Disease-enhancing antibodies improve the efficacy of bacterial toxin-neutralizing antibodies

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SUMMARY

During infection, humoral immunity produces a polyclonal response with various immunoglobulins recognizing different epitopes within the microbe or toxin. Despite this diverse response, the biological activity of an antibody (Ab) is usually assessed by the action of a monoclonal population. We demonstrate that a combination of monoclonal antibodies (mAbs) that are individually disease-enhancing or neutralizing to Bacillus anthracis protective antigen (PA), a component of anthrax toxin, results in significantly augmented protection against the toxin. This boosted protection is Fc gamma receptor (FcγR)-dependent and involves the formation of stoichiometrically defined mAb-PA complexes that requires immunoglobulin bivalence and simultaneous interaction between PA and the two mAbs. The formation of these mAb-PA complexes inhibits PA oligomerization, resulting in protection. These data suggest that functional assessments of single Abs may inaccurately predict how the same Abs will operate in polyclonal preparations and imply that potentially therapeutic mAbs may be overlooked in single Ab screens.

INTRODUCTION

Antibody-mediated immunity (AMI) is crucial for combating diseases. Hybridoma technology produces individual Abs as monoclonal preparations, which allows the study of single immunoglobulins (Kohler and Milstein, 1975). Consequently, most studies of mAb efficacy evaluate single preparations and classify the immunoglobulins as protective, indifferent, or disease-enhancing depending on how they modify the course of infection, cancer progression or toxemia (Mukherjee et al., 1992; Pethe et al., 2001; Abboud et al., 2010; Scheid et al., 2011). Several neutralizing mAbs have been developed as therapeutics (Saylor et al., 2009), and in particular, they are promising candidates to treat toxin-mediated infectious diseases (Migone et al., 2009; Lowy et al., 2010). The major effort in mAb discovery and characterization has been focused on candidates with therapeutic potential,

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but a paradox of AMI is that most antitoxin Abs are non-protective (Chow and Casadevall, 2012). Disease-enhancing mAbs have been reported pertaining to toxins (Maddaloni et al., 2004), bacteria (Mohamed et al., 2004; Little et al., 2011), and viruses (Peiris and Porterfield, 1979; Takeda et al., 1988; Dejnirattisai et al., 2010). In contrast to their protective counterparts, relatively few enhancing mAbs have been studied in detail and there is no good explanation of why such Abs are generated in an immune response or how they affect the host.

Various papers report that the potency of protective mAbs can be augmented additively or synergistically by the addition of other protective mAbs. The mixing of protective mAbs targeting different epitopes of a toxin molecule can synergize protective efficacy (Cheng et al., 2009; Demarest et al., 2010; Varshney et al., 2011; Ngundi et al., 2012). Combining neutralizing mAbs to individual toxin components boosts protection against toxicity (Brossier et al., 2004; Chen et al., 2009). The primary rationale for mixing multiple protective mAbs is to achieve additivity or synergy by targeting different epitopes of the virulence factors and reducing the potential for selecting escape variants (Logtenberg 2007), while the combinational approaches also have the potential to approximate the complexity of natural AMI. In contrast, enhancing Abs have been rarely studied in polyclonal preparations or in combination with other mAbs.

The system involves the combination of protective antigen (PA) and lethal factor (LF) to form lethal toxin (LeTx) and mAb toxin neutralizing efficacy can be easily in vitro and in vivo (Little et al., 1988; Rivera et al., 2006; Abboud et al., 2010). The macrophage cytotoxicity assay allows the assessment of Ab protection against the cytotoxic effects of LeTx in vitro (Welkos et al., 1986), and these results are often translatable to in vivo studies (Brossier et al., 2004; Rivera et al., 2006; Abboud et al., 2010). Here we report that mAbs that are disease enhancing when evaluated individually can boost the efficacy of protective mAbs against LeTx.

RESULTS

Generation of PA-specific protective, enhancing, and indifferent mAbs

Splenocytes from a BALB/c mouse immunized with GalXM-PA vaccine (Chow and Casadevall, 2011) were fused with myeloma fusion partner to generate hybridoma cells. Twenty hybridomas that secreted PA (PA83)-binding mAbs were isolated and stabilized by cloning twice in soft agar. We sequenced the mRNA coding for the variable region of the heavy and light chains to confirm that the hybridomas were unique and not clonally identical. The isotype distribution was 3 IgG2a and 17 IgG1. We then studied the binding domain of the PA molecule recognized by the mAbs by Western blotting, with the tested targets being PA83, the pre-furin-cleaved form of PA, the fragments PA63 and PA20 generated by the cleavage (Table S1). The mAbs were then characterized individually for their capacity to neutralize LeTx using MTT assays with J774.16 macrophage-like cells. Depending on their toxin neutralizing activity we categorized these mAbs as protective (10%), enhancing (30%), and indifferent (60%), and observed that mAbs with similar activity to the toxin shared higher similarity in their sequences (Table S1). Protective mAbs reduced LeTx toxicity while enhancing mAbs augmented it (Figure 1A), and indifferent mAbs had no effect. These data are consistent with our prior finding that protective mAbs are the minority among reported mAbs (Chow and Casadevall, 2012).

To examine the cytotoxic effect of LeTx in a concentration-dependent manner in the presence of a constant amount of protective or enhancing mAb, J774.16 macrophages were treated with various concentrations of PA and LF (Figures 1B and 1C). The results revealed a 10-fold difference in the LeTx amount needed to kill the macrophages between the
protective mAb- and enhancing mAb-treated conditions. To determine whether mAbs N2D6 and N1F7 mediated protection or disease enhancement in vivo, we challenged BALB/c mice with LeTx after injection of PBS control or individual mAbs (Figure 1D). N2D6 prolonged the survival of treated mice while N1F7 produced disease enhancement as manifested by reduced survival in a dose-dependent manner. The mice treated with 1 mg of N1F7 became very sick 30 h prior to the PBS control group, even though the disease enhancement was not entirely reflected by the survival curve.

Combination of protective and enhancing mAbs to PA boosted protection against LeTx

Given the complexity of AMI and its polyclonal nature, we attempted to examine our 20 PA-specific mAbs in combinations of two mAbs at one time, mimicking a controlled polyclonal environment, and measured whether the combination exhibited any changes in protective efficacy against LeTx. Indifferent mAbs did not gain protective efficacy upon mixing (Figure S1). Unexpectedly, 5 of 6 disease-enhancing mAbs manifested augmented protective efficacy against LeTx when combined with a protective mAb (Figures 2A–2C). This phenomenon was not specific to one individual mAb-mAb pair, as similar results were observed with 5 enhancing mAbs and 3 protective mAbs in our library (Figures 2A–2C). Of these mAbs, protective mAb 19D9 was previously described (Abboud et al., 2009, 2010). Protective mAb N2D6 and enhancing mAb N1F7 were selected for further study since they shared the same isotype as IgG2a and had comparable dissociation constants (Kds) (3.92 × 10^{-8} M and 1.87 × 10^{-8} M, respectively). We then studied the efficacy of protective mAb against LeTx in the presence of constant amount of enhancing mAb, and observed that protective mAbs exhibited about 100-fold more toxin neutralizing capacity and that an indifferent mAb became slightly protective (Figure 2D). The results obtained with the MTT assay (Figure 2E, top) were confirmed using lactate dehydrogenase (LDH) release as an independent measure of cytotoxicity (Figure 2E, bottom). To determine whether the mixing of protective and enhancing mAbs augmented protection in vivo, we pre-injected BALB/c mice with PBS, individual mAb (N2D6 as protective, N1F7 as enhancing, irrelevant IgG2a mAb), or mAb mixture followed by the challenge of LeTx (Figure 2F). The specific inoculum of LeTx was chosen since it is the minimum dosage that can result in 100% mortality in BALB/c mice (Moayeri and Leppla, 2009). The dosage of protective mAb was chosen based on a previous protection study against B. anthracis in a murine model (Winterroth et al., 2010). Equal amounts of enhancing mAb were used, to fulfill the condition of 1:1 molar ratio between the mAb pairs based on the peak protection observed in the MTT assay (Figures 2A–2C). With this experimental design the concentration of enhancing mAb was not optimized to kill the mice, but evidence for disease enhancement was observed as the mice treated with enhancing mAb became very sick 30 h prior to the PBS-treated mice. The mice treated with enhancing mAb without LeTx were used as negative control, indicating that the enhanced toxicity was accounted by the interaction between the toxin and enhancing mAb. The administration of protective mAb, alone, and in combination with irrelevant mAb, significantly prolonged the survival of mice compared to the control, while no difference in protection was found between the two. Remarkably, all mice injected with the protective and enhancing mAb combination survived LeTx challenge. These results indicate that the combination of protective and enhancing mAbs resulted in synergistic protection that relied on the molar ratio of the two mAbs.

Competition for binding PA molecule abrogates synergistic protection by mAb combination

The fact that the combination of multiple protective mAbs competing for epitopes diminishes the overall protective efficacy reveals the importance of simultaneous interaction between multiple mAbs and the antigen on protective synergy (Henchal et al., 1988). One of the enhancing mAbs, N5D7, did not augment protection against LeTx upon mixing with a
protective mAb (Figures 2A–2C). To determine whether the lack of protection in the N5D7 combination was caused by competition between the two mAbs, we performed a competition study by ELISA. No competition was observed for all mAb combinations except those involving N5D7, indicating different specificities (Figures 3A–3C). A difference in epitope specificity was a necessary but not sufficient condition for synergy in toxin neutralization, since the mixing of one mAb with its Fab counterpart did not confer augmented protection against LeTx (Figure 3D). Hence, removal of Fc region and bivalence by papain digestion abrogated the mAb-PA interaction required for synergistic protection.

**Immunoglobulin bivalence and FcγR engagement are required for protection by mAb combination**

To determine the role of FcγR in the synergistic protection by mixing mAbs, we studied the cytotoxic effect of LeTx on bone marrow macrophages (BMMs) derived from FcγRII double knockout (DKO) (FcγRγ−/−RIIB−/−) mice on C57BL/6 background (Figure 4A). No protection was observed in the conditions of Fab/whole IgG mixture in macrophages derived from both wild type (WT) and DKO mice. In FcγR-deficient macrophages, the combination of mAbs still exhibited increased protection but to a lesser extent relative to the protection observed in WT macrophages. These results revealed a role of FcγR engagement, but the fact that depletion of FcγR did not completely abolish the synergistic protection indicated the presence of other underlying mechanism for this phenomenon. We then generated F(ab')2 of protective and enhancing mAbs with pepsin digestion, and performed MTT assay with various combinations. We found that the relative protective efficacy of mAb combination was whole IgG/whole IgG > whole IgG/ F(ab')2 > F(ab')2/ F(ab')2 (Figure 4B). In contrast to Fab (Figure 3D and 4A), F(ab')2 mixing resulted in synergistic protection, depicting the importance of immunoglobulin bivalence. To confirm these observations in vivo, we performed passive immunization experiments in BALB/c mice using F(ab')2 pair and to FcγRγ−/−RIIB−/− DKO mice using whole IgG pair, respectively. The combination of protective and enhancing F(ab')2 significantly prolonged mouse survival compared to protective mAb alone, while the presence of Fc region in whole IgG pair further boosted protection against LeTx (Figure 4C). The lack of FcγR in the DKO mice reduced the protective synergy by mAb combination (Figure 4D). Since IgG isotype can affect neutralization efficacy (Abboud et al., 2010), we generated switch variants of two enhancing mAbs, N4H3 and N5D7, from IgG1 to IgG2a and IgG2b. As shown in Fig. 2 A–C, N4H3 (IgG1), but not N5D7 (IgG1), augmented protection when in combination with protective mAbs. When tested with the MTT assay, the isotype switch variants, IgG2a and IgG2b, retained the same toxin enhancing activities as the parent IgG1 for both N4H3 and N5D7 (Figures 4E and 4F). Furthermore, the isotype switching of the two enhancing mAbs did not affect the overall protective outcome when mixed with a protective mAb, indicating the lack of Fc region preference on the synergistic protection (Figure 4G). The similar avidity of different mAb forms showed that their binding capacity was not a major factor of this phenomenon (Figure S2). These results revealed that the two arms of the immunoglobulin molecule directly determined the synergistic protection by mixing mAb, while FcγR engagement further increased the efficacy.

**Synergistic protection associated with mAb-PA complex formation at optimum molar ratio of protective and enhancing mAbs**

A sufficient amount of Ab is needed for toxin neutralization, ADCC, complement activation and opsonization, but excessive Ab concentrations may result in prozone-like effects that abrogate protection (Taborda et al., 2003). Based on the observations that the synergistic protection against LeTx required the simultaneous interactions of the two mAbs on PA and that the synergy decreased in an excess of enhancing mAbs (Figures 2A–2C and 3A–3C), we hypothesized that the augmented protection was also dependent on the concentration of
the protective mAb. We analyzed for the ability of the combination to protect macrophages using the MTT assay titrating both the protective and enhancing mAbs (Figure 5A). The protection given by the protective mAb with minimal amount of enhancing mAb (<0.2 ng/ml) was defined as the baseline protection. It was not surprising that the baseline protection increased with the amount of protective mAb in the system. Therefore, the synergistic protections given by mAb mixing in different molar ratios were normalized based on the baseline protection (Figure 5B). A three-dimensional presentation of the results revealed that the synergistic protective effect observed by combining protective and enhancing mAbs exhibited a prozone-like effect, peaking at certain molar ratios for the two mAbs and then decreasing when more mAb was added to the system (Figure 5C). To visualize how the two mAbs interacted with PA, we conjugated the protective mAb, enhancing mAb, and PA to Alexa-Fluor-568, -405, and -488, respectively, followed by mixing of the three proteins in molar ratios corresponding to X, Y, and Z, respectively (Figure 5D). The protein mixture was added to J774.16 cells and allowed to incubate for 2 h at 4 °C to allow cell-based mAb-PA interaction without macrophage ingestion (Abboud, et al., 2010). Immunofluorescence revealed the formation of mAb-PA complexes at the molar ratio that paralleled the peak protection of the three ratios shown (Y). As the concentration of enhancing mAb increased, the number and size of the complexes decreased. Flow cytometry was used to measure the uptake of mAb-PA complexes by macrophages. The results confirmed that mAb-PA complexes were most abundant in condition Y and that the large size of the complexes did not affect its binding to the cell surface receptor and subsequent uptake by macrophages (Figure 5E). These data indicate the tight relationship between the synergistic protection by mAb mixing and mAb-PA complex formation that occurred at optimum ratio of the two mAbs.

**A computation model of antibody-antigen formation approximates complex molecular mass**

To determine whether this absence of protection was associated with a lack of complex formation, we mixed mAbs in 1:1 molar ratio in the presence of PA and analyzed complex formation by native PAGE. We observed the formation of complexes with high molecular weights in the mixture of PA, N2D6 and N1F7, but not the one that involved N5D7 (Figure 6A). This result echoed the absence of immune complex for the combination of protective mAbs that compete for epitope on the PA molecule (Figure S3). We then stabilized the mAb-PA interaction using a crosslinker, and performed Western blotting probed with PA-specific IgG1 mAb 10F4 (Rivera et al., 2006). An IgG1-specific secondary Ab was used to avoid isotype cross-reactivity in blot development. The Fab of the two mAbs lacking bivalence served as a negative control for complex formation. The results revealed the presence of complexes with distinct, high order molecular weights found only in the combination of whole IgG form of the two mAbs (Figure 6B). These molecular weights corresponded to the possible combinations of the three proteins, and their distributions were quantified with densitometry (Figure 6C).

On the basis of these results we constructed a simple rule-based computational model to simulate the formation and distribution of higher-order complexes. The rules involved a PA molecule with two mAb binding sites and equilibrium dissociation constants (Kds) measured with SPR analysis to constrain the binding kinetics. Remarkably, this simple model approximated the empirical distribution of mAb-PA complexes (Figure 6C). The discrepancies for some values could reflect differing Kds for the higher order complexes and precipitation of larger complexes and thus effective degradation, whereas our model implicitly assumed no correlation between degradation rate and complex size. These findings indicate that the protective and enhancing mAbs spontaneously formed high order complexes at optimum molar ratio.
**mAb-PA complex formation inhibited oligomerization of PA**

The inactivated form of PA, PA\(_{83}\), is cleaved by furin on the host cell into two fragments, PA\(_{63}\) and PA\(_{20}\), respectively (Molloy et al., 1992), followed by the oligomerization of PA\(_{63}\) (Petosa et al., 1997; Kintzer et al., 2009) and the formation of a pore structure that translocates LF and edema factor (EF) into the cell causing subsequent intoxication (Mogridge et al., 2002). Numerous protective mAbs have been reported to inhibit different steps of anthrax intoxication including PA binding, cleavage, and ultimately oligomerization (Froude et al., 2011). To determine whether the mAb-PA complex formation resulted from the combination of protective and enhancing mAbs affected PA oligomerization, we performed a cell-mediated oligomer formation assay. The results revealed that the binding of PA by either individual mAb or a combination of mAbs influenced furin cleavage and oligomer formation differently (Figure 7A). The protective mAb inhibited furin cleavage and subsequent oligomer formation, while the enhancing mAb did the opposite. In contrast to the results observed with single mAb, the combination of the protective and enhancing mAbs did not reduce furin cleavage but still abolished oligomer formation, suggesting another mechanism of protection. In contrast to protective mAb alone, the mixing of protection and enhancing whole IgG mAbs resulted in mAb-PA complex formation that abolished PA oligomerization completely (Figure 7A). The lack of inhibition on PA oligomerization and the absence of mAb-PA complex in the Fab mixtures confirmed the importance of mAb bivalence in complex formation and protection against LeTx. The fact that all the mAb pairs that exhibited enhanced protection against LeTx bound to PA\(_{20}\) (Table S1) not only revealed the mAb-PA complex formed prior to the furin cleavage but also implied a role of steric hindrance on toxin neutralization. We therefore examined the combination of 6 enhancing mAbs with 10F4, a protective mAb that recognizes a distant epitope on domain 4 of the PA molecule (Rivera et al., 2006), and found no augmented protection (Figure 7B). These results suggest that the steric effect from multiple mAbs binding to proximal but distinct epitopes may play a role in toxin neutralization. Based on our experimental results and their congruence with the computational model we propose a mechanism to account for the unexpected protection given by the mAb combinations. The protective and enhancing mAbs recognize distinct epitopes on the PA molecules that are spatially apart to allow simultaneous binding (Figure 7C) (Potter and Lieberman, 1970). At the optimum protective ratio between PA and the two mAbs, the bivalent property of immunoglobulins favors the formation of mAb-PA complexes (Figure 7C, middle two) (Potter and Lieberman, 1970; Mouquet et al., 2010; Radjainia et al., 2010). Complex formation prior to furin cleavage sequesters PA and prevents pore formation to translocate LF abrogating LeTx toxicity. The cross-linking of Fc\(\gamma\)R further boosts the protection by increasing the uptake of neutralized PA via phagocytosis (Aderem and Underhill, 1999). At other molar ratios of protective and enhancing mAbs, the mAb-PA complex can no longer form and protection is lost (Figure 7C, top and bottom).

**DISCUSSION**

The observation that the combination of F(ab')\(_2\) exhibited significant protective advantage over the single mAb indicated that Fc\(\gamma\)R engagement was not the primary factor to determine the synergistic protection. However, Fc\(\gamma\)R engagement still played a significant role in contributing to protection, since only the whole IgG mixture provided full protection against LeTx challenge. Fc\(\gamma\)R engagement triggers signal transduction (Anderson et al., 1990) that promotes neutralization of mAb-PA complexes by macrophages (Abboud et al., 2010), and the deletion of the \(\gamma\) chain leads to the loss of function in Fc\(\gamma\)R-mediated phagocytosis in macrophages (Takai et al., 1994). The combination of protective and enhancing mAbs allowed not only toxin neutralization but also the crosslinking of Fc\(\gamma\)R, whereas the polyvalent nature of the complexes further accelerates its Fc\(\gamma\)R-mediated...
internalization and degradation inside the host cell (Mellman and Plutner, 1984). Among all the IgG subclasses, IgG1 is the most prevalent among the reported protective and indifferent mAbs, while the majority of enhancing mAbs are IgG2a (Chow and Casadevall, 2012). However, the isotype prevalence among the reported mAbs does not necessarily represent the prevalence of the most effective isotype against toxins, since the protective efficacy of a PA-specific mAb was shown to be IgG2a > IgG2b > IgG1 (Abboud et al., 2010). Indeed, the selection and engineering of isotype has been a focus in Ab therapeutic development (Salfeld, 2007), and the choice of immunization of adjuvants can skew the immune system to produce more IgG of one subclass than the others (Chow and Casadevall, 2011). In this report we observed a relatively small effect for isotype choice on overall protection when mixing the two mAbs, which could be explained by the observation that the bivalence was the dominant requirement for the effect rather than the type of Fc engagement.

Anthrax toxin was used in our study to illustrate the ability of individually characterized enhancing mAbs to benefit the host in combination with other mAbs, but we anticipate that the underlying mechanism of the protection can be applied to other microbes. Despite the fact that this study used a specific monomeric antigen, we propose that the findings here may be extrapolated to other monomeric toxins or even viruses with repetitive epitopes. Crosslinking of virions by mAbs leads to aggregation and neutralization, whereas this process can be reversed by papain digestion suggesting the critical role of mAb bivalence in forming immune complexes (Thomas et al., 1985; Colombo et al., 1989). Antibody-dependent enhancement of virus infection has been shown to be mediated by the Fc region of the immunoglobulin (Takada and Kawaoka, 2003), so the combination of F(ab’)2 may reduce enhancement while potentially providing synergistic protection by forming complexes with the virion. Our results showed that the protective efficacy of a mAb established using the immunoglobulin alone cannot be extrapolated to conditions where other Abs are present, although meeting these conditions may not be sufficient for certain Ab pairs to mediate protection. For example, the mixing of protective and non-protective Abs can abrogate protective efficacy against polysaccharide antigens that possess less defined molecular weight and structure (Nussbaum et al., 1996, De Jesus et al., 2010; Cordero et al., 2011).

Emergent properties cannot be explained by the individual components alone, and usually reflect an outcome that is greater than the sum of the parts with certain form of novelty (Ablowitz, 1939). Microbial virulence, networks of biological signaling pathways, and even evolution of life are examples of emergent properties in nature (Bhalla and Iyengar, 1999; Corning, 2002, Casadevall et al., 2011). Our findings that mAbs characterized as disease-enhancing on single Ab assays can benefit the host in combination with other Abs provides an example of emergent properties in AMI. These results demonstrate combinatorial complexity in Ab interactions challenging the view that one can assign biological activity to a single immunoglobulin molecule that retains its meaning in the context of other types of such molecules, and suggest the need for a new conceptual framework to understand AMI. At a practical level, it is possible that many Abs categorized as indifferent or enhancing when studied alone have the potential for protective functions in combination with other Abs and many potentially useful Abs are likely being discarded in the search for anti-infective and -cancer therapeutics. In the past decade the systems biology approach has become a prominent means to study emergent properties in biological sciences – it can be used to model gene expression of pathogenic microbes (Kanjilal et al., 2010), and predict immunogenicity of vaccine in humans (Querec et al., 2009). On the basis of emerging high throughput technology and systems biology platform, combination testing of mAbs could reveal new properties of previously neglected mAbs, and transform the direction and approach in therapeutics development against various infectious diseases and cancers.
EXPERIMENTAL PROCEDURES

Hybridomas and monoclonal antibodies (mAbs) to PA

BALB/c mice of 8–10 weeks old were immunized with the GalXM-PA conjugate generated from our lab (Chow and Casadevall, 2011). Hybridomas were generated by fusing splenocytes to NSO or Bcl2 myeloma fusion partner (de StGroth and Scheidegger, 1980). The specificity and isotype of mAbs were tested with ELISA.

Determination of \( V_H \) and \( V_L \) sequences

The sequencing of the heavy chain and light chain of the antibody variable region was performed as described previously with modification (Rivera et al., 2006). Briefly, total RNA of hybridoma cells was isolated, and genomic DNA was removed. cDNA was synthesized, and mAb variable domains were generated by PCR with universal 5'-end (sense) variable region and specific 3'-end (antisense) constant region primers. The amplified PCR products were sequenced at the Sequencing Core Facility at AECOM. The sequences were then compared to the mouse immunoglobulin database using IgBLAST to determine gene usage.

Cell viability assay

Cell viability of macrophages was analyzed by MTT (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide) assay as described (Abboud et al., 2010). Briefly, J774.16 cells and BMM were treated with lethal toxin (LeTx) (0.3 \( \mu \)g/ml PA and 0.3 \( \mu \)g/ml LF unless specified), in the presence or absence of specific mAb(s) for 4 h and 72 h, respectively. In the experiments of mixing mAbs, the mAb with constant concentration was at 1 \( \mu \)g/ml and the other mAb being titrated from 30 \( \mu \)g/ml, unless specified. The percentage viability was calculated as \((\text{OD}_{\text{experimental}} - \text{OD}_{\text{min viability}})/(\text{OD}_{\text{max viability}} - \text{OD}_{\text{min viability}})\) *100, whereas \( \text{OD}_{\text{max viability}} \) and \( \text{OD}_{\text{min viability}} \) were obtained from cells treated with media only and overdose of LeTx, respectively.

Cytotoxicity assay

Cell-mediated cytotoxicity was measured by CytoTox 96 non-radioactive cytotoxicity assay (Promega). Cells treated with lysis solution were used as maximum LDH release control, whereas cells with media only were used as baseline LDH release control.

Animal experiments

Eight- to ten-week-old female BALB/c mice were pre-injected with PBS, individual mAb (1 mg), or mAb mixture (1 mg each) intraperitoneally 4 h prior to LeTx challenge. LeTx (35 \( \mu \)g PA and 35 \( \mu \)g LF per mouse) was injected intravenously. Equal amount of enhancing mAb was used, in order to fulfill the condition of 1:1 molar ratio between the mAb pairs. For the survival studies that compared the protective efficacy of F(ab')\(_2\) and whole IgG in BALB/c mice, and that involved wild type C57/BL6 and FcR\( \gamma^\text{--/--}\)/RIIB\( ^\text{--/--}\) mouse experiment, LeTx (40 \( \mu \)g PA and 40 \( \mu \)g LF per mouse) was used. All experiments were done in compliance with federal laws and institutional guidelines and have been approved by the Albert Einstein College of Medicine.

Inhibition ELISA

Costar plates were coated with PA (2 \( \mu \)g/ml), followed by the addition of a protective mAb and an enhancing mAb. The concentration of the first antibody was kept constant at 2 \( \mu \)g/ml, while the other was titrated from 60 or 180 \( \mu \)g/ml, and vice versa. An alkaline phosphatase-conjugated goat anti-mouse antibody with isotype specific to the first antibody was used to detect mAb-mAb competition. The absorbance was measured at 405 nm.
Fluorescence labeling of PA and mAbs and immunofluorescence imaging

Protective mAb N2D6, enhancing mAb N1F7, and PA were conjugated to Alexa-Fluor_{568}, −405, and −488 (Molecular Probes, Invitrogen), respectively, following manufacturer’s instructions. They were mixed in molar ratios 1:0.001:1 (condition X), 1:1:1 (Y), or 1:20:1 (Z) (Concentrations of Alexa-Fluor conjugated protective mAb, enhancing mAb, and PA were 0.5 μg/ml, 5 × 10^{-3}/0.5/10 μg/ml, and 0.5 μg/ml, respectively), and pre-incubated with J774.16 macrophage-like cells at 4 °C for 2 h to allow interactions and receptor binding (Abboud et al., 2010). Cells were washed, fixed with 4% paraformaldehyde, and imaged by epifluorescence microscopy.

Measurement of PA uptake by flow cytometry

The procedures were similar to that of fluorescence labeling, except that after 2 h incubation at 4 °C the cells were washed to remove unbound complex/mAbs/PA, followed by incubation at 37 °C for 30 min to allow phagocytosis of the antibody-antigen complex. The internalization of complex takes place within 30 min (Abboud et al., 2010), so all bound complexes should be uptaken by the macrophage before the cells were resuspended and subjected to flow cytometry.

Native gel electrophoresis

PA and mAb(s) were mixed in 1:1 (or 1:1:1) molar ratios (5.55 μM) and incubated at 37 °C for 2 h. The mixtures were subjected to native PAGE using NuPAGE Novex 3–8% Tris-Acetate Gel (Invitrogen) followed by Coomassie staining.

Crosslinking experiment and western blotting

Combinations of PA, N2D6_{whole IgG}, N2D6_{Fab}, N1F7_{whole IgG}, and N1F7_{Fab} of the same concentration (5.55 μM), at molar ratio 1:1:1 for N2D6:N1F7:PA, were incubated at 37 °C for 2 h. A water-soluble crosslinker, bis[sulfosuccinimidyl] suberate (BS³) (Thermo Scientific) was used to induce crosslinking between PA and mAb(s) following the manufacturer’s instructions. The reaction was performed at room temperature for 30 min, and terminated by the addition of Tris-HCl. The reaction mixture was subjected to SDS-PAGE, with no boiling of samples, using NuPAGE Novex 3–8% Tris-Acetate Gel followed by Western blotting. A PA-specific mAb, 10F4 (IgG1), and Alexa Fluor_{680}-conjugated goat anti-mouse IgG1 antibody were used as the primary and secondary antibodies, respectively (Rivera et al., 2006). The signal was detected by Odyssey Infrared Imager (LI-COR) at 700 nm, and analyzed with Image Studio software (LI-COR) and Image J.

Cell-mediated PA oligomer formation assay

Cell-mediated oligomer formation of PA_{63} was studied as described previously with modification (Rivera et al., 2006). Briefly, PA_{63} was pre-incubated in variation with N2D6_{whole IgG}, N2D6_{Fab}, N1F7_{whole IgG}, and N1F7_{Fab} of the same concentration (36 μM) at 37 °C for 2 h. The mixture was added to J774.16 macrophage-like cells and incubated for 45 min to allow furin cleavage, oligomer formation and uptake by the cells. The culture was washed with PBS and lysed. The reaction mixture was subjected to non-reducing SDS-PAGE and Western blotting as described above. The signal was detected by Odyssey Infrared Imager at 700 nm.

Rule-based modeling

The BioNetGen language (BNGL) was used to implement a rule-based model of complex formation due to antibody-antigen binding (Faeder et al., 2009; Chylek et al., 2011). Simulations were performed using the Gillespie stochastic simulation algorithm (Gillespie, 1977). We used a version of the KS test that is, in contrast to the most commonly used forms
that apply only to continuous distributions, applicable to discrete distributions (Gleser, 1985).

**Statistics**

The statistical significance for all in vitro assays was determined by the Student’s t test. Survival rate of the animal experiments was analyzed with the log-rank test. All the statistical analyses were performed using Prism software (GraphPad Software, Inc.).

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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S.-K.C. and A.C. designed the biological experiments, interpreted the results, produced the figures, and wrote the paper. S.-K.C. performed all the biological experiments. M.A.P. carried out the antibody sequencing, and analyzed the results. C.S., T.M. and A.B. designed and performed the rule-based modeling, interpreted the results, produced the figures, and wrote the paper.

**REFERENCES**


HIGHLIGHTS

- Disease-enhancing mAbs augment protective mAb efficacy against toxins
- Bivalence of immunoglobulin determines the synergistic protection by mAb combinations
- FcγR engagement enhances the anti-toxin efficacy of mAb combinations
- Stoichiometrically defined antibody-toxin complex formation prevents intoxication
Figure 1. see also Figure S1 and Table S1. Protective and disease-enhancing activities of PA-specific mAbs

(A) The protective efficacy of individual mAbs was measured by MTT assay with J774.16 cells. The viabilities of cells treated with media or LeTx only were used as references to determine mAbs to be protective (green), indifferent (black), or enhancing (red). The inset is the magnification of the region showing only indifferent (black) and enhancing (red) mAbs.

(B–C) The effect of protective mAb N2D6 (green) and enhancing mAb N1F7 (red) on LeTx toxicity studied by MTT assay using J774.16 macrophage-like cells, with (B) PA concentration being constant (0.5 μg/ml) and the one of LF titrated, and (C) LF concentration being constant (0.5 μg/ml) and the one of PA titrated vice versa. mAb concentration = 5 μg/ml. error bars, mean ± SD; ***P < 0.0005, **P < 0.005, *P < 0.05 protective mAb versus no mAb; ###P < 0.0005, ##P < 0.005, #P < 0.05 enhancing mAb versus no mAb, all by t test.

(D) Dose dependent survival of BALB/c mice challenged with LeTx (35 μg of PA and LF each) 4 h after pre-injection of protective mAb N2D6 or enhancing mAb N1F7. PBS control was used as reference. Data shown are representative of three independent experiments. Means and SD of triplicates are shown from one representative experiment out of three (A–C). In vivo studies are representative of two independent experiments with n = 5 mice per group; **P < 0.005, *P < 0.05 log-rank test.
Figure 2. Combination of protective and enhancing mAbs to PA boosts protection against LeTx

The protective efficacy of enhancing mAbs (red) in the presence of protective mAbs (green), (A) N2D6, (B) N5C10, (C) 19D9, respectively, measured by MTT assay, with the concentration of protective mAbs being constant (1 μg/ml). (D) The protective efficacy of protective mAbs (green) and indifferent mAb (black) was determined by MTT assay in the absence (dotted line) and presence (solid line) of enhancing mAb, N1F7 (red), with the concentration of N1F7 being constant (0.1 μg/ml). mAb 19D9 was described by Abboud et al (2010). error bars, mean ± SD; ***P < 0.0005 absence of N1F7 versus corresponding presence of N1F7 counterparts, by t test. (E) LDH measurement mirrored results from the MTT assay to show significant protection against LeTx by combining protective and enhancing mAbs. (top panel) MTT assay showed the protective efficacy against LeTx of protective mAb (green), enhancing mAb (red), and their mixture (black). (bottom panel) LDH measurement showed the same protection pattern as MTT assay. (F) Percentage survival of BALB/c mice challenged with LeTx (35 μg of PA and LF each) 4 h after pre-injection of PBS, individual mAb (N2D6 as protective, N1F7 as enhancing), or mAb mixture (1 mg mAb each per mouse). The irrelevant mAb was 18B7, an IgG1 murine mAb that did not bind to PA or LF (Mukherjee et al., 1992). In vivo studies are representative of two independent experiments with n = 5 mice per group; **P < 0.005, *P < 0.05 log-rank test. Data shown are representative of two independent experiments. Means and SD of triplicates are shown from one representative experiment out of two (A–E).
Figure 3. Epitope competition of the protective and enhancing mAbs results in no synergistic protection

Competition binding assay for PA between protective mAbs (green) and enhancing mAbs (red) as determined using inhibition ELISA. The concentration of protective mAbs (A) N2D6, (B) N5C10, (C) 19D9, respectively, was kept constant (2 μg/ml), and those of enhancing mAbs were titrated, and vice versa. (D) The protective efficacy of whole IgG mAbs compared with their Fab fragments by MTT assay. The concentration of N2D6 whole IgG and Fab is 6.67 nM. Data shown are representative of three independent experiments. Means and SD of triplicates are shown from one representative experiment out of three (A–D).
Figure 4. see also Figure S2. Immunoglobulin bivalence and FcγR engagement contribute to protective synergy

(A) The function of Fc region in Ab-mediated protection was measured by comparing LeTx cytotoxicity on bone marrow macrophages (BMMs) derived from FcγRγ chain/FcγRII double knockout (FcγRγ−/−/RIIB−/−) and wild type (WT) mice using MTT assay. Error bars, mean ± SD; **P < 0.005, *P < 0.05 WT BMM N2D6 with N1F7 whole IgG versus with N1F7Fab, & & P < 0.005, & P < 0.05 DKO BMM N2D6 with N1F7 whole IgG versus with N1F7Fab, all by t test. N2D6 concentration was kept constant (6.67 nM). (B) The protective efficacy of whole IgG mAbs was compared with their F(ab')2 fragments by MTT assay. N2D6 concentration was kept constant (6.67 nM). (C) Percentage survival of BALB/c mice challenged with LeTx 4 h after pre-injection of PBS, mAb F(ab')2, mAb whole IgG, or mAb mixture (1 mg whole IgG or 0.667 mg F(ab')2 each). Representative of two independent experiments with n = 5 mice per group; **P < 0.005, log-rank test. (D) Percentage survival of wild type C57BL/6 and FcγRγ−/−/RIIB−/− mice challenged with LeTx pre-injected with 1:1 mixture of N2D6 and N1F7 (1 mg each). P = 0.0005, log-rank test. Data shown are representative of three independent experiments. (E–G) Switching of Fc region on enhancing mAbs did not affect protective outcome when mixed with protective mAb. IgG1 enhancing mAbs, (E) N5D7 and (F) N4H3, were class switched to IgG2a and IgG2b, respectively. MTT assay indicated no significant change of LeTx enhancing activities for the switched mAbs. (G) Different classes of N5D7 and N4H3 were mixed with protective mAb, N2D6 (1 μg/ml), and the combinational protective outcome was determined by MTT assay. Data shown are representative of three independent experiments. Means and SD of triplicates are shown from one representative experiment out of three (A–B, E–G).
Figure 5. Molar ratio of protective and enhancing mAbs determine mAb-PA complex formation
Protection from combining protective and enhancing mAbs peaked at optimum molar ratio of the two. (A) The protection from N2D6 with minimal amount of N1F7 (<0.2 ng/ml) was set as baseline protection, which increased with the concentration of N2D6. (B) To reveal the synergistic protection given solely by the addition of N1F7 to the system, the baseline protection of individual N2D6 concentration was subtracted from the corresponding plot of mixing N2D6 and N1F7. The two-dimensional plots illustrated the results after normalization. (C) Three-dimensional plot of net protection given by the mixing of protective and enhancing mAbs with respect to protective mAb alone. The concentration of PA and LF used in this experiment was 0.6 μg/ml each. Data shown are representative of two independent experiments. (A–C). (D) Alexa-Fluor−568, −405, and −488 conjugated protective mAb, enhancing mAb, and PA mixed in molar ratio 1:0.001:1 (X), 1:1:1 (Y), or 1:20:1 (Z), and were added to J774.16 macrophages to allow cell-based mAb-PA interaction but not phagocytosis by the cells. The cells were imaged by epifluorescence microscopy. n > 150 cells. ***P < 0.0005 and **P < 0.005, t test. Scale bar = 30 μm (E) J774.16 macrophage-like cells were incubated with Alexa-Fluor conjugated mAbs and PA as described in (D) followed by phagocytosis of the mAb-PA complexes by macrophages. The uptake was measured by flow cytometry, and presented as total cell-associated fluorescence. Data shown are representative of three independent experiments. Means and SD of triplicates are shown from one representative experiment out of three (D and E).
Figure 6. see also Figure S3. Experimental mAb-PA complex formation with discrete molecular weights concurs with computational model

(A) Individual mAbs or their mixture were incubated with PA and subjected to native PAGE followed by Coomassie Blue staining, which aimed to study the mAb-PA complex formation with their natural conformations. The irrelevant mAb was 18B7 (Mukherjee et al., 1992). (B) Individual mAbs or their mixture were incubated with PA, and their interactions were stabilized by crosslinker BS3. The reaction mixture was subjected to SDS-PAGE followed by Western blotting. A PA-specific mAb, 10F4 (IgG1), that targeted PA63 region of the PA molecule was used to probe PA and its complexes with mAbs. (C) Schematic diagram showing the components of mAb-PA complexes with distinct molecular weight and comparison of gel band optical density to rule-based model. A Kolmogorov-Smirnov test adapted for discrete distributions did not reject the null hypothesis that the empirical and model distributions represent samples from the same underlying form. Error bars show sample SD. Data shown are representative of three independent experiments. SD of triplicates is shown from one representative experiment out of three (C).
Figure 7. see also Table S1. mAb-PA complex formation abrogates PA oligomerization
(A) PA pre-incubated with individual mAbs, or mAb mixture was added to J774.16 macrophages and incubated for 45 min to allow furin cleavage, oligomer formation and uptake by the cells. Whole cell lysate was subjected to SDS-PAGE and Western blotting probed with PA-specific mAb 10F4 (IgG1) that recognized PA83, PA63, and the PA oligomers. Data shown are representative of three independent experiments. (B) Protective efficacy of enhancing mAbs (red) in the presence of protective mAbs 10F4 (green) measured by MTT, with the concentration of 10F4 being constant as 1 μg/ml. 10F4 binds to a distant epitope from the PA20 region of PA83, as described by Rivera at al. (2006). Data shown are representative of two independent experiments. SD of triplicates are shown from one representative experiment out of two. (C) Proposed mechanism for synergistic protection against LeTx involving the combination of protective and enhancing mAbs.