Anthrax toxin-neutralizing antibody reconfigures the protective antigen heptamer into a supercomplex

Mazdak Radjainia, Jae-Kyung Hyuna, Clinton E. Leysathb, Stephen H. Lepplab, and Alok K. Mitraa,1

aSchool of Biological Sciences, University of Auckland, 1010 Auckland, New Zealand; and bNational Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

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The tripartite protein exotoxin secreted by Bacillus anthracis, a major contributor to its virulence and anthrax pathogenesis, consists of binary complexes of the protective antigen (PA) heptamer (PA63h), produced by proteolytic cleavage of PA, together with either lethal factor or edema factor. The mouse monoclonal anti-PA antibody 1G3 was previously shown to be a potent antidote against infection. It was suspected that 1G3 reacts to the same affinity reactions of host cell intoxication (6–10). Several well-characterized neutralizing antibodies, e.g., 1B7 (9, 11), act by binding to epitopes that are identical to or overlap with the surfaces that recognize the cellular receptor. In contrast, the neutralizing mouse monoclonal antibody 1G3 (6) was found to react only with activated PA63h, and not with inactive monomeric PA (12). This economy in function leads in part to the high potency of this antibody (13) in blocking the toxin in vitro and protecting against infection. It was suspected that 1G3 reacts to the same newly created interface between two monomers on the “top” of PA63h as LF (6, 14, 15).

Results and Discussion

We find that 1G3, apart from interfering with PA63h and LF association, appears to profoundly affect the heptamer structure itself. Our analyses of transmission electron micrographs and single-particle images demonstrate that the heptamer structure is compromised and replaced by a uniformly larger, mostly dodecameric structure for the antibody-PA63 complex (Fig. 1). This rearrangement into a supercomplex is specific for 1G3. We tested the effect of two other neutralizing antibodies, 1B7 and 2D3 (11). For both of these antibodies, no dodecameric complexes were observed (Fig. S1). In particular, with 2D3, the arm-like extensions of the protective antigen heptamers appeared to be extended consistent with the putative binding region of 2D3 in domain III of the known x-ray structure. Incubation with 1B7 produced a phenotype that is very distinct from the other two (i.e., 1G3 and 2D3) and is characterized by a preponderance of largely nonspecific aggregation products.

The reaction product of protective antigen heptamers and 1G3 monoclonal antibody displays a preferred en face view characterized by an inner lumen and arm-like extensions such as that seen for isolated PA63h (Fig. 1). A representative field of view of the PA63 oligomer preparation prior to incubation with 1G3 consists of largely nonspecific aggregation products.


The authors declare no conflict of interest.

Data deposition: The image reconstruction has been deposited in the EM Data Bank, www.em databank.org (entry code EMD-5215).

1To whom correspondence should be addressed. E-mail: a.mitra@auckland.ac.nz.

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the relative contrast variations of the arm-like extensions (Fig. 2). Although homooligomeric protective antigen is an approximately circular ring (inner diameter ∼3.5 nm; outer diameter ∼16 nm) composed usually of seven monomers (2), the 1G3 · PA63 supercomplex appears in the preferred orientation approximately in the form of a rhombus with significantly larger inner dimensions of ∼9 × 13 nm and outer dimensions of ∼18 × 21 nm and an apparent C2 symmetry. Although not firmly established due to the low resolution, it is very likely that the twofold symmetry is perpendicular to the plane of the molecule. This symmetry ensures that the monomer–monomer interaction remains unaltered across the entire complex.

For specimens that assume preferred orientation on the EM grid, i.e., normal to the viewing direction, and display no other views, tilting the specimen stage in the microscope generates a cone of the given tilt angle defined by the azimuthal angle for the various in-plane rotations. The three-dimensional (3D) structure can be generated from such a random-conical tilt procedure by determining the geometrical relationship between tilted projections (17). Multiple such 3D reconstructions of the 1G3 · PA63h supercomplex were generated and simultaneously refined to take into account the possible structural heterogeneity (18) reflected in the class averages mentioned before. All of these reconstructions indicated a common feature in the presence of 12 arms that are divided into two continuous segments of density that could each include six PA63 subunits (Fig. 2). Details of how these two segments are joined are not clarified in this modest, ∼33 Å resolution reconstruction. A volume of density is located between the two arms at the center of each continuous segment suggesting the location of 1G3.

Size-exclusion chromatography (SEC) was used to isolate PA63 · 1G3 or PA63 · 1G3 F(ab′)2 supercomplexes from larger aggregation products and for molecular mass estimation (Fig. 3). The molecular mass of the PA63 · 1G3 supercomplex was estimated to be ∼1.1 MDa and the presence of 1G3 as part of the complex was confirmed by SDS-PAGE analysis of SEC fractions (Fig. S2). In line with this observation, when 1G3 F(ab′)2 was used in complex formation, the assembled product eluted at a volume corresponding to a lower molecular mass (Fig. 3). Projection classes and random-conical tilt 3D reconstructions of negatively stained PA63 · 1G3-F(ab′)2 specimens shared similar, overall features with those seen for PA63 · 1G3 (Fig. S3).

When suspended in vitrified buffer, the 1G3 · PA63 supercomplex again displayed strong preferential orientation (Fig. S4). The calculated projection classes for the nominally untilted views (Fig. 2) are consistent with a large oligomeric structure and largely conform to the reprojections of the 3D reconstructions produced from the negatively stained specimens (Fig. 2). There are some differences in the morphology between the stained and unstained-vitrified specimens in terms of the ascribed number of constituting PA63 monomers and the density corresponding to the antibody, as well as in the more discernible C2 symmetry in the latter case. These differences may have arisen from slightly varying viewing directions, uneven stain penetration, and/or better preservation of finer details in the cryospecimens. Based on the results described above, it is clear that irrespective of whether the 1G3 full antibody or F(ab′)2 is used, the protective
antigen heptamer structure is replaced by a significantly larger oligomer comprised of 10 or more PA63 monomers, suggesting that the reaction itself is not an Fc-domain-dependent process. This observation could be of considerable interest because the neutralizing efficiency of human therapeutic antibody MDX-1303 (19) and even polyclonal anti-PA sera has been shown to depend on the Fc receptors (20). The antibody 1G3 is unique in that it remains highly potent in neutralizing the toxic activity of PA that is bound to its cell receptor, whereas other neutralizing antibodies such as 2D3, which also act by preventing LF binding, are relatively less effective on cellular targets (6). In this context, we confirmed the formation of a receptor-bound PA63·1G3 complex on the cells by Western blot analysis (Fig. S5). This observation suggests that the specific structural alterations instigated by 1G3 might be representative of what occurs on the cell surface.

Next we asked whether the observed reorganization of the protective antigen heptamer is due to the bivalent nature of the antibody. For this purpose, we examined the complex produced by mixing PA63h with recombinant single chain 1G3 scFv. Analysis of the recorded images of the reaction product showed that the heptameric structure predominated with a minor fraction of larger oligomeric structures similar to those produced by the bivalent form of the antibody (Fig. 4). Thus, in a typical field of view, ~90% of the particles correspond to the large oligomeric structures when full-length 1G3 or 1G3 F(ab′)2 fragment was used, whereas only ~10% of the particles displayed the supercomplex morphology when 1G3 scFv was used instead. It appears therefore that the bivalent nature or some other inherent difference between mono- and bivalent antibody such as greater size or avidity enhances the reaction, although the observed macromolecular reorganization apparently does not proceed to completion even when 1G3 was in excess as judged by SEC.

We then examined the effect of full-length LF on the bivalent antibody-instigated heptamer reorganization. On a size-exclusion column, the elution volume of the PA63·1G3 macromolecular complex remained unaltered regardless of the order in which 1G3 and LF were added to PA63h, in agreement with the competitive inhibition by 1G3 (Figs. S6 and S7). However, the peak height of the macromolecule fraction was reduced upon addition of LF. In agreement with this observation, examination of such samples in the electron microscope indicated a larger population of apparently unperturbed heptamers, the proportion of which, in typical fields of view, appeared to be slightly more when LF was added first. This difference in the relative population of heptamers in the reaction product suggests a small level of “protection” of the protective antigen heptamer by LF against the structural reorganization precipitated by the addition of 1G3.

Based on the 3D reconstruction and supported by molecular mass measurements, the supercomplex is likely a dodecamer produced by the molecular stitching of PA63 monomers derived from two “parental” heptamers. However, we note that the projection classes and 3D reconstructions point to a level of structural heterogeneity that precludes the derivation of an exact composition. Such heterogeneity could result from the recent observation that PA63 can exist both as a heptamer as well as an octamer (21) in solution, pointing to a polymorphism in intermonomer interactions. In order to rationalize the assembly mechanism of the observed octameric PA63, Kintzer et al. 2009 (21) suggested dimers of PA63 to be intermediates in the oligomerization process, especially in the presence of ligands such as the PA receptor or recombinant N-terminal domains of LF. Such dimeric PA63 structures or multiples of these could also be stabilized by 1G3 and contribute as building blocks for the formation of the dodecameric complex. Precise mechanistic knowledge of how this supercomplex is formed could require designing of PA63 mutants to identify intermediate states.

We show that the Fc-domain is not essential for this mechanism because F(ab′)2 fragments produce essentially similar oligomeric structures as does the full-length IgG. This finding is in line with prior observations that Fc-domain dependency stems from Fc-receptor binding (20). Also, the potential to reorganize the heptamer is greatly reduced in the presence of monovalent 1G3 scFv domains. This observation could mean that the characteristic large bivalent immunoglobin structure is chemically critical to heptamer disruption and supercomplex formation as mentioned above. In this context, 1G3 could be thought as deviating from canonical antibody functions.

The observed capability of 1G3 to rupture the PA63 heptameric arrangement and lead to the formation of a supercomplex is a behavior that, to the best of our knowledge, is unreported in the field of immunology. The fact that 1G3 precipitates drastic

Fig. 4. Bivalent 1G3 and 1G3 F(ab′)2 are more potent in converting PA63h into supercomplexes than 1G3-scFv. (Upper) Electron micrographs of negatively stained specimen produced from incubation of PA63h with 1G3 (Left), 1G3 F(ab′)2 (Center), and recombinantly expressed single chain 1G3 scFv (Right). Typically, only ~10% of particles in 1G3-scFv samples display the large oligomeric structure compared to ~90% in the case of 1G3 and 1G3 F(ab′)2. (Scale bar: 50 nm.) (Lower) Sets of the three prominent projection classes corresponding to the images in the top panel. (Scale bar: 10 nm.).
structural reorganization of PA63h, which appears to be in line with our previous results that show large-scale morphological changes for the PA63 heptamer upon ligand (LF) binding (22), may underpin and possibly enhance the neutralizing effect of the antibody. Overall, based on our study, we draw two general conclusions regarding antibody–antigen complexation. First, the study demonstrates that antibodies directed against active sites may exert their effect as inhibitory agents through direct structural perturbation. Second, electron microscopy can be used as a tool in drug discovery and vaccine design to screen for antibodies that strongly impact the morphology of the target protein.

Methods

Protein Purification, Ligand Binding, and Size-Exclusion Chromatography. Protective antigen heptamer PA63h and LF were purified as described by Park and Leppila (23) and Kliment et al. (24) and stocks were stored at −80°C. Monoclonal antibodies 1G3, 2D3, and 14B7 were produced from the hybridoma cells in high-density cultures. For ligand binding, freshly thawed PA63h, mAb, and LF stocks were used. A mouse IgG1, Fab and F(ab')2 preparation (Pierce) that employs immobilized ficin, was used to cleave 1G3. Cleavage product of 100 μL of 5.7 mg/mL 1G3 in PBS was concentrated and buffer exchanged into 100 μL of 150 mM NaCl, 5 mM 2-(W-cyclohexylamino)ethanesulfonic acid (CHEs), pH 9, using a 10-kDa cutoff Centricon (Millipore). SDS-PAGE analysis on a nonreducing 10% gel was used to confirm completion of the cleavage (Fig. S8). The 1G3 scFV DNA sequence was synthesized by Blue Heron Biotechnology and cloned into pAK400. The plasmid was transformed into BL21-DE3 cells (Stratagene). Expression of the scFV was performed at 37°C in 1 L of well-aerated Terrific Broth (Difco) by inducing exponentially growing bacteria with 1 mM IPTG for 3 h. Cells were centrifuged, resuspended in 100 mL of immobilized metal affinity chromatography (IMAC) buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 10 mM imidazole, and 0.1% Tween 20), and lysed using an EmulsiFlex C3 high pressure homogenizer (Avestin, Inc.). After centrifugation, the supernatant was incubated with Ni-nitriotropic agarose (Qiagen, Inc.) for 1 h on ice. Beads were washed twice with 100 mL IMAC buffer and protein was eluted in 4 mL of 50 mM Tris, pH 8.0, 500 mM NaCl, and 250 mM imidazole. Eluate was concentrated to 1 mL using a Amicon Ultra centrifugal filter device (10,000 molecular weight cutoff, Millipore) and subjected to SEC on a Superdex 200 10/30 column using an Äkta FPLC (GE Healthcare). Running buffer was 10 mM Tris, pH 8.0, 100 mM NaCl, and 0.5 mM EDTA. Fractions containing scFV were pooled, sterile filtered, lyophilized in 0.5 mL aliquots, and frozen at −80°C until use.

In order to generate the PA63-1G3 complex, typically 45 μL of PA63h at a concentration of 0.56 mg/mL in 150 mM NaCl, 50 mM CHEs, pH 9 buffer was incubated for 1 h at room temperature with 5 μL of 5.7 mg/mL 1G3 in PBS, i.e., at −3.3 (1G3):(1PA63h) stoichiometric ratio. Aliquots of concentrated 1G3:F(ab')2 were mixed with typically 35 μL of 0.56 mg/mL PA63h in the CHEs buffer until the peak for excess 1G3 Fab was seen in a SEC run (see Fig. 2). Lyophilized 1G3 scFV was reconstituted at 0.74 mg/mL in 100 mM NaCl, 10 mM Tris, pH 8.0, 0.5 mM EDTA and typically 15 μL was incubated with 35 μL of the PA63h at 0.56 mg/mL in CHEs buffer. This mixture corresponded to −9.1(scFV) : 1 (PA63h) molar ratio. The lyophilized control antibodies 2D3 and 14B7 were reconstituted, respectively, at 3.9 mg/mL and 1.9 mg/mL in PBS. After dilution in CHEs buffer to a concentration of −0.4 mg/mL, 2 μL of control antibody was incubated with 18 μL PA63h at 0.01 mg/mL in CHEs buffer corresponding to a −10 (mAb):(1PA63h) molar ratio.

SEC was primarily used for isolation and purification of the various complexes from the incubation mixtures described above, which were then utilized for specimen preparation for electron microscopy. Typically, 30 μL of the sample solutions were applied to a Superdex 200 26/30 column controlled by an Äkta FPLC system to purify the complexes with 1G3, 1G3 Fab(ab')2, or 1G3 scFV in CHEs buffer. In buffer, for analyzing the post- or preaddition effect of LF on the 1G3-protective antigen supercomplex, SEC was carried out using a Superose 6 HR 10/30 column calibrated with dextran, thyroglobulin, ferritin, and amylase that were used to determine molecular mass estimates of the generated PA.1G3 complex (Fig. S9). An 80 μL 0.56 mg/mL aliquot of PA63h was incubated for 1 h with 10 μL 5.7 mg/mL 1G3 and then for 1 h with 5 μL 12 mg/mL LF or vice versa. Samples for the two cases were applied onto a Superose 6 10/30 column and four fractions under the peak corresponding to the species with the largest molecular mass analyzed by 10% SDSPAGE under reducing conditions.

Sample Preparation, Electron Microscopy, and Image Analysis. Negatively stained specimens were prepared on EM grids overlaid with a perforated carbon film as follows. Typically, 6 μL of the protein sample in the chosen fraction after an SEC run was applied to glow-discharged 1.2 μm holey carbon film grids (Quantifoil) for 45 sec, blotted with a filter paper (Whatman No. 1) and immediately stained with 1% uranyl acetate or Nano-W stain (Nanoprobes) solution. After 60 sec, excess stain was removed using filter paper and the grid air dried. For cryoelectron microscopy, protein samples were concentrated using 10-kDa cutoff Centricon units (Millipore), applied to a glow-discharged 2 μm holey carbon film grids (Quantifoil), and plunge frozen using Vitrobot Mark IV (FEI).

Low-dose electron micrographs at a nominal magnification of 50,000 and 1–2 μm underfocus were recorded on Kodak SO-163 films using an FEI Tecnai12 electron microscope equipped with a LaB6 filament and operated at 120 kV. For 3D reconstructions, image pairs at tilt angles of 45° and 0° were recorded for random-conical tilt analysis. Micrographs were examined on an optical diffractometer, and those with minimal artefactism or specimen drift were selected using a Nikon Super CoolScan 9000 ED at an effective pixel size of 2.5 Å on the specimen.

Manual selection of tilted pairs of images of complexes of 1G3 or 1G3 (Fab')2 with PA63 and random-conical tilt analysis was carried out using XMIPP v2.1 (25) package. XMIPP mark was used to manually select 3,459 and 8,446 tilted image pairs of PA63-1G3 and PA63-1G3 (Fab')2 supercomplexes, respectively, and windowed to size of 160 × 160 pixels. The images were 2 × 2 pixels binned, 20–220 Å bandpass filtered, masked, and normalized. Preprocessed tilted images were subjected to maximum-likelihood classification and grouped in five classes. Using the assigned images for each projection class average, a preliminary reconstruction was carried out using the random-conical tilt approach. These models served later as initial model for refinement. For each of the two types of supercomplex, the three reconstructions deemed most suitable with highest signal-to-noise were selected. These models were then simultaneously subjected to 25 rounds of maximum-likelihood three-dimensional refinement, where the whole set of tilted single-exposure images was used, with and without imposing the C2 rotational symmetry. The resolution of the reconstruction was determined by 0.5 Fourier shell correlation criterion. The EMAN v1.9 (26) suite was used for automated particle selection and projection classification of images of 1G3scFv – PA63 complex and for reprojections of reconstructed 3D models. EMAN was also used for automated particle selection and projection classification of images of freeze-frozen PA63-1G3 and negatively stained PA63-1G3 scFv particles. The selected projections were preprocessed as described above for negatively stained specimens. PA63-1G3 projections were submitted to maximum-likelihood classification in XMIPP and grouped into 20 classes. PA63-1G3 scFv particles were classified using the refine2d.py routine in EMAN and grouped to 100 classes (Fig. 4). Visualization of 3D reconstructions and manual docking of atomic-resolution X-ray models in the density map envelopes were carried out using UCSF Chimera (27).

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