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Abstract. A panel of human anti-anthrax protective antigen IgG1 monoclonal antibodies were evaluated to determine the mechanism of toxin neutralization. AVP-22G12, AVP-1C6 and AVP-21D9 bound to the protective antigen with picomolar affinities to distinct non-overlapping linear epitopes. Two of the antibodies neutralized the anthrax toxin by completely inhibiting the protective antigen oligomer assembly process *in vitro*.

Keywords: Human monoclonal antibodies, bacillus anthracis, protective antigen

1. Introduction

Inhalation of anthrax spores can be fatal if not treated immediately with antibiotics. The interval between the onset of symptoms and death can be very short, hours rather than days in situations where aggressive treatment is not applied. This is in part due to the pathogen *Bacillus anthracis*, which utilizes two distinct strategies to evade immune surveillance, thus facilitate dissemination throughout the body and a rapid rise in bacteremia. Initially, upon exposure to spores, the capsule composed of poly-D-glutamic acid provides a physical barrier to circumvent phagocytosis. Secondly, via the concerted effect of three proteins, protective antigen (PA), lethal factor (LF), and edema factor (EF) [1–3] the immune response against the invading bacteria is compromised. PA is a 83 kD protein that binds to tumor endothelial marker-8 (TEM-8) [4] or human capillary morphogenesis protein –2 (CMG-2) [5], collectively called the anthrax toxin receptors (ATRs). The receptors are found on both macrophages and endothelial cells. The membrane bound PA83 is cleaved by a furin or furin like protease to PA63, which spontaneously assembles into a 7-member oligomer. A hypothetical model of the receptor bound membrane-inserted PA pore has recently been described [6]. This PA63 heptamer facilitates the entry of LF/EF, which then exert an initial immunosuppressive [7] and ultimately toxic effect [8]. LF is a protease that inhibits mitogen-activated protein kinase-kinase [9], which reduces the cytokine production by macrophages and ultimately leads to cell death. EF is an adenylate cyclase that generates cyclic AMP in eukaryotic cells [10] and impairs the ability of neutrophils to engulf bacteria.

The anti-toxin activity of both polyclonal and monoclonal anti-PA antibodies is well documented [11–13]. Moreover it has been shown that the combination of toxin neutralization with antibodies and inhibition of bacterial growth by antibiotics is very effective in a small animal anthrax challenge model [14]. Anti-anthrax toxin antibodies, which neutralize the immunosuppressive effects of PA/LF/EF, working in concert

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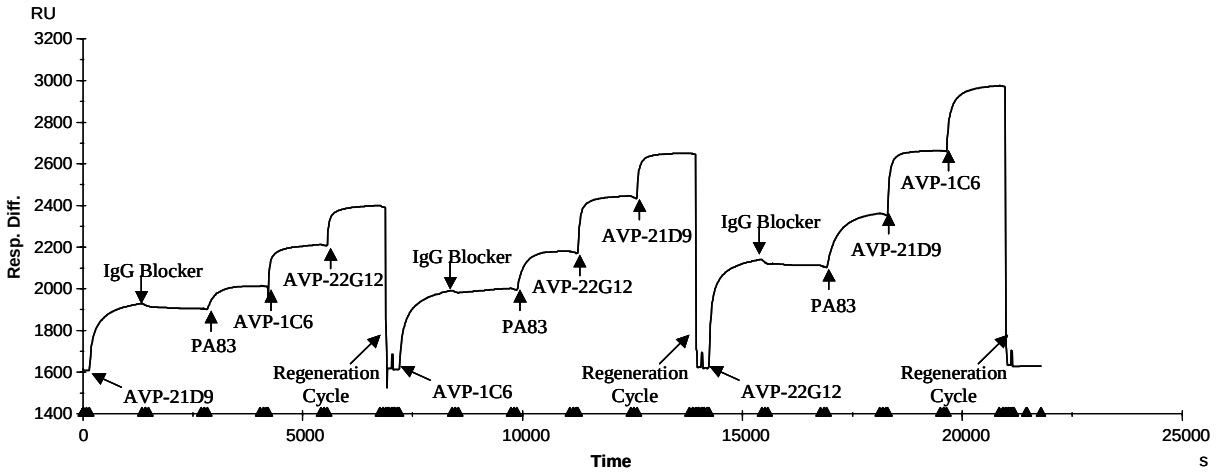

Fig. 1. Sensogram of sequentially bound anti-PA antibodies, demonstrating that irrespective of the order of binding all three human monoclonal anti-PA83 antibodies (AVP-21D9, 22G12 and 1C6) can bind to a single PA83 molecule. The CM-5 chip was coated with a anti human IgG capture antibody, first antibody was applied, followed by pooled human IgG blocking antibody, PA83 followed sequentially with the 2nd and 3rd antibodies. The order in which the antibodies were applied was changed in subsequent runs to cover all permutations.

with conventional antibiotics may provide additional protection in the event of an anthrax spore exposure.

A panel of 3 high affinity fully human anti-anthrax PA antibodies that neutralize anthrax toxin *in vitro* and *in vivo* generated from anthrax vaccine adsorbed (AVA) vaccinated donors have been described [15]. Here the mechanism of antibody mediated toxin neutralization is described.

2. Materials and methods

The binding of the monoclonal antibodies to distinct or overlapping epitopes on PA83 was determined by surface plasmon resonance. Anti-human IgG capture antibody (goat anti-human Fc gamma specific, Jackson Immuno Research, PA) was coupled to a CM-5 chip through standard amine chemistry using an immobilization guide provide by the Biacore (Biacore Inc, NJ), whereby a response unit (RU) value of 10,000 units was approached. The first human monoclonal antibody was applied, followed by pooled human (non-immune) IgG blocking antibody, PA83 (PA83, and LF both purchased from List Biological Labs, CA) followed sequentially with the 2nd and 3rd monoclonal antibodies. The order in which the antibodies were applied was changed in subsequent runs to cover all permutations. The test reagents (PA, MAbs) were applied at 20 μg/ml in HBS-EP buffer provide by Biacore, the blocking antibody human IgG1/κ (Sigma Co, MO) was used at 40 μg/ml. The resulting binding data is presented in a sensogram Fig. 1.

To map the antibody recognition to distinct portion of the PA83, western blot analysis was undertaken. In Fig. 2(a), a schematic of fragments of PA83 generated by trypsin and chymotrypsin digestion based on the sequences and mapping studies previously described [16, 17] is shown. Intact PA83, trypsin, chymotrypsin or combination of trypsin and chymotrypsin generated PA fragments were probed with human monoclonal antibodies AVP-1C6, AVP-22G12 and AVP-21D9 in a western blot (Fig. 2(b)).

To investigate whether the antibody bound to PA83 blocked subsequent processing, PA83 was pre-incubated with antibodies, then, treated with trypsin, and the resulting mixtures were analysed by SDS-PAGE and Coomassie staining (Fig. 2(c)).

The role of antibodies in inhibiting the binding of lethal factor to PA 63 oligomer was again investigated by surface plasmon resonance. PA63 (PA63 oligomer, List Laboratories, CA) was immobilized on a BiaCore CM5 chip, essentially as described above, antibody captured and lethal factor applied. The binding events were monitored and are presented in a sensogram (Fig. 3(a)). Also the role of antibodies in blocking binding of the anthrax toxin PA83 to its receptor was investigated in a similar manner. Anti-human Fc gamma was conjugated to the CM5 chip, human monoclonal anti-PA antibody captured, PA83 bound and soluble anthrax toxin receptor applied, again each binding event was monitored by surface plasmon resonance (Fig. 3(b)).

Finally, to determine if the antibodies inhibited the formation of PA63 heptamer equimolar amounts of

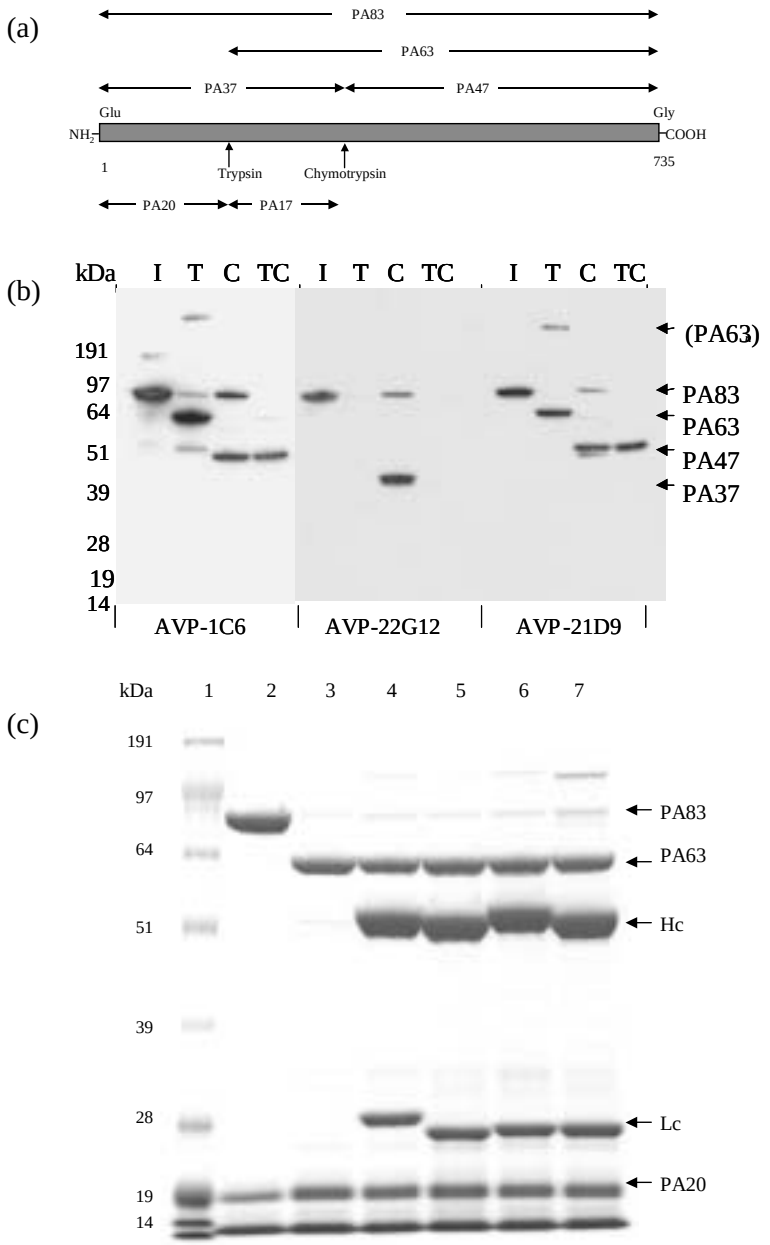

Fig. 2. Identification of protective domains on PA83. (a) A schematic of fragments of PA83 generated by trypsin and chymotrypsin digest based on the sequences and mapping studies [16,17]. (b) Western blot analysis of intact (I), trypsin (T), chymotrypsin (C) and combination of trypsin and chymotrypsin (T+C) generated PA fragments probed with AVP-1C6, AVP-22G12 or AVP-21D9. (c) Coomassie stained SDS-PAGE of antibody bound PA83 treated with trypsin. Lane (1) Molecular weight markers; (2) PA83 no trypsin; (3) no antibody; (4) AVP-22G12; (5) AVP-21D9; (6) AVP-1C6; (7) AVP-1451 isotype matched human IgG anti-tetanus control.

PA83 (0.25 nmol) and anti-PA antibody (0.25 nmol) were mixed in 70 μl of PBS. After 30 minutes incubation at room temperature the mixture was transferred to 4°C and 10 μl of ice-cold trypsin (50 μg/ml) was added for 5 minutes. Trypsin was inactivated by the addition of 5 μl trypsin and chymotrypsin inhibitor (10 mg/ml). Citric phosphate buffer (115 μl of 0.1 M, pH5.0) was then added to bring the pH to 5.0 to facilitate PA63 oligomerization. SDS loading buffer was added and the mixtures placed on boiling water for 10 minutes. Polypeptides were separated in a 10% Bis-Tris gel under reducing condition. Protein bands were visualized

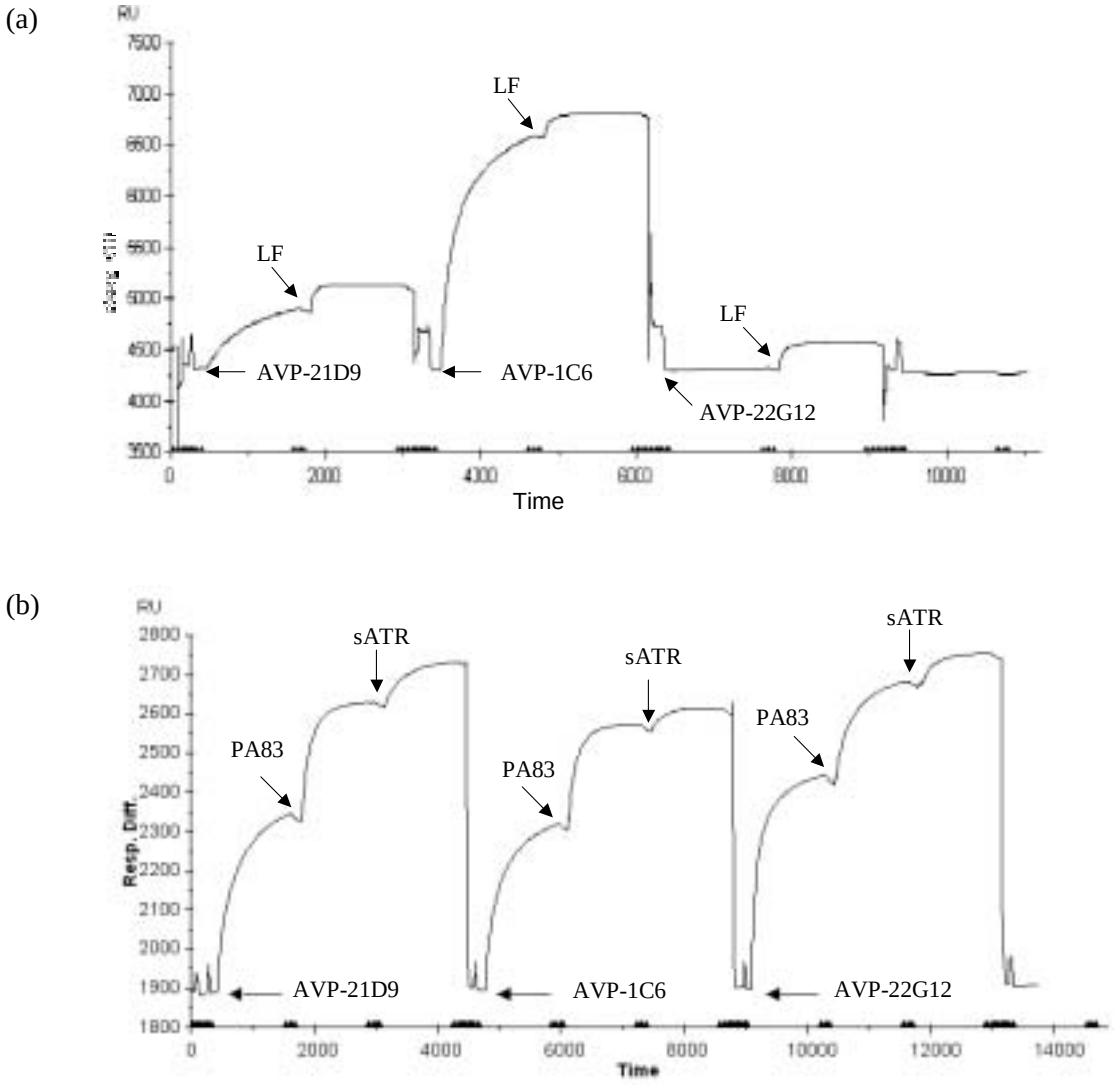

Fig. 3. Interaction of human anti-anthrax PA antibodies with (a) PA63 and lethal factor and (b) PA83 and soluble anthrax toxin receptor by surface plasmon resonance. (a) PA 63 oligomer was immobilized on a BiaCore CM5 chip, antibody captured and lethal factor applied. (b) Anti-human Fc gamma conjugated to BiaCore CM5 chip, human monoclonal anti-PA83 antibody captured, PA83 bound and soluble anthrax toxin receptor applied (plasmid encoding full-length ATR a kind gift from Dr. Ken Bradley, UCLA).

by Coomassie blue staining (Fig. 4).

3. Results and discussion

Sequential binding of anti-PA antibodies to PA83 indicated that they bound to distinct non-overlapping epitopes (Fig. 1). Western blot analysis of intact PA 83 indicated all three antibodies recognized linear epitopes on PA83 (Fig. 2(a,b)). AVP-21D9 and AVP-1C6 mapped to the carboxyl domain PA47 generated by chymotrypsin digest. The binding of AVP-22G12 mapped to the chymotrypsin generated fragment PA37 which contains the natural furin cleavage site [17]. Treating PA83 with trypsin abolished AVP-22G12 binding in the western blot (Fig. 2(b)). Initially this suggested that AVP-22G12 itself might act by inhibiting the cleavage of PA83 to PA63. To test this hypothesis, PA83 was pre-incubated with antibody prior to trypsin addition, the resulting mixture was analysed. Surprisingly, AVP-22G12 bound to PA83 permits cleavage by trypsin, implying that it binds close to (and possibly spans) the accessible cleavage site (Fig. 2(c)).

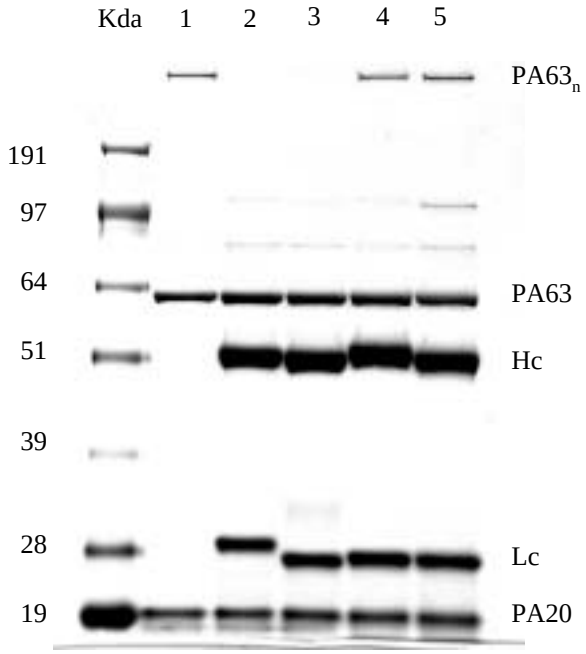

Fig. 4. Effects of Anti-PA antibodies on PA63 oligomer formation. Coomassie stained SDS-PAGE of antibody bound PA83 treated with trypsin. Lane assignment: Molecular weight markers; (1) PA83 trypsin treated no antibody; (2) AVP-22G12; (3) AVP-21D9; (4) AVP-1C6; (5) AVP-1451 isotype matched human IgG anti-tetanus control.
Equal molar of PA83 (0.25 nmol) and PA-ab (0.25 nmol) were mixed in 70 μl of PBS. After 30 minutes incubation at room temperature the mixture was transferred to 4°C and trypsin (50 μg/ml) 10 μl of ice-cold added for 5 minutes. Trypsin was inactivated by the addition of 5 μl trypsin and chymotrypsin inhibitor (10 mg/ml). Citric phosphate buffer (115 μl of 0.1 M, pH5.0) was then added to bring the pH to 5.0 to facilitate PA63 oligomerization. SDS loading buffer was added and the mixtures boiled for 10 minutes. Polypeptides were separated in a 10% Bis-Tris gel running under reducing condition. Protein bands were visualized by Coomassie blue staining.

To determine whether the antibodies efficacy was in part due to inhibiting LF binding to PA63 oligomer, the interactions between PA63 oligomer, antibody and LF were investigated. AVP-22G12 did not bind to preformed PA63 oligomer, thus by default did not appear to compete for EF/LF binding; though AVP-21D9 had very weak binding (possibly due to the presence of a small amount of PA63 monomer) and AVP-1C6 bound to the PA 63 oligomer, neither inhibited LF binding (Fig. 3(a)). All the antibodies bound PA83, which subsequently bound soluble ATR (sATR) (Fig. 3(b)). However partial inhibition of sATR binding was observed on AVP-1C6 captured PA83, hinting at a possible mode of action.

AVP-21D9 and AVP-22G12 did not inhibit PA83 sATR interaction, nor did they appear to prevent subsequent processing to PA63 or the binding of LF to PA 63 oligomer, yet paradoxically they were the two most potent inhibitors of anthrax lethal toxin (PA/LF) *in vitro* and *in vivo* in rats [15]. AVP-22G12 bound to native PA83, denatured PA83 and PA37, but not to the preformed heptamer or monomer PA63. These observations implied that at least for AVP-22G12 the step of toxin neutralization probably occurred prior to heptamer assembly. In a natural exposure to anthrax, upon cleavage of PA83 to PA63 and the release of PA20, the PA63 spontaneously forms a heptamer [8].

An oligomer of PA63 can be formed *in vitro* by treating PA83 with trypsin and is stable in the presence of sodium dodecyl sulphate (SDS) as shown in lane 1 of Fig. 4. Antibody bound PA83 was cleaved by trypsin to mimic the natural furin like protease, to generate PA63-PA20. pH was adjust to 5.0 to facilitate heptamer assembly. The mixtures were examined by SDS-PAGE. In the absence of anti-PA antibody (lane 1) or in the presence of an isotype matched control antibody (lane 5), the PA63 oligomer formed readily. Both AVP-22G12 and AVP-21D9 (lanes 2 and 3) completely inhibited heptamer formation (Fig. 4). Since western blot analysis had shown that the two antibodies bind to distinct regions of PA83, clearly the antibodies prevented the oligomer formation via distinct mechanisms. It was demonstrated that AVP-22G12 binds to a linear epitope on PA83 that possibly spans PA63 and PA 20 cleavage site, but still permits access to protease site and the clipped molecule retains PA20. It is plausible that the retention of PA20 on the antibody-antigen complex may hinder the subsequent heptamer formation. Whereas, AVP-21D9 bound to a distal linear epitope within the PA47 polypeptide and prevented PA oligomer formation possibly by masking potential assembly interfaces. It has previously been demonstrated that correct pore assembly is absolutely essential to facilitate LF and EF entry into cells [18], thus blocking this portal molecule effectively protects against the effects of both EF and LF anthrax toxin(s).

This is the first report of fully human antibodies generated in response to AVA vaccination that neutralize anthrax exotoxin PA by preventing PA63 oligomer assembly. Identification of the epitope for AVP-21D9 may further add to the understanding of the self-assembly process and the development of site directed therapeutic interventions (such as AVP-21D9). The 3 antibodies described may also be therapeutically useful against anthrax infection and may have utility as prophylactic agents for at risk non-vaccinated individuals.

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Competing interests

All authors were employees of Avanir Pharmaceuticals during this study.

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