Human IgG Fc domain engineering enhances antitoxin neutralizing antibody activity

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Introduction

The effector activity of antibodies is dependent on engagement with Fcγ receptors (FcγRs) and activation of the associated intracellular signaling pathways. Preclinical evaluation of therapeutic humanized or chimeric mAbs to study the interactions of their Fc regions with FcγRs is hampered by substantial structural and functional FcγR diversity among species. In this report, we used mice expressing only human FcγRs to evaluate the contribution of FcγR-mediated pathways to the neutralizing activity of an anti-anthrax toxin chimeric mAb. We observed that the protective activity of this mAb was highly dependent upon FcγR engagement, with minimal protection against anthrax toxin observed in FcγR-deficient mice following mAb administration. We generated anti-anthrax toxin mAbs with specific Fc domain variants with selectively enhanced affinity for particular human FcγRs and assessed their activity in FcγR-humanized mice. We determined that Fc domain variants that were capable of selectively engaging activating FcγRs substantially enhanced the in vitro and in vivo activity of anthrax toxin-neutralizing antibodies. These findings indicate that the application of Fc domain engineering is a feasible strategy to enhance toxin-neutralizing activity and suggest that engineered antitoxin antibodies will have improved therapeutic efficacy.

Results and Discussion

Toxin neutralization was previously thought to be the sole result of blocking toxin activity through Fab-antigen interactions. However, our recent observations indicate that FcγR-mediated path-
ways, such as Fc-mediated toxin uptake by effector cells like macrophages, contribute substantially to the neutralizing activity of anti-anthrax toxin mAbs (1, 10). Given the previously described requirement for FcγRs for the activity of anti-anthrax mouse mAb 19D9 (1), we have generated a mouse-human chimeric IgG form of this antibody as a necessary precursor to a fully human therapeutic antibody. The constant regions of the heavy and light chains (mouse IgG1,κ) were replaced with those of human IgG1 (hIgG1) and κ constant regions, respectively (Figure 1A).

Similarly, we have shown previously that generation of switch variants of the parental 19D9 mAb and comparison of their neutralization potencies resulted in more effective neutralizing antibodies, while not affecting their binding affinity or specificity, such that their relative efficacy was IgG2a>IgG2b>IgG1 (1). This hierarchy of mouse IgG subclass activity results from the differential binding of these subclasses to specific mouse FcγRs, as previously determined (7). Mouse IgG2a binds with a log higher affinity to the activation FcγRIIb (CD16b) (19). To determine the contribution of FcγR-mediated pathways to the activity of the chimeric 19D9 hIgG1 mAb, bone marrow–derived macrophages (BMDMs), the main target of the anthrax lethal toxin (LeTx) during anthrax infection, were obtained from mice lacking all genes encoding for FcγRs (FcγR-null) and from FcγR-null mice expressing human FcγRs (FcγR-humanized mice). Comparison of the in vitro neutralization activity of the chimeric hIgG1 19D9 mAb in FcγR-null and FcγR-humanized cells revealed significantly decreased protective activity in the absence of FcγRs, as evidenced by enhanced anthrax LeTx-mediated cytotoxicity in FcγR-null BMDMs compared with that in FcγR-humanized BMDMs (Figure 2A) (hIgG1 19D9 IC50 in FcγR-null and FcγR-humanized cells were 0.21 μg ml−1; hIgG1 19D9 IC50 in FcγR-null and FcγR-humanized cells were 21.45 μg ml−1). Analysis of the expression of human FcγRs of BMDMs obtained from FcγR-humanized mice showed an expression pattern similar to that of primary human macrophages (18). In particular, FcγR-humanized BMDMs expressed FcγRIIa (CD32a), FcγRIIb (CD32b), and FcγRIIIa (CD16a), while they lacked FcγRI (CD64) and FcγRIIIb (CD16b) (Figure 2B).

Since the main determining factor for the Fc-FcγR interactions is the amino acid backbone of the Fc region, we generated specific mutants of hIgG1 19D9 within the Fc domain region that participates in the Fc-FcγR interface. We characterized the effect of different mutations on the affinity for the different human FcγR classes and identified variants that exhibit differential binding affinities for the various human FcγR classes. These Fc domain variants had the capacity for enhanced engagement of either activating FcγRs, such as FcγRIIa and FcγRIIb (G236A/S239D/A330L/I332E), or the inhibitory FcγRIIb (S267E/L328F) (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI72676DS1). Additionally, an Fc domain variant exhibiting minimal binding (N297A) for all classes of human FcγRs was generated (Supplemental Table 1).

Wild-type hIgG1 and Fc domain variants of 19D9 were expressed in mammalian cells, and their specificity and affinity to the antigen, protective antigen (PA) of Bacillus anthracis, were determined by ELISA (Figure 1B) and surface plasmon resonance (Figure 1C and Supplemental Table 2). While these Fc domain variants exhibited differential binding capacity for human FcγRs, no differences in their affinity and specificity for PA were evident. This observation is consistent with a prior observation that isotype switching of their relative efficacy was IgG2a>IgG2b>IgG1 (1). This hierarchy of mouse IgG subclasses to engage activating or inhibitory FcγRs, such as FcγRIIa and FcγRIIb, while mouse IgG1 preferentially engages the inhibitory FcγRI (S267E/L328F) (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI72676DS1). Additionally, an Fc domain variant exhibiting minimal binding (N297A) for all classes of human FcγRs was generated (Supplemental Table 1).
with *B. anthracis* Sterne strain (10⁴ cells), no activity was observed in FcγR-deficient mice (Figure 2C). These findings clearly suggest the requirement of FcγR engagement in the protective activity of hlgG1 19D9 against anthrax toxin.

Since the protective activity of the mAb 19D9 to LeTx is dependent upon FcγR engagement, we hypothesized that alterations in its capacity to engage particular classes of FcγRs could modulate its neutralizing potency. To test this concept, the in vivo neutralizing activity of different Fc domain variants of hlgG1 19D9 was compared using FcγR-humanized mice. These variants have been characterized in terms of FcγR binding, and they presented enhanced affinity for either activating or inhibitory human FcγRs (Supplemental Table 1). When compared with wild-type hlgG1 19D9, the G236A/S239D/A330L/I332E variant, which has increased affinity for activating FcγRs, including FcγRIIa (13-fold increase) and FcγRIIIa (15-fold increase), demonstrated substantially augmented protective activity in vitro (10-fold improved potency — wild-type hlgG1 1C10: 0.31 μg ml⁻¹; G236A/S239D/A330L/I332E IC₅₀, 0.03 μg ml⁻¹).

To assess whether the G236A/S239D/A330L/I332E variant also displayed augmented activity in vivo, we selected a suboptimal dose (350 μg), at which the wild-type hlgG1 19D9 was ineffective (Figure 3B). As is evident from Figure 3B, >70% survival was achieved with 350 μg G236A/S239D/A330L/I332E 19D9, while no significant protective activity was observed with wild-type hlgG1 19D9 at this particular dose, suggesting that administration of the G236A/S239D/A330L/I332E variant lowered the threshold of mAb required to fully confer in vivo protection.

In contrast, no major difference was observed when we compared the activity of wild-type IgG1 19D9 with that of the S267E/L328F variant, which binds with enhanced affinity to FcγRIIa and the inhibitory FcγRIIb (44-fold and 113-fold increase for FcγRIIa and FcγRIIb, respectively; Supplemental Table 1) (Figure 3, A and C). Minimal in vitro and in vivo neutralizing activity was observed for the FcγR null binding mutant (N297A), further supporting the requirement for FcγR for the mAb-mediated protection against *B. anthracis* infection (Figure 3, A and C).

These findings suggest that preferential engagement of FcγR does enhance the neutralization activity of the 19D9 mAb in humans and are consistent with our previous observations in wild-type mice, in which the mouse IgG2a 19D9 iso-type variant was more effective compared with mouse IgG1 (1). In contrast, minimal contribution of the inhibitory FcγRIIb to the mAb-mediated toxin neutralization was evident, as the FcγRIIb-enhanced binding Fc domain variant of 19D9 (S267E/L328F) displayed activity comparable to that of wild-type hlgG1. Although this variant (S267E/L328F) also displayed enhanced binding to the activating FcγRIIa, its substantially augmented binding capacity for FcγRIIb is likely to overcome any cellular activation signals following FcγRIIa coengagement, especially given the higher expression levels of FcγRIIb compared with FcγRIIa in all leukocyte types (18, 19). This assumption is further strengthened by our previous observations from mAb-mediated cellular cytotoxicity models in FcγR-humanized mice, in which the S267E/L328F variant failed to display enhanced in vivo cytotoxic activity, despite its capacity to engage FcγRIIa (19).

As we have previously demonstrated (1), mouse 19D9 mAb (irrespective of IgG subclass) failed to protect against *B. anthracis* infection in mice deficient in activating FcγRs (FcR-γ chain knockout [Fcer1g⁻/⁻] mice) or in all classes of FcγRs (FcγRIγ⁻ and FcγR2b⁻/⁻ mice), further excluding a role of FcγRIIb in the in vivo neutralizing activity. It is likely that increased mAb interactions...
with activating FcγRs lead to substantially enhanced protection during *B. anthracis* infection through modulation of the phagocytic activity of FcγR-expressing effector cells, augmenting the clearance of PA–anti-PA immune complexes and preventing the formation of anthrax LeTx. Additionally, since the G236A/S239D/A330L/I332E IgG1 Fc variant (and particularly the A330L mutation; ref. 21) is deficient in mouse and human C1q binding and complement activation, a role for complement-mediated pathways in the in vivo mAb-mediated neutralization activity of 19D9 could be excluded.

In summary, we have shown that the toxin neutralization activity of a mouse-human antibody against the anthrax LeTx is dependent on interactions with human FcγRs and identified an optimized human FcγR binding profile for toxin-neutralizing efficacy. In the past, the evaluation of the in vivo activity in wild-type mice of human mAbs intended for human therapeutics, like 19D9, was hampered by the substantial interspecies differences both in structure and in the function of FcγRs as well as their expression pattern. In this study, we have circumvented that prior limitation by using FcγR-humanized mice, which faithfully recapitulate the unique pattern of human FcγR expression observed in the various human tissues. So, our findings, obtained with the use of FcγR-humanized mice, are physiologically relevant to the context of human *B. anthracis* infection. Additionally, we showed that the in vivo neutralization activity of mouse-human chimeric 19D9 mAb offers significant advantages over conventional strategies using wild-type mice, thus allowing the precise characterization of the activity of Fc domain variants with altered capacity to engage specific classes of human FcγRs. Most importantly, our findings establish that it is possible to enhance the toxin-neutralizing activity of an antibody by manipulating Fc-mediated interactions. Indeed, increased endocytic uptake of mAb-opsonized PA by augmented engagement of activating FcγRs on effector cells could prevent furin-mediated cleavage of PA and the subsequent assembly of the anthrax LeTx (1, 10). That finding in turn suggests strategies for designing more effective therapeutic antibodies for toxin neutralization and vaccines that elicit superior toxin-neutralizing responses through the production of antibodies that engage activating FcγRs.

**Methods**

A detailed description of the methods is provided in the Supplemental Methods.
Mice and in vivo protection experiments. Mice deficient for all classes of murine FcγRs (FcγRedefned) and mice reconstituted with humanized FcγRs have been previously characterized (19). For in vivo protection experiments, 19D9 or isotype antibody was administered i.p. 3 hours prior to i.v. challenge with B. anthracis Sterne strain (104 cfu).

Statistics. Quantitative data from multiple experiments are presented as mean ± SEM. Two-way ANOVA with Bonferroni post-hoc t test (2 tailed) was used to test for differences in in vitro protection. For in vivo protection experiments, survival rates were analyzed with the log-rank test. P values of less than 0.05 were considered to be statistically significant.

Study approval. All in vivo experiments were performed in compliance with US federal laws and institutional guidelines of The Rockefeller University Institutional Animal Care and Use Committee.

Acknowledgments

The authors wish to thank Johanna Rivera and all the members of the Laboratory of Molecular Genetics and Immunology for the helpful discussions and support. This work was supported by grants from the NIH and the Northeast Biodefense Center (NBC) (to J.V. Ravetch). S. Bournazos was supported by an NBC Career Development Award (SU54AI057158).

Received for publication August 12, 2013, and accepted in revised form October 30, 2013.

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