Reactive Oxygen Species and the MEK/ERK Pathway Are Involved in the Toxicity of *Clostridium perfringens* α-Toxin, a Prototype Bacterial Phospholipase C

Laura Monturiol-Gross,1 Marietta Flores-Díaz,1 Cindy Araya-Castillo,1 María-José Pineda-Padilla,1 Graeme C. Clark,4 Richard W. Titball,4,5 and Alberto Alape-Girón1,2,3

1Instituto Clodomiro Picado, Facultad de Microbiología, 2Departamento de Bioquímica, Escuela de Medicina, and 3Centro de Investigaciones en Estructuras Microscópicas, Universidad de Costa Rica, San José, 4Microbiology group, Biomedical Sciences, Defence Science and Technology Laboratory, Porton Down, Salisbury; and 5Biosciences, University of Exeter, United Kingdom

*Clostridium perfringens*, the most broadly distributed pathogen in nature, produces a prototype phospholipase C, also called α-toxin, which plays a key role in the pathogenesis of gas gangrene. α-Toxin causes plasma membrane disruption at high concentrations, but the role of intracellular mediators in its toxicity at low concentrations is unknown. This work demonstrates that α-toxin causes oxidative stress and activates the MEK/ERK pathway in cultured cells and furthermore provides compelling evidence that O$_2^-$, hydrogen peroxide, and the OH$^-$ radical are involved in its cytotoxic and myotoxic effects. The data show that antioxidants and MEK1 inhibitors reduce the cytotoxic and myotoxic effects of α-toxin and demonstrate that edaravone, a clinically used hydroxyl radical trap, reduces the myonecrosis and the mortality caused by an experimental infection with *C. perfringens* in a murine model of gas gangrene. This knowledge provides new insights for the development of novel therapies to reduce tissue damage during clostridial myonecrosis.
The structure of α-toxin shows 2 domains: the α-helical catalytically active N-terminal domain and the β-sandwich-type C-terminal domain, which binds membranes [11]. α-Toxin hydrolyses mainly phosphatidylcholine and sphingomyelin, although it has broad substrate specificity [12]. It is hemolytic and cytotoxic to cultured cells whereas when injected intramuscularly alters leukocyte adherence and induces aggregation of platelets and neutrophils causing vascular occlusion [5]. Considerable progress has been made in the knowledge of the mechanism by which α-toxin induces hemolysis and platelet aggregation [13, 14]. However, the understanding of the mechanism of toxicity in nucleated cells is incomplete [15]. At high concentrations, α-toxin causes membrane disruption and cytolysis [15]. However, at low concentrations, α-toxin leads to the unregulated generation of second messengers [15]. In epithelial cells α-toxin activates arachidonic acid metabolism [16], whereas in neutrophils it activates Protein kinase C and the Mitogen-activated protein kinase cascade, leading to superoxide production (O₂⁻) [17]. However, it remains unknown whether these and/or other signaling pathways play a role in the toxic effects of α-toxin.

The aim of this work was to clarify whether ROS and the MEK/ERK pathway play a role in α-toxin-induced cytotoxicity. Two cell lines hypersensitive to α-toxin due to its ganglioside deficiency were used as a model [18]. Muscle fibers have the lowest ganglioside concentration among mammalian tissues, which can explain their high susceptibility to α-toxin [18]. In addition, experiments were performed to evaluate the role of ROS in myonecrosis induced either by α-toxin or by an experimental infection with C. perfringens.

MATERIALS AND METHODS

Toxin and Chemicals
C. perfringens α-toxin from the strain NCTC 8237 expressed in Escherichia coli was purified as described elsewhere [9]. Chemicals were obtained from Sigma or Biomol.

Cell Culture
Chinese hamster fibroblasts (Don Q) [18] and mouse melanoma cells (GM-95) [19] were cultivated in Eagle’s Minimal essential medium with 10% fetal bovine serum, l-glutamine (5 mmol/L), penicillin (100 U/mL), and streptomycin (100 μg/mL) in a atmosphere containing 5% carbon dioxide.

ROS and Cellular Glutathione Level
After 7 hours of incubation in the absence or presence of C. perfringens α-toxin (4 ng/mL), Don Q cells were loaded with 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) (5 μmol/L) for 30 minutes, washed with phosphate-buffered saline (PBS) and pictured randomly using an Olympus BX-51 microscope and a Cool SNAP digital camera (Media Cybernetics). For flow cytometry analysis Don Q cells were incubated in the absence or presence of C. perfringens α-toxin (4 ng/mL), for different time periods, loaded with DCFDA (5 μmol/L), washed with PBS, tripiplined, and stained with propidium iodine. Some cells were coincubated with α-toxin and edavarone (0.25 μmol/L) or tiron (2 mg/mL). A FACSCalibur flow cytometer (Becton Dickinson) was used to collect data for 5 × 10⁵ cells, and analysis was performed using CellQuest (Becton Dickinson) to determine the percentage of fluorescent living cells. Reduced glutathione was determined using a glutathione detection kit (Chemicon International).

Cytotoxicity Assays
Don Q and GM95 cells were incubated in the absence or presence of C. perfringens α-toxin (4 ng/mL) in 100 μL of medium per well and viability assessed 18 hours later using a neutral red assay [18]. Cell survival was expressed as a percentage, considering as 100% the value of parallel cultures incubated only with medium. Assays were performed with 3–6 replicates.

To evaluate the effect of antioxidants or MEK1 inhibitors in cytotoxic effect of α-toxin, cells were incubated with each drug during exposure to serial dilutions of α-toxin. The drugs and concentrations used were as follows: the cell-permeable glutathione monoethyl ester (GSH-MEE), 2–6 mg/mL [19]; the glutathione precursor N-acetyl-L-cysteine (NAC), 2–6 mg/mL [20]; the O₂⁻ dismutase mimic manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP), 0.3–1.9 mg/mL [21]; the free radical scavengers 4,5-dihydroxy-1,3-benzene disulfonic acid (tiron), 0.16–2 mg/mL [22]; 3-methyl-1-phenyl-2-pyrazolin-5-one (MCI-186 or edaravone), 0.25–1 mmol/L [23]; the hydrogen peroxide (H₂O₂)–decomposing enzyme catalase, 1400–2800 U/mL [24]; the iron chelators N, N’-di(2-hydroxybenzyl)ethylenediamine-N, N’-diacetic acid (HBED), 10–80 μmol/L [25]; dipiridil, 0.3–1.4 mg/mL [26]; deferoxamine, 3–15 mg/mL [27]; apoferritin or ferritin, 0.08–0.33 mg/mL [28]; and the MEK1 inhibitors PD98057 (12.5–50 μg/mL) [29] or U0126 (1.56–6.25 μg/mL) [30]. These drugs were not cytotoxic at the concentrations used, because as viability was >95% in cells exposed only to the drugs in comparison with untreated cells. Furthermore, they did not inhibit the enzymatic activity of α-toxin toward phosphatidylcholine in liposomes [18].

Western Blot Analysis
Don Q or GM95 cells were exposed to C. perfringens α-toxin (4 ng/mL) during different time periods and resuspended in lysis buffer (150 mmol/L sodium chloride, 1% Triton X-100 and 50 mmol/L Tris–hydrochloride acid, 0.1 mmol/L
phenylmethylsulfonyl fluoride (PMSF) and 10 µg/mL leupeptin), and protein concentrations in lysates were measured. To detect carbonylated proteins, derivatization with 2,4 dinitrophenyl hydrazine in 2 mol/L hydrochloric acid (0.1 mg/mL) was performed. Equal amounts of total protein (20 µg per lane) were electrophoresed on polyacrilamide gels and transferred to PVDF membranes. Carbonylated proteins were detected with an anti-DNP antibody. P-ERK was detected with antibodies to ERK1/2 pTyr 185/187 and stripped for total ERK1/2 detection. Chemiluminescence substrate (Invitrogen) was used to develop the reaction on films in which bands were quantified using ImageJ 1.43m software.

Animal Studies
Mice were handled according to protocols approved by the Institutional Committee for Care and Handling of Experimental Animals of the Universidad de Costa Rica. To evaluate the myotoxicity induced by α-toxin, groups of 10 CD-1 mice of 16–18 g were injected in the right gastrocnemius with 1.1 µg of C. perfringens α-toxin. The creatine kinase (CK) activity in plasma was determined using the CK-10 assay (Sigma) [9]. After 24 hours mice were killed, and the injected muscles were dissected and processed for histological examination. For residual CK activity determination the injected muscle was dissected, weighed, and homogenized in 4 mL of PBS. Then, 1 mL of PBS containing 0.5% Triton X-100 was added. After centrifugation at 5000 g for 5 minutes, the supernatant was diluted 1:35 with distilled water, and CK activity was quantified and expressed in units per gram of muscle weight.

Treated mice received the following substances intraperitoneally: MnTMPyM (4 µg) [21] and manganese (III) tetakis (4-benzoic acid) porphyrin chloride (MnTRAP) (9 µg) [31]; edaravone (5 µg) [23], alpha-phenyl-N-t-butylnitrone (PBN) (3 µg) [32]; dimethylurea (10 mg) [33]; or tiron (14 mg) [22]; the combination of NAC and deferoxamine (500 µg each) [34]; the MEK1 inhibitors PD98057 (5 µg) [29] or U0126 (7.5 µg) in 100 µL of PBS 1 hour before and 1 hour after challenge. None of the substances had evident toxic effects in mice at the doses used, which were chosen according to the references.

Experimental infections were induced in groups of 10 CD-1 mice of 16–18 g by injection in the right gastrocnemius of C. perfringens α-toxin. After 5 hours, plasma CK activity was measured and survival recorded for 48 hours. Treated mice received intraperitoneally 30 µg of edaravone in 100 µL of PBS 1 hour before and after challenge with C. perfringens.

Statistical Analysis
Statistical significance was determined by analysis of variance and post hoc Tukey test.

RESULTS
α-Toxin Induction of Oxidative Stress
The effect of α-toxin in ROS production in cultured cells was evaluated. The intracellular content of ROS increases in Don Q cells exposed to α-toxin reaching a plateau 7 hours after exposure, as determined by flow cytometry (Figure 1A). ROS accumulate intracellularly 7 hours after exposure to α-toxin (Figure 1B), in comparison with control cells (Figure 1C), as evidenced using the membrane-permeable probe DCFDA. The ROS accumulation in cells exposed to α-toxin was preventable by the cell-permeable O2− scavenger tiron or the free radical scavenger edaravone, as determined by fluorescence microscopy (Figure 1D and 1E, respectively) or flow cytometry (Figure 1F). Accordingly, the cellular glutathione decreases progressively, falling to 40%–48% 5 hours after exposure to α-toxin, relative to that of control cells (not shown), whereas carbonylated proteins accumulate already at 7 hours after toxin exposure (not shown). Similar results were obtained in the GM-95 cell line (not shown). Taken together, these results demonstrate that α-toxin causes oxidative stress in cultured cells.

Antioxidant Protection From α-Toxin–Induced Cell Death
The effect of antioxidants in α-toxin–induced cytotoxicity was evaluated (Figure 2A and 2B). Cell death was inhibited by the cell-permeable GSH-MEE or the glutathione precursor NAC, suggesting that ROS are involved in cytotoxicity. Accordingly, MnTMPyP and tiron also exerted a dose-dependent protection from the cytotoxic effect α-toxin. Extracellularly added catalase also reduced cell death induced by α-toxin. The copper chelator neocuproine had no effect in α-toxin–induced cytotoxicity (not shown), but 4 structurally unrelated iron chelators (HBED, 2,2-dipyridyl, desferrioxamine, and apoferritin) protect from its cytotoxic effect. In contrast to apoferritin, ferritin—which has no iron-chelating capacity—had no effect in α-toxin–induced cytotoxicity. Furthermore, edaravone also reduces α-toxin–induced cytotoxicity. For all antioxidants, the protection observed was dose dependent within the range of doses indicated in Materials and Methods (not shown).

Antioxidant Reduction of α-Toxin–Induced Myonecrosis
The effect of antioxidants in α-toxin–induced myotoxicity was also evaluated (Figure 3). The CK release to the plasma induced by α-toxin was reduced in mice treated with the O2− dismutase mimics MnTMPyP and MnTRAP, in comparison with that induced in untreated mice (Figure 3A). Treatment with the free radical scavengers tiron, dimethylthiourea, or PBN also alleviates the muscle damage caused by α-toxin (Figure 3B). Furthermore, myotoxicity was attenuated by a combination of NAC and desferrioxamine or by edaravone.
(Figure 3B), further supporting the conclusion that ROS production is induced by α-toxin in vivo and that oxidative stress contributes to its myotoxicity. The residual CK activity was also measured in the gastrocnemius muscle 24 hours after toxin injection in mice untreated or treated with edaravone or tiron to evaluate the

**Figure 1.** *Clostridium perfringens* α-toxin induces reactive oxygen species (ROS) production and causes oxidative stress in cultured cells. Don Q cells were incubated at different times with α-toxin (4 ng/mL). 2′,7′-Dichlorodihydrofluorescein diacetate (DCFDA) (5 μmol/L) was added during the last 30 minutes of incubation. A, To detect ROS production by flow cytometry, the percentage of FL1 positive events among living cells was determined. Results represent mean of 3 independent experiments. B, Don Q cells were incubated in the presence of α-toxin (α-toxin, tiron, and edaravone [EDV]) or in its absence (without α-toxin) and treated with tiron (2 mg/mL) or EDV (0.25 mmol/L) during α-toxin exposure. After 6 hours, DCFDA (5 μmol/L) was added and further incubated for 1 hour, before fluorescence microscopy analysis. Results are representative of ≥2 independent experiments (bar, 30 μm). C, Don Q cells were treated with tiron (2 mg/mL) or 3-methyl-1-phenyl-2-pyrazolin-5-one (EDV) (0.25 mmol/L) and incubated along with α-toxin for 7 hours. During the last 30 minutes of each treatment, DCFDA (5 μmol/L) was added, to detect ROS production by flow cytometry. Results represent means and standard errors of ≥3 independent experiments. *P < .0001. Abbreviation: PLC, phospholipase C.

**Figure 2.** Antioxidants protect from *Clostridium perfringens* α-toxin–induced cell death. Don Q (4) and GM95 (8) cells were preincubated with glutathione monoothyl ester (GSH-MEE) 6 mg/mL, N-acetyl-L-cysteine (NAC) (6 mg/mL), manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP) (1.9 μg/mL or 0.5 μg/mL), tiron (1.5 μg/mL or 2 μg/mL), catalase (2800 U/mL), edaravone (0.5 μg/mL or 1 μg/mL), N,N′-di[2-hydroxybenzyl]ethylenediamine-N,N′-diacetic acid (HBED) (20 μmol/L), dipyridyl (0.4 μg/mL or 0.7 μg/mL), deferoxamine (DFX) (7 mg/mL), apoferritin (0.17 mg/mL), or ferritin (0.17 mg/mL), 2 hours before exposure to α-toxin. Cell viability was determined 22 hours later, using the neutral red assay. Results are expressed as the percentage of neutral red incorporated by the remaining cells, compared with that incorporated by cells incubated with the same antioxidant but not exposed to the toxin. Results represent means and standard errors of 2–3 independent experiments with 3 replicate samples. *P < .001.
remaining amount of CK in muscle cells (Figure 3C). The results showed higher CK levels in muscle of treated mice, in inverse correlation with the plasma CK levels, supporting the conclusion that muscle damage is significantly reduced by these antioxidants. Accordingly, histological analysis showed a reduction in the muscle damage induced by α-toxin in mice treated with tiron, compared with that induced in untreated mice (Figure 3D).

MEK1 Involvement in Cytotoxic and Myotoxic Effects of α-Toxin
Because α-toxin activation of MEK/ERK in rabbit neutrophils has been reported elsewhere [17] and sustained ROS production has been reported to activate the MEK/ERK cascade [35], Western blot analysis was performed to determine whether ERK is activated in Don Q and GM-95 cells exposed to C. perfringens α-toxin. This analysis showed that ERK became rapidly phosphorylated and remains activated for ≥3 hours after exposure to α-toxin (Figure 4A).

The role of the MEK/ERK pathway in the cytotoxicity induced by α-toxin was assessed by evaluating the effect of 2 MEK1 inhibitors, PD98059 and U0126 in α-toxin–induced cell death. Both drugs reduce significantly the cytotoxic effect of α-toxin (Figure 4B). Whether the myotoxic effect of α-toxin can be alleviated by inhibiting MEK1 was also investigated. Muscle damage induced by α-toxin was significantly reduced in mice treated with PD98059 or U0126, in comparison with that induced in untreated animals (Figure 4C), which indicates that MEK1 also plays a role in α-toxin–induced myonecrosis.

Edaravone Reduction of Myonecrosis and Mortality in Mouse Model of Gas Gangrene
To assess the role of ROS in a mouse model of gas gangrene, the effect of edaravone during an experimental infection with C. perfringens was evaluated. The administration of edaravone was found to reduce significantly both myotoxicity and lethality (Figure 5), suggesting that this antioxidant could be used in the treatment of patients with gas gangrene.
DISCUSSION

Understanding the molecular basis of the toxicity of *C. perfringens* α-toxin could open new opportunities for development of novel therapeutic strategies in benefit of patients with gas gangrene. This work reveals that the cytotoxic and myotoxic effects of α-toxin are mediated by ROS, clarifying this hitherto unknown mechanism of action at physiologically relevant low concentrations. In addition, the results suggest the potential therapeutic use of antioxidants to reduce tissue damage during clostridial myonecrosis.

Gas gangrene was an important disease in the battlefield before the antibiotics era and nowadays it is important in patients with deficient blood flow, including those who are

![Figure 4](image1.png)

**Figure 4.** Inhibitors of the MEK/ERK pathway protect from the cytotoxic and myotoxic effect of *Clostridium perfringens* α-toxin. α-Toxin activates MEK1 in Don Q and GM95 cells. A, B, Don Q (A) or GM95 (B) cells were treated with or without α-toxin for 5, 15, 30, 60, 120, and 180 minutes, followed by Western blot analysis using antibodies against ERK1/2 pTyr 185/187 (P-ERK) and then stripped for ERK1/2 detection. Experiments were performed ≥3 times, and representative data are shown. C, GM95 cells were preincubated with PD98059 (25 μg/mL), U0126 (3.12 μg/mL), or Minimal Essential Medium (control) before exposure to α-toxin. Cell viability was determined 18 hours later using the neutral red assay. Results are expressed as the percentage of neutral red incorporated by the remaining cells, compared with that incorporated by control cells incubated with each drug but not exposed to the toxin. Results represent the mean of 2–4 independent experiments with 3 replicate samples. *P < .01. D, Groups of 10 CD-1 mice were challenged intramuscularly with 1.1 μg of *C. perfringens* α-toxin, and after 3 hours creatine kinase (CK) activity was measured in plasma. Mice received intraperitoneally 100 μL of phosphate-buffered saline (control), PD98059 (5 μg), or U0126 (7.5 μg) 1 hour before and 1 hour after toxin injection. Results represent means and standard errors of 3 independent experiments. *P < .01.

![Figure 5](image2.png)

**Figure 5.** Edaravone reduces myonecrosis and mortality in an experimental model of gas gangrene induced by *Clostridium perfringens*. Groups of 10 CD-1 mice were challenged intramuscularly with 6 × 10⁸ colony-forming units of *C. perfringens*. Creatine kinase (CK) activity was measured in plasma after 5 hours (A) and survival was evaluated after 48 hours (B). Treated mice received intraperitoneally 30 μg of edaravone in 100 μL of phosphate-buffered saline 1 hour before and 1 hour after challenge. Results represent means and standard errors of 3–5 independent experiments. *P < .00002 for plasma CK; *P < .05 for mice survival.
elderly or diabetic [36]. Despite the use of antibiotics and intensive care, gas gangrene still has a high mortality rate and causes considerable morbidity because patients require extensive debridement and, in many cases, radical amputation to avoid shock and death [36].

Clostridium perfringens α-toxin, the major virulence factor in the pathogenesis of gas gangrene, hydrolyzes the main phospholipids of cellular membranes. Therefore, it has been assumed that cell death induced by this toxin is due to membrane disruption [37]. Indeed, we showed elsewhere that in Don Q and GM-95 cells, α-toxin causes complete plasma membrane disruption at concentrations >20 ng/mL [18]. This work reveals that α-toxin also causes oxidative stress in these cells at a concentrations of 4 ng/mL. The finding that α-toxin exposure induces oxidative stress in cultured cells suggested that ROS could play a role in myonecrosis during gas gangrene. Accordingly, the importance of ROS generation in α-toxin–induced myotoxicity was demonstrated in vivo. Interestingly, in muscular dystrophies, oxidative stress contributes to muscle fiber degeneration [35]. Mammalian skeletal muscle is particularly sensitive to ROS-mediated damage, because muscle fiber membranes have a high content of unsaturated acyl chains, highly susceptible to oxidative damage [35]. Furthermore, the high concentration of heme-containing proteins, such as myoglobin, confers greater sensitivity to ROS, because such proteins efficiently convert H$_2$O$_2$ to more reactive species [35].

Different antioxidants prevented the cytotoxic effect of α-toxin. The protection by cell-permeable glutathione or its precursor NAC indicates that disruption of thiol metabolism is causally related to cytotoxicity. Furthermore, cytoprotection by MnTMPyP and tiron, implicates O$_2^-$ in the cytotoxic effect of α-toxin. The protection by extracellularly added catalase indicates that the membrane-permeable H$_2$O$_2$ also plays a role in α-toxin–induced cell death. Fe$^{2+}$ ions catalyze the formation of OH$^-$ radical from O$_2^-$ and H$_2$O$_2$ through the Fenton reaction. Different iron chelators prevent α-toxin–induced cell death, indicating that the labile iron pool [38] is required for α-toxin to induce cytotoxicity. Taken together, these data demonstrate that the cytotoxic effect of α-toxin is mediated by ROS, involves H$_2$O$_2$, and occurs in an iron-dependent way. Accordingly, α-toxin–induced cytotoxicity was significantly reduced by edaravone, an OH$^-$ radical trap [39] used clinically in acute infarctions to reduce tissue injury caused by cerebral and myocardial ischemia or reperfusion [40, 41].

Hence, the muscular damage induced by intramuscular injection of α-toxin was reduced in mice treated with MnTBAP and MnTMPyP, 2 SOD mimics that increase free radical degradation. Furthermore, different free radical traps (including tiron, PBN, and the OH$^-$ radical scavenger dimethyldiurea) significantly alleviate myonecrosis. In addition, the combination of NAC and deferroxamine, 2 synergistically acting antioxidants, also reduced the myotoxic effect of α-toxin. Taken together, these results indicate that O$_2^-$ and the OH$^-$ radical contribute to α-toxin–induced muscle damage and suggest that antioxidants could reduce the myonecrosis during gas gangrene. Accordingly, α-toxin–induced myotoxicity was also alleviated by edaravone.

The MEK/ERK pathway is activated by ROS and regulates cell survival and growth [42]. However, this cascade could trigger cell death in response to oxidative stimuli, such as ultraviolet radiation, cobalt chloride, and chemotherapeutic agents, including taxol, cisplatin, retinoids, and doxorubicin [43]. Evidence is also accumulating that the MEK/ERK pathway contributes to cell death in vivo. MEK1 inhibitors reduce tissue injury during cerebral and renal ischemia, as well as in traumatic brain injury in experimental animals [44–47]. To evaluate the role of MEK1 in the cytotoxic effect of α-toxin, 2 inhibitors were used: PD098059, which prevents MEK1 activation, and U0126, which inhibits its enzymatic activity. These inhibitors protect cultured cells from α-toxin and, remarkably, also reduce α-toxin–induced muscle damage when administered systemically to mice, indicating a causal relationship between MEK1 activation and myonecrosis. Thus, MEK1 could represent a novel therapeutic target for mitigating muscle damage during gas gangrene.

Excessive ROS production contributes to tissue damage in a variety of diseases. Reperfusion of ischemic tissue produces an influx of oxygen and inflammatory cells, leading to oxidative stress and tissue injury, which is reduced by antioxidants [48]. ROS are involved in the pathogenesis of sepsis and endotoxemia, and antioxidants exert beneficial effects in models of endotoxic and septic shock [49].

This work provides compelling evidence for the importance of ROS in the toxicity of the key virulence factor of gas gangrene and suggests the potential use of antioxidants, such as edaravone, as a novel therapeutic approach to reduce tissue damage during clostridial myonecrosis. Phospholipases C with substrate specificity similar to that of α-toxin are secreted by several bacteria. The results of this work suggest that the role of ROS in infections by those bacteria should be evaluated.

**Notes**

**Acknowledgments.** We thank Dr Monica Thelestam (Karolinska Institutet) for providing some of the reagents used in this study and Dr Julian Rood (Monash University) for providing the C. perfringens strain JIR 325.

**Financial support.** This work was supported by Costa Rica-United States of America Foundation (grant CT13-02 to M. F. D. and A. A. G.), Vicerrectoria de Investigación, Universidad de Costa Rica (grants 741-A3-503 to M. F. D. and A. A. G. and 741-B1-601 to A. A. G., M. F. D., and L. M. G.), and the UK Ministry of Defence.

**Potential conflicts of interest.** All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.
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