Serum protease cleavage of *Bacillus anthracis* protective antigen

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The protective antigen component of anthrax lethal toxin, produced *in vitro*, has a molecular mass of 83 kDa. Cell-culture studies by others have demonstrated that upon binding of the 83 kDa protective antigen to cell-surface receptors, the protein is cleaved by an unidentified cell-associated protease activity. The resultant 63 kDa protein then binds lethal factor to form lethal toxin, which has been proposed to be internalized by endocytosis. We found that, in the blood of infected animals, the protective antigen exists primarily as a 63 kDa protein and appears to be complexed with the lethal factor component of the toxin. Conversion of protective antigen from 83 to 63 kDa was catalysed by a calcium-dependent, heat-labile serum protease. Except for being complexed to protective antigen, there was no apparent alteration of lethal factor during the course of anthrax infection. The protective antigen-cleaving protease appeared to be ubiquitous among a wide range of animal species, including primates, horses, goats, sheep, dogs, cats and rodents.

Introduction

*Bacillus anthracis*, the aetiological agent of anthrax, possesses three primary, plasmid-encoded, virulence factors; a poly-D-glutamic acid capsule, encoded by the pXO2 plasmid (Green *et al.*, 1985; Makino *et al.*, 1988, 1989; Uchida *et al.*, 1985), and lethal and oedema toxins encoded by the pXO1 plasmid (Leppla *et al.*, 1985; Mikesell *et al.*, 1983; Uchida *et al.*, 1986). Lethal toxin is composed of two proteins; lethal factor (LF, 90-2 kDa, Bragg & Robertson, 1989) and protective antigen (PA, 82-7 kDa, Welkos *et al.*, 1988). Similarly, oedema toxin is composed of a calmodulin-dependent adenylate cyclase, termed oedema factor (EF, 88-8 kDa, Robertson *et al.*, 1988) and PA (Beall *et al.*, 1962; Fish & Lincoln, 1967; Leppla, 1984; Leppla *et al.*, 1985). Earlier SDS-PAGE analyses suggested a size for PA of 85 kDa (Vodkin & Leppla, 1983; Leppla *et al.*, 1985), whereas subsequent sequence analysis revealed it to be approximately 83 kDa (Welkos *et al.*, 1988). Leppla *et al.* (1988) reported that the 83 kDa PA (then 85 kDa) does not bind LF or EF. However, upon binding to surface receptors of target cells grown *in vitro* (Friedlander, 1986, 1990; O'Brien *et al.*, 1985), an unidentified, cell-associated protease cleaves the 83 kDa PA (PA83), resulting in the release of a 20 kDa fragment. The activated 63 kDa (PA63) fragment retained at the cell surface binds LF or EF and, as proposed, the complex enters the cell by endocytosis (Leppla *et al.*, 1988). Besides its central role in the binding of the two toxins by target cells, PA also plays an important role in eliciting a protective immune response against anthrax (Ivins *et al.*, 1986; Ivins & Welkos, 1988; Ivins *et al.*, 1990; Klein *et al.*, 1962; Puziss & Wright, 1963; Turnbull *et al.*, 1988). No vaccine lacking PA has been found to be protective (Ezzell & Abshire, 1988; Hambleton *et al.*, 1984; Ivins *et al.*, 1986; Ivins & Welkos, 1988). PA bound to aluminium salts is the principal antigen in the US and UK human anthrax vaccines (Brachman *et al.*, 1962; Puziss & Wright, 1963, and has a molecular mass primarily of 83 kDa. However, veterinary vaccines are composed of viable spores of *B. anthracis* Sterne strain, a non-encapsulated toxigenic variant (Sterne, 1939). The PA produced by veterinary vaccines has also been generally assumed to have a mass of 83 kDa in vaccinated animals. Although PA in both human and veterinary vaccines is protective, studies with laboratory animals have shown no definitive correlation between antibody titres to PA and protection. For example, guinea pigs vaccinated with the human vaccine develop significantly higher titres than those receiving the veterinary vaccine, yet they are less well protected against lethal challenge (Broster & Hibbs, 1990; Ivins & Welkos, 1988; Little & Knudson, 1986).

*Abbreviations:* PAP, protective antigen protease; PA, protective antigen; LF, lethal factor; EF, oedema factor; PA83, 83 kDa form of protective antigen; PA63, 63 kDa form of protective antigen.

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These data suggest there may be differences between in the in-vitro-produced PA bound to aluminium salts (human vaccine) and the in-vitro-produced PA (veterinary vaccine) with regard to its form and/or its presentation to the host immune system. These differences may influence the type of host immune response (i.e. cell-mediated versus humoral immunity) and/or expression of the necessary antigenic epitopes required for protection.

In previous unpublished studies, we found that the majority of the PA in the culture supernatants of B. anthracis strains grown in media containing 50% or 100% guinea pig serum was 63 kDa rather than 83 kDa. Through Western blot analysis of sera from normal and infected animals, we have now detected a protease activity that cleaves PA83 to form PA63. Evidence has also been obtained that PA exists in the blood of infected animals primarily as PA63 complexed with LF (Ezzell et al., 1990b).

**Methods**

Bacterial strains. The virulent toxigenic, encapsulated Bacillus anthracis Ames strain (pXO1, pXO2) was obtained as frozen spor suspensions from the US Army Medical Research Institute of Infectious Diseases culture collection, Fort Detrick, MD. Spore suspensions were prepared (Ezzell & Abshire, 1988) and animals were infected intramuscularly with 0.1 ml of inoculum containing 1000 spores.

Sera and plasma. Blood from guinea pigs and rats was obtained by intracardiac puncture with a 23-gauge needle after animals were anaesthetized by intramuscular injection of 30 mg ketamine per kg (Vetalan) and 6 mg of xylazine per kg (Rompun). Plasma was obtained from blood of rhesus and cynomologous monkeys, horses, cows, goats and sheep was obtained by staff veterinarians from the US Army Medical Research Institute of Infectious Diseases; dog and cat sera were obtained from a local veterinary clinic. Human sera were obtained from medical research volunteer subjects who had not received anthrax vaccine.

Western blots. Analyses of serum samples containing PA were performed by diluting 1:25 in 2.5% (w/v) SDS plus 10 mM-2-mercaptoethanol, 10 mM-EDTA and 25 mM-Tris, with 0.01% bromphenol blue, (pH 6.8) boiled for 5 min, and the proteins separated by electrophoresis on the PhastGel System (Pharmacia), using 10 to 15% (w/v) gradient acrylamide SDS gels. For native gels, samples were diluted 1:25 in 25 mM-Tris with 0.01% bromphenol blue, (pH 8.8) and electrophoresis was performed using 8-25% (w/v) gradient acrylamide gels (PhastGel). After electrophoresis, proteins were transblotted (Towbin et al., 1979) for 18 to 20 h by direct contact of the plastic-backed gel with nitrocellulose (Bio-Rad). The gel and nitrocellulose were sandwiched between double layers of Whatman no. 3 filter paper and two glass plates, under a 500 g weight. For SDS gels, the nitrocellulose and filter paper were dampened with 20% (v/v) methanol in 25 mM-Tris plus 200 mM-glycine, pH 8.9; whereas for native gels, methanol was omitted. The nitrocellulose was blocked with 5% (w/v) powdered milk quench buffer, washed, and reacted with monoclonal antibody (mAb) to either PA or LF. The PA mAbs were supplied by S. Little (this Institute) as ascites fluid designated as PA11-18C2-1-1, PA1-2D3 and PA1 3B6 (Little et al., 1988) and the LF MAs as LF311-SD2 and LF311-IOG3. MAs were diluted in 10 mM-sodium-phosphate-buffered saline (PBS) supplemented with 0.3% Tween 20 and 0.5% gelatin, pH 7.3 (PBSTG). After three washes with PBS plus 0.3% Tween 20, goat anti-mouse IgG, IgA, IgM/alkaline phosphatase conjugate (Kirkegaard & Perry) was applied for 30 min at 37°C. The nitrocellulose was washed four times with PBS plus 0.3% Tween-20 (PBST), and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium stain (BCIP/NTB) (Bio-Rad) was added for 30 min.

**PA affinity column.** The PA MAs used for staining Western blots were purified from ascites fluid using Affi-Gel Blue (Ezzell et al., 1990a), and were coupled to Affi-Gel Hz gel (Bio-Rad) according to the manufacturer’s instructions. The column was controlled, and the effluent monitored and collected, with a FPLC system (Pharmacia). Samples were loaded at 0.1 ml min⁻¹ in PBS plus 0.02% sodium azide, the column was washed, and bound PA was eluted with 3 M-sodium phosphate-buffered saline (PBS) containing 0.3% Tween-20 (PBST) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium stain (BCIP/NTB) (Bio-Rad) was added for 30 min.

**Protease inhibitors.** Each inhibitor was added to fresh native guinea pig serum and incubated at 37°C for 2 h. The PA was separated by electrophoresis on a 1% (w/v) gel stained with 0.2% (w/v) bromphenol blue, (pH 6.8) for 18 to 20 h and the PA bands were visualized by staining for 30 min with 0.2% BCIP/NTB and 0.02% sodium azide. The PA was quantitated by scanning the gel and measuring the optical density at 450 nm. The PA was purified from ascites fluid using Affi-Gel Blue (Ezzell et al., 1990a), and was coupled to Affi-Gel Hz gel (Bio-Rad) according to the manufacturer’s instructions. The column was controlled, and the effluent monitored and collected, with a FPLC system (Pharmacia). Samples were loaded at 0.1 ml min⁻¹ in PBS plus 0.02% sodium azide, the column was washed, and bound PA was eluted with 3 M-sodium phosphate-buffered saline (PBS) containing 0.3% Tween-20 (PBST) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium stain (BCIP/NTB) (Bio-Rad) was added for 30 min.

**Results and Discussion**

**Analysis of infected guinea pigs**

We infected female Hartley guinea pigs (500 to 600 g) by intramuscular injection with 1000 c.f.u. of B. anthracis Ames strain spores and collected blood shortly before and at death. Guinea pigs, which overtly appeared to be normal and healthy throughout most of the infection, were judged to be approaching death (within 1 to 2 h) upon becoming inactive or lethargic. Western blot analysis of the toxin in the serum of these animals, following electrophoresis on SDS gels, revealed that only PA63 was present with no detectable levels of PA83 (Fig. 1b). In all animals where toxin was detected, LF and PA formed what appeared to be a high-molecular-mass
complex, as determined by Western blots after electrophoresis on native gels (Fig. 1c, d). The putative complex was supported by the observation that on native gels, the majority of the PA and LF did not migrate from the upper stacking gel, but instead remained at or just below the site of sample application by the PhastGel electrophoresis unit. Other material appeared at the interface (I) as denoted by the arrows. This material stained with both the PA and LF mAbs and was present in both the standards and serum samples. As discussed below, trace amounts of PA were detected that were not complexed with LF and appeared as a diffuse band on the native Western blots stained with PA mAbs (Fig. 1d).

**In-vitro analysis of PA cleavage in serum**

Studies were performed to determine the events leading to the appearance of PA63 in infected animals. Initially, we incubated purified PA83 with native serum and heat-inactivated serum (56 °C for 30 min). Samples were withdrawn at the indicated time intervals (Fig. 2) and analysed by Western blotting. As shown, in the presence of native serum most of the PA83 was converted to PA63 by 30 min. However in heated serum, PA83 was not converted, thereby demonstrating that the protease involved was heat-labile. Further experiments demonstrated that the PA protease (PAP) was inactivated between 45 and 50 °C (data not shown). As the serum used was filter-sterilized and thereby devoid of blood cells, we concluded that the PAP was not cell-associated, but rather, was free in the serum.

**Protease inhibitors**

To characterize, and possibly identify, the role of PAP in normal serum, a wide range of protease inhibitors was tested. The only two compounds that totally inhibited PA83 conversion to PA63 were EDTA and EGTA. Antipain showed approximately 50% inhibition, but only at high concentrations (1 mg ml\(^{-1}\)), whereas the calcium-activated neutral protease inhibitor was estimated to give 33% inhibition at 3 U ml\(^{-1}\). Both of these determinations were made subjectively by comparing the relative intensities of the PA63 and PA83 bands on Western blots. Several other inhibitors, at the indicated concentrations, had no effect (see Methods).
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Fig. 2. Western blot of samples taken at the indicated time intervals from reaction mixtures composed of (a) PA83 (100 μg ml⁻¹) and native serum, (b) PA83 and heated serum, (c) PA83 and 10 mM-HEPES buffer, pH 7.0. Samples were treated with SDS and separated on SDS PhastGels before transblotting and staining with PA mAbs. C, PA83 control standard.

Table 1. Retention of PA and LF by PA affinity column after incubation in native or heat-inactivated guinea pig serum

<table>
<thead>
<tr>
<th></th>
<th>Native serum</th>
<th>Heat-inactivated serum</th>
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<tbody>
<tr>
<td></td>
<td>PA83</td>
<td>PA83+LF</td>
</tr>
<tr>
<td>Final PA molecular mass (kDa)*</td>
<td>63</td>
<td>63</td>
</tr>
<tr>
<td>Retained by column†</td>
<td>PA</td>
<td>PA+LF</td>
</tr>
</tbody>
</table>

* Determined by Western blot analysis following serum incubation. † Determined by Western blot analysis of 2 mM-sodium isothiocyanate eluant.

We made several observations based on the inhibitor studies. First, blood drawn from terminally ill animals directly into EDTA contained only PA63. Second, since heparin did not inhibit PAP, its proteolytic activity could be shown in heparinized plasma. Thirdly, PAP was fully active in sera from normal, healthy animals. From these observations, we concluded that PAP does not require activation as part of a clotting cascade and is not produced in response to infection. In studies not presented, we determined that 100 mM-CaCl₂ completely reversed both EDTA and EGTA inhibition, whereas 100 mM-MgSO₄ partially restored activity. These data strongly suggest that PAP is calcium-dependent. Manganese (MnCl₂) and ferrous (FeCl₃) ions were ineffective at 100 mM, whereas copper (CuSO₄) and zinc (ZnCl₂) were, in themselves, inhibitory at 1 mM.

Fig. 3. Nitrocellulose strips printed with a band of LF and placed in reaction mixtures containing serum and PA83 at 0 μg ml⁻¹ (a), 10 μg ml⁻¹ (b), and 100 μg ml⁻¹ (c). Native guinea pig serum was used for strips 1, 3, and 5; heat-inactivated serum was used for strips 2, 4, and 6. The presence of the bound PA was detected using anti-PA mAbs (top). The molecular mass of the PA after 2 h incubation was determined by Western blot analysis as shown beneath the respective strips (bottom).
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PA protease activity in various animals

PAP activity was found in a wide range of animal species. Conversion of PA83 to PA63 was detected in serum from humans, monkeys (rhesus and cynomolgous), dogs, cats, guinea pigs (Hartley and Strain-13), rats (Fischer 344 and NIH Black), horses, goats and sheep. In every case, PAP activity in the sera from these animals was inhibited by EDTA and inactivated by heating at 56 °C for 30 min.

Interaction of PA63 with LF

The in vitro and in vivo interactions of PA63 with LF were compared. As previously reported, PA83 does not bind LF, but according to the cell-membrane model for toxin activation (LePplla et al., 1988; Singh et al., 1989) PA83 must be converted to PA63 for binding to occur. Likewise, PA in serum must also be converted to PA63 for binding of LF. As shown in Table 1, PA83 (250 μg ml⁻¹) incubated at 37 °C for 2 h in heat-inactivated serum, or PA that has been converted to PA63 in native serum, was retained by the PA affinity column, as determined by SDS-PAGE and Western blots of the 2 M-sodium isothiocyanate eluates. When LF (50 μg ml⁻¹) was added to native serum containing PA83 which was converted by PAP to PA63, the LF was also retained by the column, but it was not retained when added to PA83 in heat-inactivated serum. Since the PA mAbs used to make the affinity column do not cross-react with LF, we concluded that LF could have been retained by the column only through its association with PA63.

The requirement for PA83 to be converted to PA63 for LF binding to occur was also demonstrated using nitrocellulose strips on which purified LF had been directly printed using a PhastGel TC applicator. After the printed nitrocellulose was blocked with quench buffer (Ezzell & Abshire, 1988), the strips were incubated for 2 h at 37 °C in either native serum or heat-inactivated serum to which purified PA83 was added at 10 μg ml⁻¹ (B-strips, Fig. 3) and 100 μg ml⁻¹ (C-strips, Fig. 3). At the end of the incubation, the PA in the sera was subjected to SDS-PAGE and Western blotting using anti-PA antibodies to determine any alterations in molecular mass (Fig. 3, bottom). PA that had become bound to the LF band was detected with PA mAbs and stained with goat anti-mouse/alkaline phosphatase conjugate and substrate (Fig. 3, top). The PA83, converted to PA63 in native serum, reacted with the printed LF band (strips B3 and C5). Conversely, the PA83 in the heat-inactivated serum (strips B4 and C6) was stained at background levels (strips A1 and A2). In subsequent experiments, we detected trace amounts of PA in the LF preparations used to print the strips which may account for the faint staining of strips A1 and A2.

Studies were conducted to characterize the interaction of PA63 and LF. When PA83 and LF were incubated in native serum, the PA63 resulting from PAP activity interacted with LF to form a high-molecular-mass complex, which remained at the site of application on native PhastGels. Both PA and LF mAbs stained this
complex, as shown in lane 1 of Fig. 4(a) and 4(b), respectively. However, when PAP activity was destroyed by heat inactivation, the PA83 was not converted to PA63, and both PA and LF migrated as separate bands (lane 2, Fig. 4a, b). When incubated in fresh serum without LF and analysed on native gels, the resulting PA63 did not form a distinct band (Fig. 1, lanes 2 through 5; Fig. 4, lane 3). This may indicate that the PA molecules do not exist as a single charged species or, more likely, that the PA molecules interact to form complexes. Originally observed by S. Leppla (personal communication), PA63 tends to aggregate, except at high pH (≥9). Therefore, the smeared or diffuse band on native gels may stem from gradual disassociation of the aggregates during electrophoresis at pH 8-8. To date, we have not detected any alteration of LF (Fig. 1a) in serum other than being complexed to PA63.

The results of the in vitro studies with PA and LF are consistent with observations made during analysis of sera from infected animals, which indicated that LF and most of the PA molecules do not exist individually in serum, but rather as a complex. In all cases, both the PA and LF were associated with the high-molecular-mass complex at the site of application to the gel (Fig. 1c, d).

The finding that PA and LF exist in the serum of infected animals as a complex poses questions regarding the role of the complex in pathogenesis and in the immune response of the host. One should not conclude from these studies that the proteolytic cleavage of PA83 in the serum and the formation of PA63/LF complexes negate the model proposed by Leppla et al. (1988), in which PA83 first binds to a host-cell receptor before it is cleaved by an unidentified cell-associated protease. Although the cell-surface model is based entirely on in vitro studies with cell cultures and not on in vivo studies with infected animals, it may be the principal means by which PA is activated in tissues where access to the serum protease is minimal. Based on our studies, we propose the following model for formation of the complexes in serum. As PA83 emerges from the bacilli, it is rapidly converted to PA63, and an undetermined number of PA63 molecules either complex with LF or EF, or remain free in the form of PA-aggregates or single molecules.

The PA/LF complexes may represent an alternative means of antigen presentation to the host which has not been previously considered and may be relevant to which strategies are used to design new anthrax vaccines. It is well known that veterinary live spore anthrax vaccines (Sterne strain) provide better and longer lasting protection than vaccines composed of PA bound to aluminium compounds (US and UK human vaccines). This is true even through the latter usually elicit a higher antibody titre to PA (Ivins & Welkos, 1988). Other than the fact that veterinary vaccines also elicit antibody titres to LF and EF, the vaccinated host may develop an immune response to the PA component of sublethal quantities of lethal toxin and/or oedema toxin. The superior protection afforded by the vaccines may reflect differences in antigenic epitopes expressed by PA when it is complexed to LF and EF as opposed to PA bound to aluminium adjuvants.

Historically, differences between anthrax toxin produced in vitro and in vivo were suggested repeatedly during the 1950's and 1960's. Smith et al. (1956) found that even though the immunizing antigen purified from in vitro sources by Strange & Belton (1954) could replace Factor II (PA) of the toxin from guinea pig plasma, it was not identical to it. In their review of literature pertaining to anthrax toxin, Lincoln & Fish (1970) stated that while in vivo and in vitro toxins are at least quite closely related (Harris-Smith et al., 1958; Smith et al., 1956; Stanley & Smith, 1963), they differ in their serological characteristics (Sargeant et al., 1960; Smith & Gallop, 1956; Smith et al., 1956; Stanley & Smith, 1963) and the rapidity with which they kill (Fish & Lincoln, 1968). Fish & Lincoln (1968) were clearly correct when they concluded that the "in vivo toxin exists as an aggregate whose biological and serological activity depends upon its particular composition or configuration, or both".

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