after the injection of alpha-toxin. The survival rate of mice preinjected with ERM and AZM was about 80 and 70%, respectively, 24 h after the administration of the toxin, showing that ERM and AZM inhibited the toxin's lethal effect. These results show that the 14-ring and the 15-ring macrolides have inhibitory effects on the lethality of alpha-toxin, but the 16-ring macrolides do not. The result coincided with that reported previously (Oda et al. 2008).

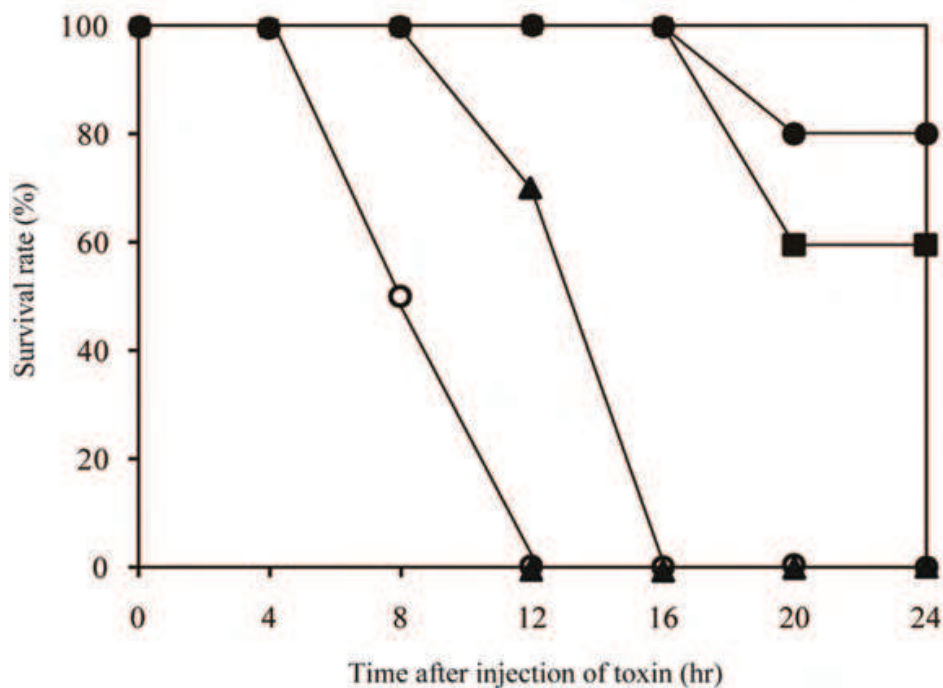


Fig. 5. Effect of macrolide antibiotics on the lethality of alpha-toxin  
Mice received 15 mg/kg of ERM, AZM, or KTM for 5 days every 24 h, and were then injected intravenously with 20 ng of alpha-toxin. Survival of the mice was monitored every 4 hr after the injection of the toxin. Symbols: Saline + alpha-toxin, ○; ERM + alpha-toxin, ●; AZM + alpha-toxin, ■; KTM + alpha-toxin, ▲.

4.2 Effect on systemic hemolysis induced by alpha-toxin

Massive intravascular hemolysis is reported to be diagnostic of *C. perfringens* septicemia (Alvarez et al. 1999). Bunderen et al. reported that the disease occurs in immunocompromised patients and patients with underlying malignancy, or in otherwise healthy individuals with abdominal surgery or following abortion (van Bunderen et al. 2010). Combination therapy with penicillin and clindamycin has been shown to minimally improve survival in animal studies (Stevens et al. 1987). However, at present, the treatment of choice for *C. perfringens* septicemia is intravenously administered high-dose penicillin and surgical debridement of infectious focus. It is reported that early treatment can rescue



patients from an otherwise rapidly fatal outcome, and that the disease with massive hemolysis leads to death within a few hours (Alvarez et al. 1999). Bunderen et al. also found that the toxin-induced hemolysis is an additional prominent factor in the pathogenesis of septicemia caused by *C. perfringens* (van Bunderen et al. 2010). We have reported the relationship between the toxin-induced hemolysis and activation of phospholipid metabolism via pertussis toxin-sensitive GTP-binding protein (Gi) (Sakurai, Ochi and Tanaka 1994, Ochi et al. 1996, Ochi et al. 2004, Oda et al. 2008). Intravenous injection of alpha-toxin in mice resulted in massive intravascular hemolysis (Sugahara and Osaka 1970, Kreidl, Green and Wren 2002). However, little is known about the mechanism of hemolysis induced by the toxin in vivo. We investigated the effect of cytokines on the hemolysis induced by alpha-toxin. Mouse erythrocytes were treated with a sub-hemolytic dose of alpha-toxin at 37°C for 30 min, and then incubated with various concentrations of TNF- $\alpha$ , IL-1 $\beta$  or IL-6 for 60 min. Fig. 6 shows that TNF- $\alpha$  enhanced the toxin-induced hemolysis of mouse erythrocytes in a dose-dependent manner, but IL-1 $\beta$  and IL-6 did not.

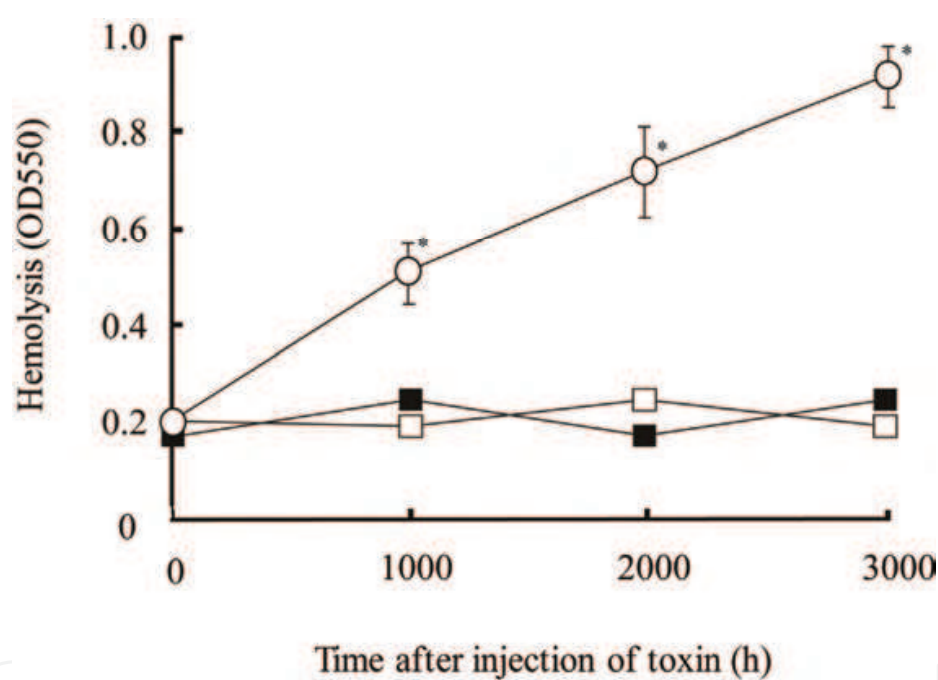


Fig. 6. Effect of cytokines on hemolysis induced by alpha-toxin in mouse erythrocytes The washed erythrocytes of mice were incubated with 20 ng/mL of alpha-toxin at 37°C for 30 min, and then treated with various concentrations of cytokines at 37°C for 60 min. Symbols: TNF- $\alpha$ ,  $\circ$ ; IL-1 $\beta$ ,  $\square$ ; IL-6;  $\blacksquare$ . \*,  $p < 0.01$ .

To investigate the effect of ERM and KTM on the hemolysis induced by alpha-toxin in vivo, mice preinjected with ERM and KTM were intravenously administered the toxin. The alpha-toxin-induced hemolysis was markedly decreased in the ERM- or AZM-injected mice, but not KTM-injected mice (Fig. 7). Blockage of TNF- $\alpha$ 's release by ERM or AZM in vivo paralleled the reduction in hemolysis caused by the toxin. It therefore appears that TNF- $\alpha$  enhances the toxin-induced hemolysis in vivo, suggesting that ERM and AZM are effective against the systemic hemolysis induced by alpha-toxin. The result suggests that the antibiotics may prevent hemolytic anemia induced by the toxin.

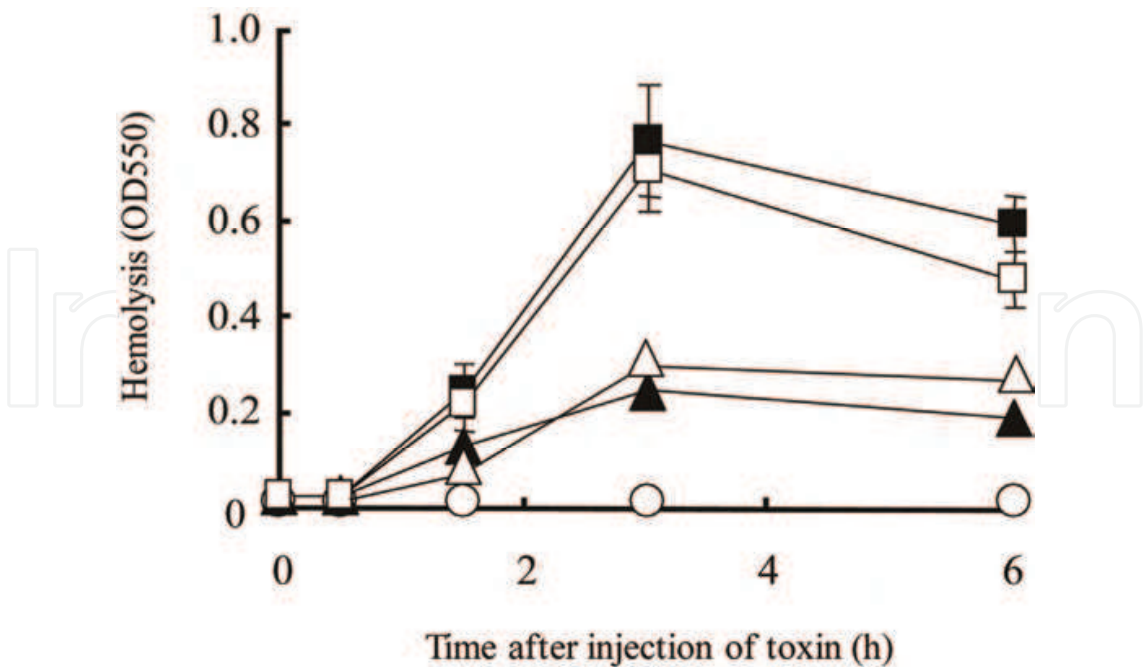


Fig. 7. Hemolysis induced by alpha-toxin in mice pretreated with macrolide antibiotics. Mice received various concentrations of ERM or KTM for 5 days every 24 h, and were then injected intravenously with 20 ng of alpha-toxin. The amount of hemoglobin in the blood was measured spectrophotometrically. Symbols: control, ○; Saline + alpha-toxin, □; ERM (0.5 mg/mouse) + alpha-toxin; ▲; AZM (0.5 mg/mouse) + alpha-toxin; Δ, KTM (0.5 mg/mouse) + alpha-toxin, ■.

#### 4.3 The effect on alpha-toxin-induced activation of neutrophils

Alpha-toxin induced the release of TNF- $\alpha$  from neutrophils and macrophages. The 14- and 15-membered macrolides inhibited the toxin-induced release of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in vivo, as mentioned above. Furthermore, the macrolides prevented the toxin-induced activation of phagocytes and release of TNF- $\alpha$  from cells in vitro. We investigated the effect of the toxin on neutrophils isolated from the macrolide-preinjected mice. The release of TNF- $\alpha$  induced by the toxin from neutrophils prepared from ERM- or AZM-preinjected mice was about 20% of that from neutrophils of untreated mice (Fig. 8A). Little significant reduction in the amount of TNF- $\alpha$  released was observed in neutrophils from mice pretreated with KTM, compared with the control (Fig. 8A). Furthermore, the pretreatment of macrophages with ERM and AZM in vivo resulted in a reduction in the toxin-induced release of TNF- $\alpha$ , but that with KTM did not. It therefore appears that the treatment of these phagocytes with ERM and AZM resulted in a reduction in the response to the toxin. We have reported the mechanism for activation of neutrophils by the toxin as follows (Fig. 8B). Alpha-toxin stimulated the generation of O<sub>2</sub><sup>-</sup> in rabbit neutrophils in vitro (Ochi et al. 2002, Oda et al. 2006). Treatment of neutrophils with the toxin resulted in tyrosine phosphorylation of a protein of about 140 kDa (Fig. 8C). The protein reacted with an anti-tyrosine kinase A (TrkA) antibody and bound nerve growth factor (NGF). The anti-TrkA antibody inhibited the toxin-induced production of O<sub>2</sub><sup>-</sup> from the cells and binding of the toxin to the protein. In addition, the toxin did not bind to PC12 cells treated with TrkA-siRNA, which did not express TrkA. The observations show that the TrkA is a receptor of

alpha-toxin. The toxin induced phosphorylation of 3-phosphoinositide-dependent protein kinase 1 (PDK1), which functions as a downstream mediator of TrkA. K252a, an inhibitor of TrkA, and LY294002, an inhibitor of phosphatidylinositol 3-kinase (PI3K), reduced the toxin-induced production of O<sub>2</sub><sup>-</sup> and phosphorylation of PDK1, but not the formation of diacylglycerol (DG) (Fig. 8D). These inhibitors inhibited the toxin-induced phosphorylation

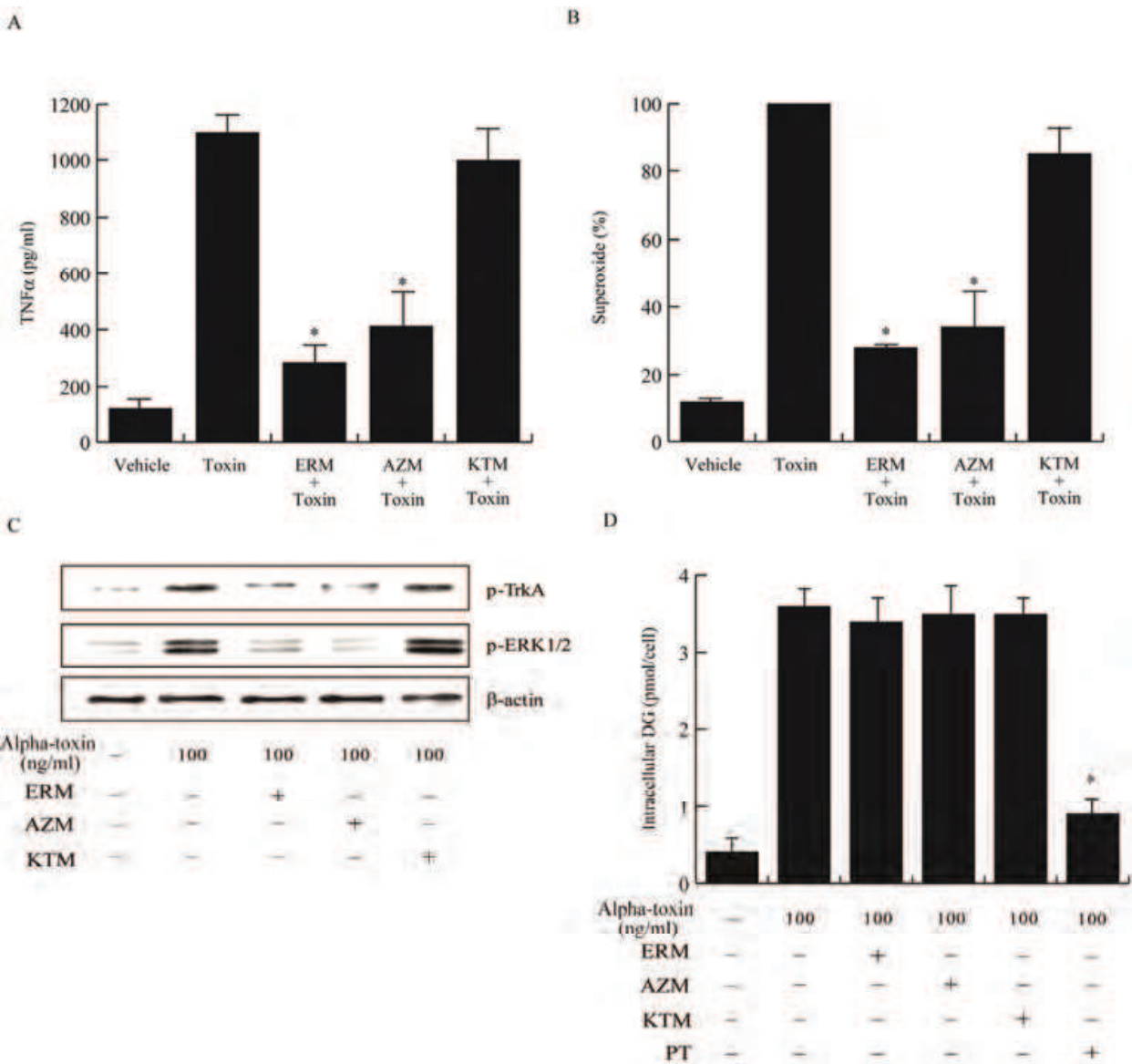


Fig. 8. Effect of alpha-toxin on TrkA-mediated signal transduction in neutrophils prepared from mice preinjected with macrolides

Neutrophils were prepared from mice injected with ERM, AZM, or KTM. A) The neutrophils were incubated with 100 ng of alpha-toxin at 37°C for 3 h. The release of TNF- $\alpha$  was assayed with an ELISA kit. \*,  $p < 0.01$ . B) Production of O<sub>2</sub><sup>-</sup> was monitored for 15 min based on MCLA-chemiluminescence. O<sub>2</sub><sup>-</sup> production induced by alpha-toxin alone was set as the maximal response (100%). \*,  $p < 0.05$ . 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. D) The neutrophils were incubated with 100 ng/ml of alpha-toxin at 37°C for 10 min, and DG in the cells was measured. \*,  $p < 0.01$ .

of protein kinase C  $\theta$  (PKC $\theta$ ). On the other hand, U73122, a PLC inhibitor, and pertussis toxin inhibited the toxin-induced generation of  $O_2^-$  and formation of DG, but not the phosphorylation of TrkA and PDK1 (Fig. 8D). These observations show that the toxin independently induces production of DG through activation of endogenous phosphatidylinositol PI-PLC via  $G_i$  and phosphorylation of PDK1 via the TrkA signaling pathway and the two events synergistically activate PKC $\theta$  which is involved in the generation of  $O_2^-$  through the stimulation of mitogen-activated protein kinase (MAPK)-associated signaling events (Fig. 9).

Macrolide antibiotics have been reported to inhibit effects through signaling pathways including NF- $\kappa$ B and AP-1. It has been reported that ERM, clarithromycin, and

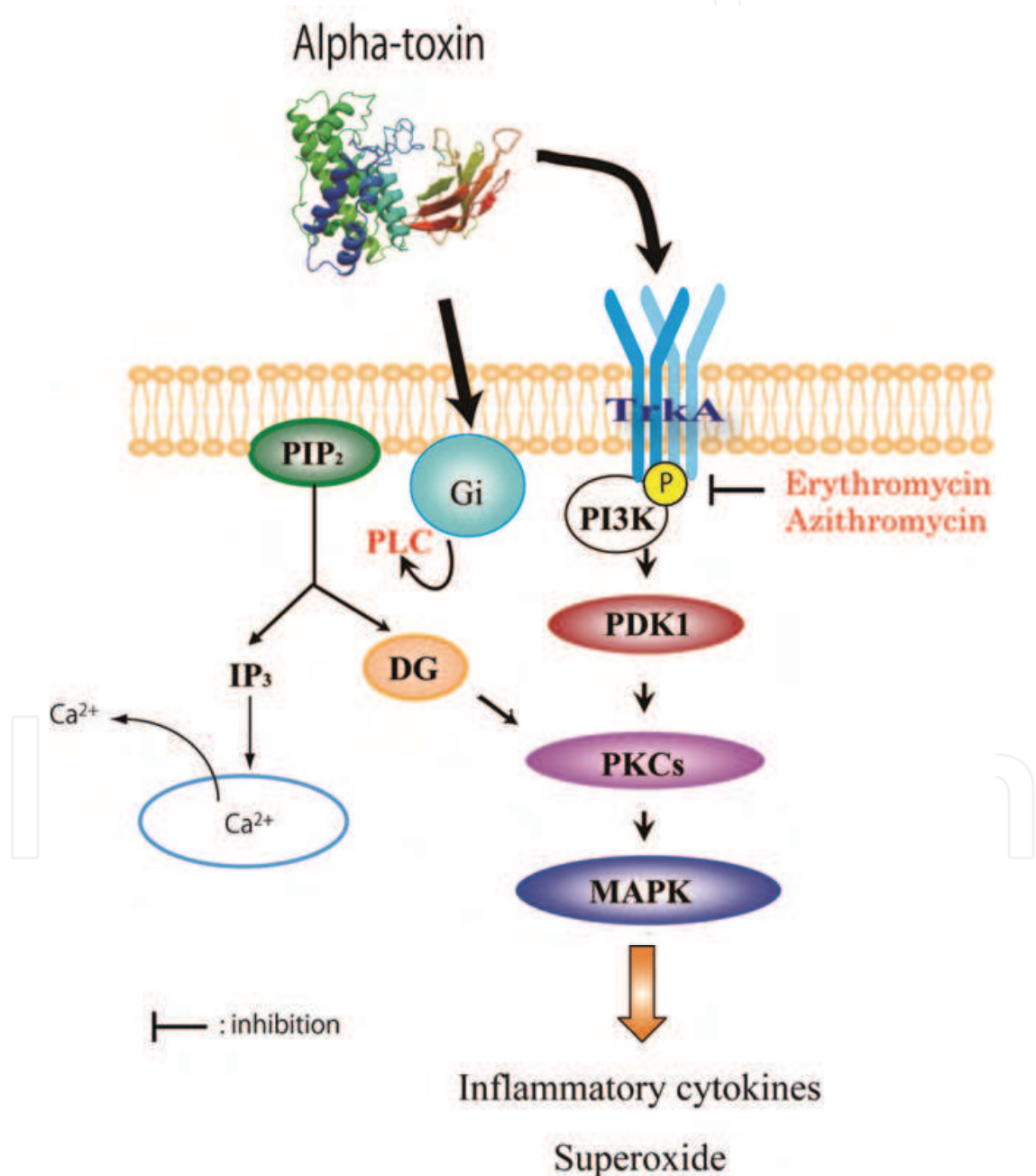


Fig. 9. Effect of ERM and AZM on alpha-toxin-activated signal transduction



roxithromycin inhibit the generation of  $O_2^-$  by stimulus-activated neutrophils (Anderson 1989, Hand and King-Thompson 1990). However, little is known about the inhibitory mechanism of the antibiotics. Recently we revealed that macrolides prevent the toxin-induced death, production of  $O_2^-$ , and release of TNF- $\alpha$  in neutrophils through inactivation of TrkA (Oda et al. 2008). ERM and AZM inhibited the toxin-induced phosphorylation of TrkA under conditions in which the toxin inhibits biological activities; production of  $O_2^-$  and release of TNF- $\alpha$  (Fig. 8). On the other hand, treatment with ERM and AZM had no effect on the formation of DG via Gi in rabbit neutrophils treated with alpha-toxin. KTM, which does not inhibit the biological activities of the toxin, did not prevent them. These observations provided evidence that inhibition of the toxin-induced phosphorylation of TrkA by ERM and AZM results in suppression of activation of neutrophils, formation of  $O_2^-$ , and release of TNF- $\alpha$ . Therefore, the results show that 14- and 15-membered macrolides specifically inhibit the phosphorylation of TrkA, viz. activation of the protein.

## 5. Discussion

*C. perfringens* alpha-toxin causes death, hemolysis, the activation of neutrophils, and the release of TNF- $\alpha$  through activation of MAPK-associated signaling via two pathways, activation of endogenous PI-PLC via Gi and PDK1 via phosphorylation of TrkA. The 14- and 15-membered macrolides specifically inhibited the phosphorylation of TrkA, preventing the activation of the MAPK signaling pathway present in the downstream region of TrkA. Therefore, it was found that the antibiotics specifically block these activities of the toxin by inhibiting the phosphorylation of TrkA.

TNF- $\alpha$  appears to play an important role in the lethal effect of the toxin, because 1) the anti-TNF- $\alpha$  antibody prevented death caused by the toxin, 2) TNF- $\alpha$ -knockout mice were resistant to the toxin, and 3) the 14- and 15-membered macrolides, which prevent the release of TNF- $\alpha$  induced by the toxin, inhibited the lethal and hemolytic effects of the toxin. However, Wiersnga and Poll reported that trials with an anti-TNF- $\alpha$  antibody and recombinant IL-1 receptor antagonist for clinical septicemia failed, and many other anti-inflammatory strategies were not successful in altering the outcome of patients with septicemia (Anas et al. 2010). Furthermore, the fallacy of the notion that excessive inflammation is the main or sole underlying cause of an adverse outcome in septic patients has been pointed out in the review by Wiersnga and Poll (Wiersinga and van der Poll 2007). On the other hand, considering that bacteria grow in patients with septicemia, there are many unanswered questions about the immune functions of the host, clearance of bacteria in vivo by antimicrobial agents, and surgical resection of foci and so on. Therefore, our findings on the role of TNF- $\alpha$  in diseases caused by *C. perfringens* should be considered significant. Certainly, TNF- $\alpha$  alone in the range of concentrations found in mice treated with alpha-toxin was not lethal. Thus, our results seem that TNF- $\alpha$  promotes lethality and massive intravascular hemolysis caused by the toxin. Fourteen- and the 15-membered macrolides have been shown to exert immunomodulatory effects on a wide range of cells; epithelial cells, macrophages, monocytes, eosinophils, neutrophils, and lymphocytes. The antibiotics are known to inhibit adherence, oxidative burst, cytokine expression and mobility. The administration of the 14- and 15-membered macrolides resulted in a drastic reduction in alpha-toxin-induced release of proinflammatory cytokines and systemic



hemolysis and death, and increases in Th 1 and 2 cytokines in mice treated with the toxin. Yan et al. reported that lysophosphatidylcholine (LPC) induced a modest and transient change in the levels of certain cytokines and that the Th 1 cytokines, IFN- $\gamma$ , IL-2 and IL-12, were increased in response to LPC, whereas the proinflammatory cytokines, TNF- $\alpha$  and IL-1 $\beta$ , were decreased (Yan et al. 2004), showing that the pattern of cytokine regulation induced by alpha-toxin in mice treated with the macrolides is similar to that observed in the LPC-treated LPS model. Increases in Th 1 cytokines, as well as the combined decrease of TNF- $\alpha$  and IL-1 $\beta$ , have been reported to have beneficial effects in sepsis (Weighardt et al. 2002, Nakahata et al. 2001). Yan et al. did not exclude the possibility that the combined effect of all these changes in cytokines may contribute to improved survival (Yan et al. 2004). Our result concerning the behavior of proinflammatory cytokines was consistent with that reported by others. On the basis of these findings, a careful balance between the inflammatory and anti-inflammatory response appears to be significant for survival in patients with septicemia. Therefore, it is possible that macrolides are valuable in patients with septicemia caused by *C. perfringens*.

The 14- and 15- member-macrolide antibiotics are effective in the treatment of infectious diseases caused by *C. perfringens*, but not in excluding *C. perfringens* from foci under the conditions. Highly effective antimicrobial agents against *C. perfringens* are required for treatment of the diseases.

## 6. Conclusion

*C. perfringens* alpha-toxin, the main agent involved in the development of gas gangrene and septicemia, induces death, hemolysis, necrosis and the activation of macrophages and neutrophils. The toxin elicits these activities through the activation of an intracellular signaling pathway involving MAPK-associated signal transduction from Gi and/or TrkA. The 14- and 15-membered macrolides specifically blocked the phosphorylation of TrkA, inhibiting these toxin-induced activities. It therefore appears that the macrolide antibiotics are effective in an improvement in clinical symptoms caused by *C. perfringens*. However, growth rates of *C. perfringens* are known to be significantly higher in infectious foci without neutrophils. The macrolides are likely not to be effective in inhibiting the growth of *C. perfringens* under the conditions. Penicillins are known to be highly effective in preventing the growth of microorganisms. In conclusion, treatment with 14- and 15-membered macrolides, an inhibitor of alpha-toxin, and high doses of penicillins, antimicrobial agents, would be effective against diseases caused by *C. perfringens*.

## 7. References

- Abe, S., H. Nakamura, S. Inoue, H. Takeda, H. Saito, S. Kato, N. Mukaida, K. Matsushima & H. Tomoiike (2000) Interleukin-8 gene repression by clarithromycin is mediated by the activator protein-1 binding site in human bronchial epithelial cells. *Am J Respir Cell Mol Biol*, 22, 51-60. ISSN pp. 1044-1549
- Alape-Giron, A., M. Flores-Diaz, I. Guillouard, C. E. Naylor, R. W. Titball, A. Rucavado, B. Lomonte, A. K. Basak, J. M. Gutierrez, S. T. Cole & M. Thelestam (2000) Identification of residues critical for toxicity in *Clostridium perfringens*

- phospholipase C, the key toxin in gas gangrene. *Eur J Biochem*, 267, pp. 5191-5197. ISSN 0014-2956
- Aldridge, A. J. (2002) Role of the neutrophil in septic shock and the adult respiratory distress syndrome. *Eur J Surg*, 168, pp. 204-214.
- Alvarez, A., S. Rives, B. Nomdedeu & A. Pereira (1999) Massive hemolysis in *Clostridium perfringens* infection. *Haematologica*, 84, pp. 571-573. ISSN 0390-6078
- Anas, A. A., W. J. Wiersinga, A. F. de Vos & T. van der Poll (2010) Recent insights into the pathogenesis of bacterial sepsis. *Neth J Med*, 68, pp. 147-152. ISSN 1872-9061
- Anderson, R. (1989) Erythromycin and roxithromycin potentiate human neutrophil locomotion in vitro by inhibition of leukoattractant-activated superoxide generation and autooxidation. *J Infect Dis*, 159, pp. 966-973.
- Awad, M. M., D. M. Ellemor, R. L. Boyd, J. J. Emmins & J. I. Rood (2001) Synergistic effects of alpha-toxin and perfringolysin O in *Clostridium perfringens*-mediated gas gangrene. *Infect Immun*, 69, pp. 7904-7910. ISSN 0019-9567
- Beutler, B., I. W. Milsark & A. C. Cerami (1985) Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. *Science*, 229, pp. 869-871. ISSN 0036-8075
- Bryant, A. E., R. Y. Chen, Y. Nagata, Y. Wang, C. H. Lee, S. Finegold, P. H. Guth & D. L. Stevens (2000) Clostridial gas gangrene. I. Cellular and molecular mechanisms of microvascular dysfunction induced by exotoxins of *Clostridium perfringens*. *J Infect Dis*, 182, pp. 799-807.
- Bryant, A. E. & D. L. Stevens (1996) Phospholipase C and perfringolysin O from *Clostridium perfringens* upregulate endothelial cell-leukocyte adherence molecule 1 and intercellular leukocyte adherence molecule 1 expression and induce interleukin-8 synthesis in cultured human umbilical vein endothelial cells. *Infect Immun*, 64, pp. 358-362.
- Bunting, M., D. E. Lorant, A. E. Bryant, G. A. Zimmerman, T. M. McIntyre, D. L. Stevens & S. M. Prescott (1997) Alpha toxin from *Clostridium perfringens* induces proinflammatory changes in endothelial cells. *J Clin Invest*, 100, pp. 565-574.
- Calandra, T., P. Y. Bochud & D. Heumann (2002) Cytokines in septic shock. *Curr Clin Top Infect Dis*, 22, pp. 1-23.
- Desaki M., H. Takizawa, T. Ohtoshi, T. Kasama, K. Kobayashi, T. Sunazuka, S. Omura, K. Yamamoto & K. Ito (2000) Erythromycin suppresses nuclear factor-kB and activator protein-1 activation in human bronchial epithelial cells. *Biochem Biophys Res Commun*. 267, pp. 124-128.
- Dinarello, C. A. (2004) Therapeutic strategies to reduce IL-1 activity in treating local and systemic inflammation. *Curr Opin Pharmacol*, 4, pp. 378-385.
- Ellemor, D. M., R. N. Baird, M. M. Awad, R. L. Boyd, J. I. Rood & J. J. Emmins (1999) Use of genetically manipulated strains of *Clostridium perfringens* reveals that both alpha-toxin and theta-toxin are required for vascular leukostasis to occur in experimental gas gangrene. *Infect Immun*, 67, pp. 4902-4907. ISSN 0019-9567
- Fujii, T., J. Kadota, K. Kawakami, K. Iida, R. Shirai, M. Kaseda, S. Kawamoto & S. Kohno (1995) Long term effect of erythromycin therapy in patients with chronic *Pseudomonas aeruginosa* infection. *Thorax*, 50, pp. 1246-1252.

- Gilmore, M. S., A. L. Cruz-Rodz, M. Leimeister-Wachter, J. Kreft & W. Goebel (1989) A *Bacillus cereus* cytolytic determinant, cereolysin AB, which comprises the phospholipase C and sphingomyelinase genes: nucleotide sequence and genetic linkage. *J Bacteriol*, 171, pp. 744-753. ISSN 0021-9193
- Guillouard, I., P. M. Alzari, B. Saliou & S. T. Cole (1997) The carboxy-terminal C2-like domain of the alpha-toxin from *Clostridium perfringens* mediates calcium-dependent membrane recognition. *Mol Microbiol*, 26, pp. 867-876. ISSN 0950-382X
- Hand, W. L. & N. L. King-Thompson (1990) Uptake of antibiotics by human polymorphonuclear leukocyte cytoplasts. *Antimicrob Agents Chemother*, 34, pp. 1189-1193. ISSN 0066-4804
- Hough, E., L. K. Hansen, B. Birknes, K. Jynge, S. Hansen, A. Hordvik, C. Little, E. Dodson & Z. Derewenda (1989) High-resolution (1.5 Å) crystal structure of phospholipase C from *Bacillus cereus*. *Nature*, 338, pp. 357-360. ISSN 0028-0836
- Kadota, J., O. Sakito, S. Kohno, H. Sawa, H. Mukae, H. Oda, K. Kawakami, K. Fukushima, K. Hiratani & K. Hara (1993) A mechanism of erythromycin treatment in patients with diffuse panbronchiolitis. *Am Rev Respir Dis*, 147, pp. 153-159.
- Khan, A. A., T. R. Slifer, F. G. Araujo & J. S. Remington (1999) Effect of clarithromycin and azithromycin on production of cytokines by human monocytes. *Int J Antimicrob Agents*, 11, pp. 121-132.
- Kikuchi T, K. Hagiwara, Y. Honda, K. Gomi, T. Kobayashi, H. Takahashi, Y. Tokue, A. Watanabe & T. Nukiwa. (2002) Clarithromycin suppresses lipopolysaccharide-induced interleukin-8 production by human monocytes through AP-1 and NF-κB transcription factors. *J Antimicrob Chemother*, 49, pp.745-55.
- Kreidl, K. O., G. R. Green & S. M. Wren (2002) Intravascular hemolysis from a *Clostridium perfringens* liver abscess. *J Am Coll Surg*, 194, pp. 387. ISSN 1072-7515
- Lum, L., B. R. Wong, R. Josien, J. D. Becherer, H. Erdjument-Bromage, J. Schlondorff, P. Tempst, Y. Choi & C. P. Blobel (1999) Evidence for a role of a tumor necrosis factor-α (TNF-α)-converting enzyme-like protease in shedding of TRANCE, a TNF family member involved in osteoclastogenesis and dendritic cell survival. *J Biol Chem*, 274, pp. 13613-13618.
- Nagahama, M., K. Michiue & J. Sakurai (1996a) Membrane-damaging action of *Clostridium perfringens* alpha-toxin on phospholipid liposomes. *Biochim Biophys Acta*, 1280, pp. 120-126.
- Nagahama, M., M. Mukai, S. Morimitsu, S. Ochi & J. Sakurai (2002) Role of the C-domain in the biological activities of *Clostridium perfringens* alpha-toxin. *Microbiol Immunol*, 46, pp. 647-655.
- Nagahama, M., T. Nakayama, K. Michiue & J. Sakurai (1997) Site-specific mutagenesis of *Clostridium perfringens* alpha-toxin: replacement of Asp-56, Asp-130, or Glu-152 causes loss of enzymatic and hemolytic activities. *Infect Immun*, 65, pp. 3489-3492.
- Nagahama, M., S. Ochi, K. Kobayashi & J. Sakurai (1996b) The relationship between histidine residues and various biological activities of *Clostridium perfringens* alpha toxin. *Adv Exp Med Biol*, 391, pp. 251-255. ISSN 0065-2598

- Nagahama, M., Y. Okagawa, T. Nakayama, E. Nishioka & J. Sakurai (1995) Site-directed mutagenesis of histidine residues in *Clostridium perfringens* alpha-toxin. *J Bacteriol*, 177, pp. 1179-1185.
- Nakahata, E., Y. Shindoh, T. Takayama & C. Shindoh (2001) Interleukin-12 prevents diaphragm muscle deterioration in a septic animal model. *Comp Biochem Physiol A Mol Integr Physiol*, 130, pp. 653-663. ISSN 1095-6433
- Naylor, C. E., J. T. Eaton, A. Howells, N. Justin, D. S. Moss, R. W. Titball & A. K. Basak (1998) Structure of the key toxin in gas gangrene. *Nat Struct Biol*, 5, pp. 738-746. ISSN 1072-8368
- Ochi, S., K. Hashimoto, M. Nagahama & J. Sakurai (1996) Phospholipid metabolism induced by *Clostridium perfringens* alpha-toxin elicits a hot-cold type of hemolysis in rabbit erythrocytes. *Infect Immun*, 64, pp. 3930-3933.
- Ochi, S., T. Miyawaki, H. Matsuda, M. Oda, M. Nagahama & J. Sakurai (2002) *Clostridium perfringens* alpha-toxin induces rabbit neutrophil adhesion. *Microbiology*, 148, pp. 237-245.
- Ochi, S., M. Oda, H. Matsuda, S. Ikari & J. Sakurai (2004) *Clostridium perfringens* alpha-toxin activates the sphingomyelin metabolism system in sheep erythrocytes. *J Biol Chem*, 279, pp. 12181-12189.
- Oda, M., S. Ikari, T. Matsuno, Y. Morimune, M. Nagahama & J. Sakurai (2006) Signal transduction mechanism involved in *Clostridium perfringens* alpha-toxin-induced superoxide anion generation in rabbit neutrophils. *Infect Immun*, 74, pp. 2876-2886.
- Oda, M., A. Kihara, H. Yoshioka, Y. Saito, N. Watanabe, K. Uoo, M. Higashihara, M. Nagahama, N. Koide, T. Yokochi & J. Sakurai (2008) Effect of erythromycin on biological activities induced by *Clostridium perfringens* alpha-toxin. *J Pharmacol Exp Ther*, 327, pp. 934-940.
- Palsson-McDermott, E. M. & L. A. O'Neill (2004) Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology*, 113, pp. 153-162. ISSN 0019-2805
- Riedemann, N. C., R. F. Guo & P. A. Ward (2003) Novel strategies for the treatment of sepsis. *Nat Med*, 9, pp. 517-524.
- Rubin, B. K. & J. Tamaoki (2000) Macrolide antibiotics as biological response modifiers. *Curr Opin Investig Drugs*, 1, pp. 169-172.
- Sakurai, J., M. Nagahama & M. Oda (2004) *Clostridium perfringens* alpha-toxin: characterization and mode of action. *J Biochem (Tokyo)*, 136, pp. 569-574.
- Sakurai, J., S. Ochi & H. Tanaka (1994) Regulation of *Clostridium perfringens* alpha-toxin-activated phospholipase C in rabbit erythrocyte membranes. *Infect Immun*, 62, pp. 717-721. ISSN 0019-9567
- Sato, K., M. Suga, T. Akaike, S. Fujii, H. Muranaka, T. Doi, H. Maeda & M. Ando (1998) Therapeutic effect of erythromycin on influenza virus-induced lung injury in mice. *Am J Respir Crit Care Med*, 157, pp. 853-857.
- Schmid, E. E. (1971) The macrolide group of antibiotics. Mode of action. *Antibiot Chemother*, 17, pp. 52-66.
- Schultz, M. J., P. Speelman, S. Zaat, S. J. van Deventer & T. van der Poll (1998) Erythromycin inhibits tumor necrosis factor alpha and interleukin 6 production induced by heat-



- killed *Streptococcus pneumoniae* in whole blood. *Antimicrob Agents Chemother*, 42, pp. 1605-1609. ISSN 0066-4804
- Simpson, J. L., H. Powell, M. J. Boyle, R. J. Scott & P. G. Gibson (2008) Clarithromycin targets neutrophilic airway inflammation in refractory asthma. *Am J Respir Crit Care Med*, 177, pp. 148-155. ISSN 1535-4970
- Stevens, D. L. (2000) The pathogenesis of clostridial myonecrosis. *Int J Med Microbiol*, 290, pp. 497-502.
- Stevens, D. L. & A. E. Bryant (1997) Pathogenesis of *Clostridium perfringens* infection: mechanisms and mediators of shock. *Clin Infect Dis*, 25, pp. 160-164.
- Sugahara, T. & A. Osaka (1970) Two molecular forms of *Clostridium perfringens* alpha-toxin associated with lethal, hemolytic and enzymatic activities. *Jpn J Med Sci Biol*, 23, pp. 61-66.
- Stevens, D.L., B.M. Laine, and J.E. Mitten. (1987) Comparison of single and combination antimicrobial agents for prevention of experimental gas gangrene caused by *Clostridium perfringens*. *Antimicrob. Agents Chemother*. 31, pp. 312-316.
- Titball, R. W. (1993) Bacterial phospholipases C. *Microbiol Rev*, 57, pp. 347-366. ISSN 0146-0749
- Titball, R. W., S. E. Hunter, K. L. Martin, B. C. Morris, A. D. Shuttleworth, T. Rubidge, D. W. Anderson & D. C. Kelly (1989) Molecular cloning and nucleotide sequence of the alpha-toxin (phospholipase C) of *Clostridium perfringens*. *Infect Immun*, 57, pp. 367-376. ISSN 0019-9567
- Tracey, K. J., Y. Fong, D. G. Hesse, K. R. Manogue, A. T. Lee, G. C. Kuo, S. F. Lowry & A. Cerami (1987) Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. *Nature*, 330, pp. 662-664.
- Tso, J. Y. & C. Siebel (1989) Cloning and expression of the phospholipase C gene from *Clostridium perfringens* and *Clostridium bifermentans*. *Infect Immun*, 57, pp. 468-476. ISSN 0019-9567
- Vallee, B. L. & D. S. Auld (1993) New perspective on zinc biochemistry: cocatalytic sites in multi-zinc enzymes. *Biochemistry*, 32, pp. 6493-6500. ISSN 0006-2960
- van Bunderen, C. C., M. K. Bomers, E. Wesdorp, P. Peerbooms & J. Veenstra (2010) *Clostridium perfringens* septicaemia with massive intravascular haemolysis: a case report and review of the literature. *Neth J Med*, 68, pp. 343-346. ISSN 1872-9061
- van Setten, P. A., L. A. Monnens, R. G. Verstraten, L. P. van den Heuvel & V. W. van Hinsbergh (1996) Effects of verocytotoxin-1 on nonadherent human monocytes: binding characteristics, protein synthesis, and induction of cytokine release. *Blood*, 88, pp. 174-183.
- Vazquez-Boland, J. A., C. Kocks, S. Dramsi, H. Ohayon, C. Geoffroy, J. Mengaud & P. Cossart (1992) Nucleotide sequence of the lecithinase operon of *Listeria monocytogenes* and possible role of lecithinase in cell-to-cell spread. *Infect Immun*, 60, pp. 219-230. ISSN 0019-9567
- Weighardt, H., C. D. Heidecke, A. Westerholt, K. Emmanuilidis, S. Maier, M. Veit, K. Gerauer, E. Matevossian, K. Ulm, J. R. Siewert & B. Holzmann (2002) Impaired monocyte IL-12 production before surgery as a predictive factor for the lethal outcome of postoperative sepsis. *Ann Surg*, 235, pp. 560-567. ISSN 0003-4932



- Wiersinga, W. J. & T. van der Poll (2007) Is the septic response good or bad? *Curr Infect Dis Rep*, 9, pp. 366-373. ISSN 1523-3847
- Yan, J. J., J. S. Jung, J. E. Lee, J. Lee, S. O. Huh, H. S. Kim, K. C. Jung, J. Y. Cho, J. S. Nam, H. W. Suh, Y. H. Kim & D. K. Song (2004) Therapeutic effects of lysophosphatidylcholine in experimental sepsis. *Nat Med*, 10, pp. 161-167. ISSN 1078-8956
- Yoshimura, A., Y. Hara, T. Kaneko & I. Kato (1997) Secretion of IL-1 beta, TNF-alpha, IL-8 and IL-1ra by human polymorphonuclear leukocytes in response to lipopolysaccharides from periodontopathic bacteria. *J Periodontal Res*, 32, pp. 279-286.



## **Gangrene - Current Concepts and Management Options**

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Gangrene is the term used to describe the necrosis or death of soft tissue due to obstructed circulation, usually followed by decomposition and putrefaction, a serious, potentially fatal complication. The presented book discusses different aspects of this condition, such as etiology, predisposing factors, demography, pathologic anatomy and mechanisms of development, molecular biology, immunology, microbiology and more. A variety of management strategies, including pharmacological treatment options, surgical and non-surgical solutions and auxiliary methods, are also extensively discussed in the book's chapters. The purpose of the book is not only to provide a reader with an updated information on the discussed problem, but also to give an opportunity for expert opinions exchange and experience sharing. The book contains a collection of 13 articles, contributed by experts, who have conducted a research in the selected area, and also possesses a vast experience in practical management of gangrene and necrosis of different locations.

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