The cytotoxic activity of *Bacillus anthracis* lethal factor is inhibited by leukotriene A_4 hydrolase and metallopeptidase inhibitors

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The lethal factor of *Bacillus anthracis* is central to the pathogenesis of anthrax. Its mechanism of action is still unknown. Recently, on the basis of sequence similarities, we suggested that lethal factor might act similarly to leukotriene A_4 hydrolase (LTA_4), a bifunctional enzyme also endowed with a metallopeptidase activity. Here we show that some inhibitors of the LTA_4 hydrolase and metallopeptidase activities of LTA_4 hydrolase also affect the cytotoxicity of the anthrax lethal factor on macrophage cell lines, without interfering with the ability of the lethal factor to enter cells. These results support the proposal that anthrax lethal factor might display in the cytosol of intoxicated cells a peptidase activity similar to that of LTA_4 hydrolase.

INTRODUCTION

Bacillus anthracis secretes three distinct proteins: protective antigen (PA, 83 kDa), oedema factor (EF, 89 kDa) and lethal factor (LF, 83 kDa), none of which is toxic if tested separately [1,2]. After association with the cell surface, PA becomes able to mediate the binding, endocytosis and translocation into the cytosol of either EF or LF [1,2]. EF is a calmodulin-dependent adenylate cyclase [3]. LF is essential for the animal death induced by *B. anthracis* [1]. The anthrax lethal toxin (LeTx, composed of PA and LF) causes rapid death in laboratory animals and the cytolysis of peritoneal macrophages and macrophage cell lines [1,2,4–6]. It has been reported that macrophage death follows the formation of reactive oxygen intermediates, including superoxide anions, and that sublytic amounts of LeTx induce the release of interleukin 1 and tumour necrosis factor [6,7]. This suggests that the induction of an inflammatory response with a release of toxic compounds and mediators might be part of the mechanism of action of LF.

LF is a zinc-binding protein [8,9] that contains the His-Glu-Xaa-Xaa-His zinc-binding motif of zinc-endopeptidases. On this basis it was suggested that LF might act via a metalloproteinase activity [8,9]. More recently, we suggested that LF might act similarly to the leukotriene A_4 (LTA_4) hydrolase, following the identification of several sequence similarities between these two proteins [10]. LTA_4 hydrolase is a bifunctional enzyme that converts the unstable allylic epoxide LTA_4 [5(*S*)-*trans*-5,6-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid, produced by metabolism of arachidonic acid through the 5-lipoxygenase pathway] into leukotriene B4 [LTB_4; 5(*S*),12(*R*)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid] [11]. LTB_4 is present in many cells, including granulocytes, monocytes, macrophages, tracheocytes and synoviocytes, and is a potent activator of neutrophils and polymorphonuclear leucocytes. LTB_4 is an important mediator of inflammation: it stimulates aggregation and degranulation of human neutrophils, induces chemotaxis of leucocytes and promotes superoxide generation [12]. Moreover, leukotrienes regulate the expression of interleukin 1 [13].

LTA_4 hydrolase also has a clearly documented arginine aminopeptidase activity (EC 3.3.2.6) [14,15], dependent on the zinc-binding motif [16–18]. Because metallopeptidase inhibitors such as bestatin and captopril inhibit LTA_4 hydrolase activity, and, reciprocally, the natural substrate LTA_4 blocks the aminopeptidase activity [17–20], it has been proposed that LTA_4 hydrolase contains two partly overlapping active sites which influence each other [14].

To test the hypothesis that LF might be similar to LTA_4 hydrolase we have assayed the effect of inhibitors of LTA_4 hydrolase and of metallopeptidase activities on the cytotoxicity of LF on a well-characterized macrophage cell line.

MATERIALS and METHODS

Chemicals and reagents

Amino acid β-hydroxamates, bestatin, amastatin {[(2*S*,3*R*)-3-amino-2-hydroxy-5-methylhexanoyl]-Val-Val-Asp}, 5-aminosalicylic acid (5-amino-2-hydroxybenzoic acid), arphamenin A [(2*R*,5*S*)-5-amino-8-guanido-4-oxo-2-phenylmethyloctanoic acid] and arphamenin B [(2*R*,5*S*)-5-amino-8-guanido-4-oxo-2-*p*-hydroxyphenylmethyloctanoic acid] were from Sigma (Milan, Italy). The inhibitors of LTA_4 hydrolase activity {16: RP64485 (6-{[5-(3-phenylpropyl)-2-thienyl]thio}hexanoic acid); 33: RP64966 [2-({4-[5-(3-phenylpropyl)-2-thienyl]butyl}oxy)-acetic acid]; 62: RP65316 {7-[4-(4-aminobenzyl)phenyl]heptanoic acid}; 65: RP68129 {7-[4-(4-ureidobenzyl)phenyl]heptanoic acid}; 72: RP64122 {7-[4-(3-quinolylmethyl)phenyl]heptanoic acid}; 74: RP64693 {7-[4-(2-pyridylmethyl)phenyl]heptanoic acid}} and the LTB_4 antagonists RP66364 [2,4,5-(3-phenylpropyl)-2-thienylbutyoxyacetic acid] and RP69698 [5,6-bis-4,5-(4-methoxyphenyl)-2-oxazolylhexyltetrazol] were gifts from Rhône-Poulenc Rorer Recherche-Développement (Vitry/Seine, France). Their synthesis and purification have been described previously [21–23]. Inhibitors of 5-lipoxygenase activity L-739,010 {3-[1-(4-chlorobenzyl)-3-t-butylthio-t-isopropylindol-2-yl]-

Abbreviations used: EF, oedema factor; LeTx, anthrax lethal toxin composed of PA and LF; LF, lethal factor; LTA_4, leukotriene A_4 [5(*S*)-*trans*-5,6-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid]; LTB_4, leucotriene B_4 [5(*S*),12(*R*)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid]; PA, protective antigen.

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2-dimethylpropanoic acid} and L-663,536 {MK886; 3-[1-(4-chlorobenzyl)-3-t-butylthio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid}, whose preparation and characterization have been described previously [24,25] were kindly supplied by Merck Frosst (Pointe Claire-Dorval, Canada). Metalloproteinase inhibitors JMV 390-1 [$HONHCH_2CH(CH_2C_6H_5)$CO-Ile-Leu-OH], JMV 531-1 and JMV 531-2 [$HONHCH_2CH(CH_2C_6H_5)$CO-Val-Leu-OH (*R*- and *S*- isomers)], and JMV 550-1 and JMV 5-2 [$HONHCH_2CH(CH_2C_6H_5)$CO-Ile-Ala-OH (*R*- and *S*- isomers)], whose characterization is described in [26], were kindly provided by Dr. A. Fehrentz (CNRS, Montpellier, France). These compounds were solubilized in 50 mM Tris/HCl, pH 7.4, or in DMSO. Acetorphan {*N*-[(*R*,*S*)-acetylmercapto-2-benzoylpropanoyl]glycine} was obtained from Bioproject Laboratories (Paris, France) and was made 0.1 M in chloroform/cremophor A11/water (1:1:8, by vol.).

Cells

The toxin-sensitive murine macrophage-like cell line J774.A1 was obtained from the American Type Culture Collection (ATCC TIB 67, Rockville, MD, U.S.A.) and maintained in RPMI 1640 supplemented with 10 % (v/v) heat-inactivated fetal calf serum, 200 μg/ml penicillin and 100 μg/ml streptomycin, and kept at 37 °C in an air/CO_2 (19:1) atmosphere.

Toxin

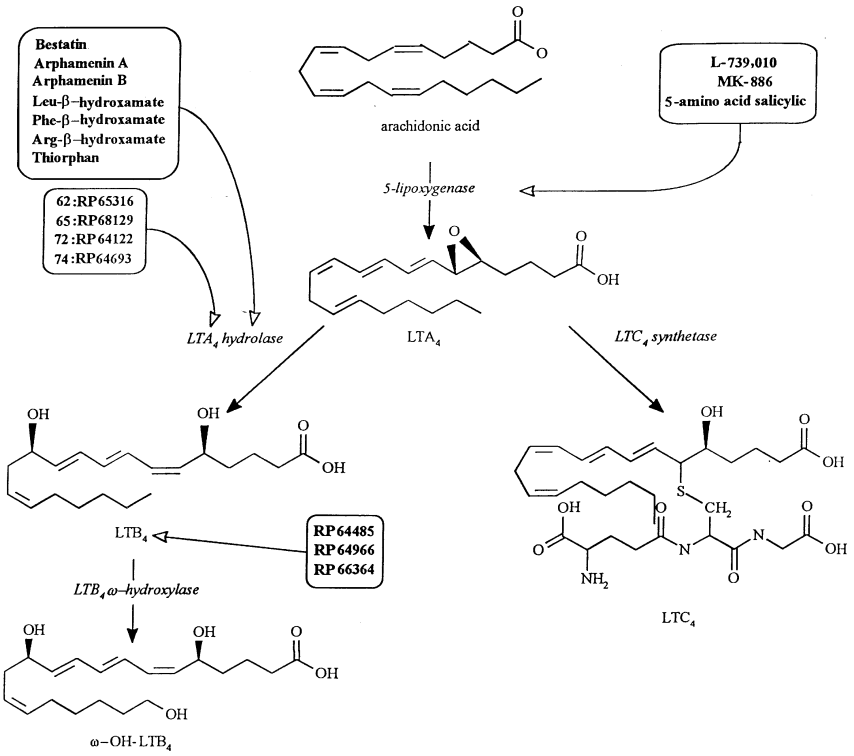
PA and LF were obtained from the culture supernatant fluids from *Bacillus anthracis* strains RP42 (PA^+, EF^-, LF^-) and RP4 (PA^-, EF^-, LF^+) respectively [27] and purified as previously described [28]. LF was radiolabelled with ^{125}I with the IODOGEN method according to a described procedure [29]: the specific activity of ^{125}I-labelled LF was 10^7 c.p.m./μg. The cytotoxicity of LF was not affected by this iodination procedure.

Cell binding and internalization of ^{125}I-labelled LF

Experiments were performed at 4 °C in Dulbecco's modified Eagle's medium, pH 7.2, without sodium bicarbonate and with 10 % (v/v) fetal calf serum. J774.A1 cells were incubated for 1 h with each inhibitor (concentration given in the text) and with PA (500 ng/ml). Cells were then washed with medium and incubation was continued for 1 h at 4 °C with the inhibitors and ^{125}I-labelled LF (50 ng/ml). Cells were washed again and incubated with prewarmed medium for 30 min at 37 °C. After washing, the cell monolayer was detached and recovered by centrifugation, and cell-associated radioactivity was measured.

Assay of LF cytotoxicity

The method of Hansen et al. [30] was followed with minor adaptations. Briefly, cells were harvested by scraping with a rubber 'policeman' and plated at a density of 5×10^4 cells per well in 96-well microtitre plates. When macrophages had reached confluence (after 2 or 3 days), PA (500 ng/ml) and various amounts of LF or LF plus inhibitor or inhibitor alone were added, and incubation was continued for 4 h. Tetrazolium salt (30 μl; 4 mg/ml in PBS) was added; after a 1.5 h incubation the reaction was stopped by the addition of 100 μl of 20 % (w/v) SDS, 50 % (v/v) dimethylformamide, pH 4.7. Plates were shaken



Scheme 1 Leukotriene biosynthetic pathway and sites of action of inhibitors and antagonists used in the present study

Enzyme names are written in italics, and enzyme inhibitors and LTB_4 antagonists are grouped in boxes: arrows indicate their sites of action.

Table 1 Inhibition of LF cytotoxicity by inhibitors of the leukotriene metabolic pathway

This table shows the protection of J774A.1 cells from the cytopathic effect of LF by using inhibitors and antagonists of the leukotriene pathway. Cells were preincubated with inhibitors for 30 min before the addition of LeTx. After 4 h, cytotoxicity was assayed as described in the Materials and methods section. IC_{50} values were determined as described in the legend to Figure 1. Abbreviation: n.i., no inhibition.

Inhibitor	Specificity	IC_{50} (M)
5-Aminosalicylic acid	5-Lipoxygenase	n.i.
L-739,010	5-Lipoxygenase	n.i.
L-663,536 (MK886)	5-Lipoxygenase	n.i.
16:RP64485	LTA_4 hydrolase	n.i.
33:RP64966	LTA_4 hydrolase	n.i.
62:RP65316	LTA_4 hydrolase	7×10^{-4}
65:RP68129	LTA_4 hydrolase	9×10^{-5}
72:RP64122	LTA_4 hydrolase	10^{-3}
74:RP64693	LTA_4 hydrolase	n.i.

overnight at room temperature, after which absorbance was measured at 534 nm in a multiwell plate reader.

RESULTS

Effect of inhibitors and antagonists of the leukotriene biosynthesis pathway

LTA_4, the substrate of LTA_4 hydrolase, is a product of arachidonic acid metabolism via the 5-lipoxygenase pathway (see Scheme 1). L-739,010, L-663,536 (MK886) and 5-aminosalicylic acid are potent inhibitors of 5-lipoxygenase *in vivo* and *in vitro* [24,25,31,32]. L-663,536 inhibits leukotriene biosynthesis in polymorphonuclear neutrophils [24] and the A23187-induced LTB_4 formation in leucocytes [25]. Here, these inhibitors were used to probe the involvement of leukotriene biosynthesis in the intoxication of macrophages by LF. None of these inhibitors was found to protect cells from the cytopathic effect of LF, even after a long preincubation time (Table 1).

LTA_4 is hydrolysed to LTB_4 or it is conjugated with glutathione to yield LTC_4 (see Scheme 1) and its metabolites (LTD_4 and LTE_4). Labaudinière et al. [21] have described a new class of specific LTA_4 hydrolase inhibitors: the ω-[(ω-arylalkyl)aryl]-alkanoic acids. Several compounds of this class were found to inhibit LTB_4 production in pig leucocytes at micromolar concentrations. Inhibition of LTA_4 hydrolase arrests the production of LTB_4, leaving unaffected both the synthesis of cysteinyl leukotriene C_4 and the spontaneous non-enzymic hydroxylation of LTA_4 to biologically inactive *trans*-LTB_4 isomers [21]. Table 1 and Figure 1 show that compounds 62:RP65316 and 65:RP68129 are effective inhibitors of LF cytotoxicity on macrophagic cells. Compound 72:RP64122, containing a quinoyl group, was less effective, and a derivative with a pyridyl group (74:RP64693) had no inhibitory effect on LF activity. No inhibition was also observed with molecules 16:RP64485 and 33:RP64966. To test the possibility that the toxicity of LF is mediated by an increased production of LTB_4, we used RP66364 and RP69698, two LTB_4 antagonists that act by inhibiting the binding of LTB_4 to its receptor [21–23]. None of them protected cells from the effect of LF. Control experiments showed that none of these inhibitors of leukotriene production affected cell binding and internalization of ^{125}I-labelled LF (see Table 3). Hence it appears that LF does not cause an increased release of LTB_4 from cells.

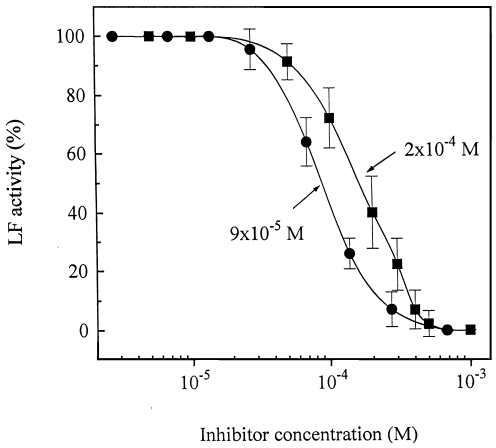


Figure 1 Inhibition of LF cytotoxicity

J774.A1 cells were preincubated for 30 min with the inhibitor and then treated with PA (500 ng/ml) and LF (100 ng/ml) in the presence of the indicated concentrations of 65:RP68129 (●) or phenylalanine β-hydroxamate (■). After 4 h, cell death was assayed as described in the Materials and methods section; data are expressed as a percentage of the effect of LF in cells not treated with inhibitors. Values and bars are the means ± S.E.M. for three experiments run in triplicate. Lines are data-fitting curves obtained, together with IC_{50} values, with the fit function of the program Origin.

Table 2 Inhibition of LF cytotoxicity by inihibitors of metalloproteases

Inhibition was tested as in Table 1, and IC_{50} values were determined as described in the legend to Figure 1. Abbreviations: n.i., no inhibition; n.d., not determined.

Inhibitor	Specificity	IC_{50} (M)
Bestatin	Aminopeptidases	$\sim 7 \times 10^{-4}$
Acetorphan	Aminopeptidases	n.d.
Amastatin	Aminopeptidase	n.i.
Arphamenin A	Aminopeptidase B	n.i.
Arphamenin B	Aminopeptidase B	$\sim 10^{-3}$
L-Leu β-hydroxamate	Metallopeptidase	$\sim 6 \times 10^{-4}$
D,L-Phe β-hydroxamate	Metallopeptidase	1.6×10^{-4}
Arg β hydroxamate	Metallopeptidase	$> 10^{-2}$
JMV 390-1	24.11, 24.15, 24.16 aminopeptidase	n.i.
JMV 531-1	24.11, 24.15, 24.16 aminopeptidase	n.i.
JMV 531-2	24.11, 24.15, 24.16 aminopeptidase	n.i.
JMV 550-1	24.11, 24.15, 24.16 aminopeptidase	3×10^{-3}
JMV 550-2	24.11, 24.16 aminopeptidase	n.i.

Metallopeptidase inhibitors

Bestatin, an inhibitor of various leucine and arginine aminopeptidases, is also a potent inhibitor of LTA_4 hydrolase [17,33]. This compound inhibits both the hydrolase and the aminopeptidase activities of the purified enzyme with IC_{50} values of 4 and 0.3 μM respectively, whereas bestatin analogues amastatin and epibestatin are ineffective [17]. Bestatin prevented intoxication of the macrophagic J774.A1 cells by PA and LF with an IC_{50} of 700 μM, whereas amastatin was ineffective (Table 2), a result that is in good agreement with those obtained with the isolated LTA_4 hydrolase enzyme. Arphamenins A and B, two inhibitors of arginine aminopeptidase [34], block the aminopeptidase activity of recombinant purified LTA_4 hydrolase at doses one-tenth of those needed to prevent the formation of

Table 3 Effect of inhibitors of LF cytotoxicity on the association of LF with cells

The amount of ^{125}I-labelled LF bound and internalized by J774.A1 cells in the absence or presence of PA was determined as described in the Materials and methods section in the presence of the indicated concentration of inhibitors of LF activity. Results are expressed in c.p.m. and are means ± S.E.M. for two different experiments run in triplicate.

Inhibitor ...	None (–)	Leucine β-hydroxamate (1 mM)	Phenylalanine β-hydroxamate (500 μM)	62:RP65316 (1 mM)	65:RP68129 (130 μM)
LF	1137 ± 305	1590 ± 75	1060 ± 80	1136 ± 450	984 ± 65
LF + PA	22727 ± 1220	18180 ± 1290	21212 ± 2650	22500 ± 230	17878 ± 797

LTB_4 [14]. In the macrophage cell line J774.A1, arphamenin B weakly inhibited LF cytotoxic activity (IC_{50} 1 mM) and arphamenin A had no effect. This might be due to lack of binding to the active site of LF and/or to poor cell permeability of these compounds.

Thiorphan, a general inhibitor of metallopeptidases, is also a good inhibitor of LTA_4 hydrolase (IC_{50} 5 μM and 10 mM for the aminopeptidase and hydrolase activity respectively) [17], but it is poorly permeable to cell membranes. In contrast, acetorphan is a lipophilic derivative of thiorphan that enters the cell cytosol, where it generates thiorphan [35]. At a concentration of 5 μM acetorphan inhibits 20 % of the LF effect on macrophages. It was not possible to determine the IC_{50} of acetorphan because at higher concentrations the chloroform and cremophor A11 necessary to solubilize acetorphan become toxic to cells.

Amino acid β-hydroxamates inhibit metallopeptidases and LTA4. Orning et al. [14] have shown that leucine and phenylalanine β-hydroxamates have a high affinity for LTA_4 hydrolase, whereas arginine β-hydroxamate has a lower affinity. Table 2 and Figure 1 show that leucine and phenylalanine β-hydroxamates are good inhibitors of LF cytotoxicity, whereas the arginine derivative is ineffective, which is similar to what was observed with isolated LTA_4 hydrolase.

JMV 390-1, JMV 531-1, JMV 531-2, JMV 550-1 and JMV 550-2 are novel metallopeptidase inhibitors whose synthesis and characterization have been recently described [26]. JMV 390-1 inhibits endopeptidases 24.11, 24.15, 24.16 and leucine and aminopeptidase N with K_i values of approx. 50 nM [26]. These compounds were ineffective on LF, indicating that LF does not belong to endopeptidase classes 24.11, 24.15 or 24.16.

Cell penetration of LF in the presence of inhibitors

LF consists of at least three parts [1]. The N-terminal portion is required for PA binding, the central domain contains four imperfect repeats of 19 amino acid residues, and the C-terminal domain presents the zinc-binding motif His-Glu-Xaa-Xaa-His, characteristic of zinc-dependent metallopeptidases [8]. To test the possibility that inhibitors of LF could act by interfering with its cell association, LF was radiolabelled with ^{125}I and its interaction with J774.A1 cells was studied. Table 3 shows that the specific association of ^{125}I-labelled LF to J774.A1 cells mediated by PA was not significantly affected by inhibitors that interfere with the cytotoxicity of LF.

DISCUSSION

LTA_4 hydrolase is a bifunctional enzyme containing two different active sites that may partly overlap, as indicated by the fact that metallopeptidase inhibitors inhibit LTA_4 hydrolase activity and LTA_4 inhibitors interfere with the aminopeptidase activity of the enzyme. Five categories of metallopeptidase inhibitor were reported to inhibit LTA_4 hydrolase: bestatin, arphamenins A and B, thiorphan, captopril and some amino acid hydroxamates [14,17,18,33]. Such inhibitors protect macrophage cells from the death induced by LF. Captopril, which inhibits both activities of isolated LTA_4 hydrolase [17,18] inhibits LF cytotoxicity poorly [8], most probably because of its low cell permeability. In the present study we show that analogues of LTA_4, which are specific LTA_4 hydrolase inhibitors, efficiently protect macrophages from LF cytotoxicity. A simple explanation of these results is that LF cytotoxicity is mediated by the activation of endogenous LTA_4 hydrolysis followed by overproduction of LTB_4 and, as a consequence, of interleukin 1 and tumour necrosis factor. However, this possibility seems unlikely because inhibitors of the biosynthesis of LTA_4 or of LTB_4 metabolism do not interfere with the action of LF. An alternative possibility is that LF is an LTA_4 hydrolase-like enzyme endowed with an aminopeptidase activity, responsible for the modification of an as yet unidentified intracellular protein target. This hypothesis establishes a parallelism with the action of other bacterial toxins produced by anaerobes, such as tetanus and botulinum neurotoxins. These clostridial neurotoxins, which share with LF the zinc-binding motif, were recently demonstrated to be intracellular zinc endopeptidases very specific for three components of the neuroexocytosis apparatus [37]. As with LF, these neurotoxins do not exhibit any proteolytic activity against a variety of peptides and peptide analogues. The demonstration of the proteolytic activity of LF might require the identification of the cellular proteolytic substrate of LTA_4 or that of the factor(s) that control the release of cytokines in macrophages.

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