Nonclinical Evaluation of GMA161—An Antihuman CD16 (FcγRIIIa) Monoclonal Antibody for Treatment of Autoimmune Disorders in CD16 Transgenic Mice

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Fc receptors are a critical component of the innate immune system responsible for the recognition of cross-linked antibodies and the subsequent clearance of pathogens. However, in autoimmune diseases, these receptors play a role in the deleterious action of self-directed antibodies and as such are candidate targets for treatment. GMA161 is an aglycosyl, humanized version of the murine antibody 3G8 that targets the human low-affinity Fcγ receptor III (CD16). As CD16 expression and sequence have high species specificity, preclinical assessments were conducted in mice transgenic for both isoforms of human CD16, CD16A, and CD16B. This transgenic mouse model was useful in transitioning into phase I clinical trials, as it generated positive efficacy data in a relevant disease model and an acceptable single-dose safety profile. However, when GMA161 or its murine parent 3G8 were dosed repeatedly in transgenic mice having both human CD16 isoforms, severe reactions were observed that were not associated with significant cytokine release, nor were they alleviated by antihistamine administration. Prophylactic dosing with an inhibitor of platelet-activating factor (PAF), however, completely eliminated all signs of hypersensitivity. These findings suggest that (1) GMA161 elicits a reaction that is target dependent, (2) immunogenicity and similar adverse reactions were observed with a murine version of the antibody, and (3) the reaction is driven by the atypical hypersensitivity pathway mediated by PAF.

Key Words: Fcγ receptor; PAF; transgenic model.

Cellular receptors for immunoglobulins (Igs) play a key role in innate immunity (Jovanovic et al., 2009; Lisi et al., 2011; Nimmerjahn and Ravetch, 2008), and immune complexes of the Ig G (IgG) class that bind to pathogens are one of the first levels of host defense (Ravetch, 2003). The immune system depends on the presence of receptors recognizing the constant region of IgG to properly contain infection via activation of cell types such as neutrophils, macrophages/monocytes, and natural killer (NK) cells. There are four such Fcγ receptors in mice—high-affinity FcγRI, FcγRII, FcγRIII, and FcγRIV (an isoform present only in mice)—each with unique properties (Dijstelbloem et al., 2001; Nimmerjahn et al., 2005). FcγRIII, CD16, exists as the A and B isoforms where CD16A is the signaling form of the receptor (Edberg et al., 1989; Ravetch and Perussia, 1989) and is expressed on NK cells, monocytes, macrophages, mast cells, and certain T cells. On the other hand, CD16B is a truncated form of the receptor with no transmembrane or intracellular domain and is attached to the cell membrane via a glycosylphosphatidylinositol anchor (Scallon et al., 1989; Ueda et al., 1989). This form is present on neutrophils and basophils (Meknache et al., 2009). Considering cell density of neutrophils compared with cell types expressing CD16A, the CD16B isoform receptor is the most predominant. The CD16B isoform is also unique in that it is only expressed in humans and higher primates such as chimpanzee and sooty mangabey—lower primates and other species only express CD16A (Clarkson et al., 1986b; Rogers et al., 2006). The function of the CD16B isoform is still unclear, with conflicting reports on its ability to activate cells (Nagarajan et al., 1995; Unkeless et al., 1995).

Studies in knockout mice have indicated a role for CD16 in diseases resulting from immune system dysfunction (Clynes and Ravetch, 1995; Hazenbos et al., 1996; Sylvestre and Ravetch, 1994). Deletion of the common γ chain results in broad-based defects in phagocytosis of immune complexes,
antibody-dependent cytotoxicity, and general inflammatory responses. Elimination of just CD16 (alpha chain knockout) has a more specific effect, resulting in the downregulation of IgG1-driven immune responses. Models of arthritis, glomerulonephritis, IgG-dependent anaphylaxis, IgG-mediated hemolytic anemia, and idiopathic thrombocytopenia purpura (ITP) are markedly less severe or nonexistent in a CD16-deleted mouse. These findings clearly suggest a potential therapeutic strategy in human diseases of blocking CD16 by biologic or pharmaceutical means.

The monoclonal antibody (mAb) 3G8 was first described by Fleit and colleagues as a reagent identifying Fcγ receptor distribution, and it was determined to have specific affinity for the CD16 receptor (Fleit et al., 1982). 3G8 was subsequently used to study the function of CD16 and its role in immune complex clearance in chimpanzees (Clarkson et al., 1986a). When IgG-coated red blood cells were coinjected into chimpanzees with 3G8, they remained in circulation substantially longer than when coadministered with IgG as a control, suggesting that CD16 was central to immune complex clearance. Interestingly, in these chimpanzees, neutrophil counts decreased rapidly after infusions of 3G8 and remained depressed for 5 days, in parallel to serum 3G8 levels; however, there was no decrease in neutrophils after infusions of 3G8 Fab fragment, indicating that the Fc is involved in the clearance. Similarly, a decrease in circulating monocytes was reported in this study. Adverse events, including cytokine elevation. One death was reported in this study.

In the development of GMA161, the murine 3G8 was humanized to reduce human anti-mouse antibody responses in order to allow for repeat dosing. Additionally, several publications (Shields et al., 2001; Tao and Morrison, 1989) have shown that aglycosyl antibodies possess markedly diminished capacity for cross-linking to effector cells and therefore minimizing antibody-dependent cell-mediated cytoxicity (ADCC) and complement-dependent cytoxicity (CDC) activity. Therefore, the humanized 3G8 was aglycosylated to eliminate FcγR binding via its Fc region in an attempt to minimize (1) neutrophil and monocyte depletion and (2) cytokine release by widespread activation of cytokine-releasing cells via the Fc. In vitro studies showed that GMA161 did not bind to soluble form of cell-bound FcγR but did bind with reduced affinity to C1q; however, in CDC activity assays, GMA161 never elicited a positive response. Evaluation of GMA161 in ADCC activity assays showed that GMA161 had significantly reduced activity compared with a wild-type control IgG antibody.

The humanized, aglycosyl antibody, GMA161, and the aglycosylated murine 3G8 were all tested in several nonclinical experiments, including a model of ITP, prior to and during clinical development of the antibody.

MATERIALS AND METHODS

Antibodies. GMA161 is a humanized anti-human CD16 mAb of the IgG1 subclass derived from the 3G8 murine antibody (Johnson et al., 2008). Refinement of the GMA161 structure involved modification of a single framework residue to restore CD16 binding affinity and mutation of Asn 297 to glutamine (N297Q) to remove the consensus N-linked glycosylation site in order to reduce or eliminate the effector function of the mAb. Aglycosyl-3G8 is a modified form of 3G8 containing the same Fc mutation as GMA161. Both antibodies are produced in a mammalian Chinese hamster ovary cell culture and purified by filtration and affinity chromatography. Antibodies are provided as a liquid formulation with a protein concentration of approximately 5 mg/ml in a buffer composed of 5mM sodium phosphate and 1.7mM potassium phosphate at pH 7.2, containing 154mM sodium chloride and 0.005% polysorbate 80 (PBS-T). The anti-mouse platelet antibody, 6A6 (Mizutani et al., 1993), was used for depletion of platelets in animal models, mimicking ITP, and was produced at MacroGenics. This antibody was chimerized to express a human IgG1 Fc region in order to effectively bind hCD16 in the Tg model of ITP.

Flow cytometry. Blood cells from a human donor, cynomolgus macaque, and dog were prepared and red blood cells lysed. White blood cells were incubated with 50 μg of GMA161 and then stained with an anti-CD16 antibody that binds CD16 in a noncompetitive manner, relative to GMA161 (D1130c, Dako Cytomation, Carpenteria, CA), and an anti-idiotypic antibody against GMA161, 3C11. Granulocyte populations were identified based on size and granularity and analyzed for fluorescence.

Hypersensitivity clinical scoring system for nonclinical testing. Reactions thought to be mediated through hypersensitivity- or anaphylactoid-like reactions were scored on a five-point system.

Score 0—Animal appears to have normal clinical observations and is bright, alert, and responsive.
Score 1—Animal is in a hunched position, moving less frequently and slower but will move if stimulated.
Score 2—Animal is in a hunched position with scruffy fur. The animal is not moving and will not respond to stimulation.
Score 3—Animal is recumbent, cold to touch, and is having difficulty breathing.
Score 4—Animal is dead.

Anti-GMA161 and 3G8 analysis. A colorimetric sandwich ELISA using GMA161 and goat anti-mouse IgG horseradish peroxidase conjugate was employed to detect the amount of anti-GMA161 or anti-3G8 antibody in mouse serum. Because GMA161 was used to screen for anti-3G8 antibodies, only antibodies directed against the 3G8 complementarity-determining regions, which are shared with GMA161, were detected. 3,3’,5,5’-Tetramethylbenzidine was added to produce color for the reaction, and the plates were read at 450 nm.
Platelet-activating factor inhibitors. Platelet-activating factor (PAF) inhibitors CV-6209 and CV-3398 (Biomol, Plymouth Meeting, PA) were received as powders and solubilized in ethanol. These solutions were diluted with isotonic saline to yield appropriate concentrations.

RESULTS

Evaluation of Species for Nonclinical Study

As mentioned above, some publications have suggested that only higher primates express CD16 on the same cell types as those observed in humans. Flow cytometry was used to evaluate GMA161 binding capability and binding distribution in human, cynomolgus monkey, dog, and marmoset. Human, cynomolgus monkey, and dog whole blood were incubated with GMA161 and then stained with a noncompetitive anti-CD16 antibody and an anti-idiotypic anti-GMA161 antibody to detect CD16-positive granulocytes and GMA161-bound cells, respectively. A similar analysis was also conducted in human, cynomolgus monkey, and marmoset, where only GMA161 binding was evaluated. Whereas human granulocytes bound to GMA161, cynomolgus monkeys, marmosets, and dogs were negative (Fig. 1). These data align with those published by Rogers et al. (2006) who found only chimpanzees and sooty mangabeys expressing CD16 similar to humans compared with other nonhuman primates such as cynomolgus monkeys. Although some reports in the literature have suggested that CD16 is expressed on neutrophils in canines (Trowald-Wigh et al., 1993), additional evaluation of blood samples from dog also found no staining on neutrophils.

An approach was taken to utilize a transgenic mouse model, where murine CD16 was knocked out and human CD16 was expressed on a transgene. This model was transgenic for both human CD16 isoforms, where A and B were expressed from the native human promoters (Li et al., 1996). Expression of CD16 was confirmed on the appropriate cell types and at densities similar to expression on human cells using the murine parent 3G8 antibody. These mice were further analyzed for their ability to respond to GMA161 dosing.

Characterization of the hCD16 Transgenic Mouse

Previous experience with the murine parent of GMA161, 3G8, in chimpanzees and humans suggested that a pharmacodynamic effect of this antibody was a rapid clearance of circulating neutrophils followed by repopulation (Clarkson et al., 1986a,b). hCD16Tg mice were dosed iv with GMA161 at doses of 0.2–20 mg/kg and analyzed for neutrophil counts over the course of 8 days. At doses of 2 and 20 mg/kg, neutrophil counts decreased and repopulated in a dose-

FIG. 1. White blood cells from a human donor (A and D), a cynomolgus macaque (B and E), canine (C), and marmoset (F) were incubated with 50 μg GMA161 and then stained for GMA161 binding with the anti-idiotypic 3C11 and (A–C only) also for CD16. Cells were gated for granulocytes by size and granularity, and fluorescence was measured by flow cytometry.
dependent fashion. In the mouse, the magnitude of the neutrophil decrease was less than what had been observed in the chimpanzee with 3G8, likely due to the deglycosylation of the antibody (Fig. 2A), as intended. Clarkson et al. (1986a,b) also showed that 3G8 was able to reverse clearance of platelets opsonized with antiplatelet antibodies in both a model of ITP in the chimpanzee and in human patients with the disease.

In our studies, hCD16Tg mice were dosed with the chimeric anti-mouse platelet antibody, 6A6, to induce thrombocytopenia, followed by a range of doses of GMA161 3 h following 6A6 administration. In control animals, receiving only 6A6, platelet counts show effective clearance that slowly rebounded to predose levels over the course of 2 days. However, in animals dosed with GMA161, platelet levels rebound significantly faster, suggesting that in this animal model GMA161 acted similar to the preclinical and clinical data generated earlier with 3G8 (Fig. 2B). Thus, effects of GMA161 in the hCD16Tg mouse largely reflect the historic results of 3G8 in chimpanzees and humans.

Pharmacokinetics of GMA161 were also analyzed in the hCD16Tg mouse and compared with nontransgenic mice. A 1 mg/kg iv dose of GMA161 was administered to hCD16Tg and Balb/c mice, and serum concentrations were analyzed over the course of 14 days. Clearance of GMA161 from circulation occurred much more rapidly in the hCD16Tg mouse compared with Balb/c, indicating that target-mediated clearance is especially active in the transgenic mouse (Fig. 2C). This result is not entirely surprising given the abundance of hCD16 target in the circulating blood, providing a sink for the antibody.

Multiple tissues in the hCD16 Tg mouse were analyzed for the presence of hCD16 by immunohistochemistry using GMA161 as a staining reagent, in order to determine if hCD16 is expressed on appropriate cell types and if it is expressed elsewhere outside of peripheral blood. Monocytes and neutrophils in peripheral blood stained positive, as did intravascular and interstitial monocytes and neutrophils in various tissues, including the lung (Figs. 3A–C). Kupffer cells (macrophages) in the liver also stained positive, an expected finding as macrophages are derived from monocytes.

A tissue pharmacokinetic study was performed to observe residence time of GMA161 in the tissues identified as targets above. hCD16Tg mice were dosed with saline, 20 mg/kg IgG1 control antibody, or GMA161 at 2 or 20 mg/kg. Lung and liver were harvested 1, 3, 7, and 14 days postinjection and subsequently stained for the presence of GMA161. Although some background staining was observed in nonspecific IgG-dosed groups, intense and specific staining of GMA161 was observed in macrophages of both the lung and the liver at days 1 and 3 postinjection. Staining returned to background levels by days 7 and 14 in the 2 and 20 mg/kg dosing groups, respectively (Fig. 3D).
These results confirm that GMA161 is pharmacologically active in this strain of mouse and affecting multiple cell types. As such, preclinical safety assessments of GMA161 were conducted in this animal model.

Single-Dose Toxicology

A single-dose toxicology study was initiated with GMA161 in hCD16Tg mice. Animals were dosed with vehicle, 0.2, 2, or 20 mg/kg GMA161 or left untreated. Mice were sacrificed on days 2 and 17 posttreatment and necropsied for histopathological assessments, and blood samples were taken for clinical pathology evaluation. All GMA161-treated mice survived dosing, and there were no adverse clinical signs or changes in body weight during the course of the study.

The only significant finding on clinical pathology was a statistically significant, dose-dependent drop in neutrophil counts on day 2. These numbers rebounded to levels comparable to vehicle-treated control animals by day 17 (Fig. 4), consistent with earlier evaluations in the characterization of the mice. Bone marrow smears of GMA161-treated animals complemented these findings in the periphery with a loss of segmented neutrophils in the marrow of both males and females as well as a generalized myeloid count drop in females on day 2; these findings were reversed by day 17. Liver weights were increased in males on day 2, and spleen weights were decreased on day 17 in both sexes—this result may reflect hematopoiesis occurring in

![Image](https://example.com/image1.png)

**FIG. 3.** Tissue staining of CD16 with GMA161 in the hCD16Tg mouse. Frozen tissues were sectioned and stained with 10 μg/ml GMA161-fluorescein isothiocyanate (FITC) and an anti-FITC-horseradish peroxidase–labeled antibody. Tissues analyzed and cells identified included (A) neutrophils in the peripheral blood, (B) neutrophils and monocytes in the spleen, and (C) macrophages in the liver. (D) Histologic sections of liver from mice at various time points postinjection of 2 mg/kg GMA161.

![Image](https://example.com/image2.png)

**FIG. 4.** Neutrophil counts after dosing with GMA161. GMA161 was dosed at 0.2, 2.0, or 20.0 mg/kg into hCD16Tg mice. Mice were sacrificed and terminally bled to allow for total hematology counts.
these organs in the face of neutrophil depletion or repeated bleeding from the experiment.

Based on the reversibility and minor nature of these effects, the no observable adverse effect level of this study was set at 20 mg/kg.

**Repeat Dosing of GMA161**

A repeat dose toxicology study was initiated with GMA161 in hCD16Tg mice. Animals were to be dosed with vehicle, 0.2, 0.6, 2, or 10 mg/kg GMA161 every other week for a total of 13 weeks. Dosing human proteins into mice can lead to hypersensitivity- or anaphylactoid-like responses (Finkelman et al., 2005); however, previous work suggested that dosing of a histamine blocker such as diphenhydramine can alleviate these effects enabling the continuation of dosing (McVie-Wylie et al., 2008). As such, mice were scheduled to also receive diphenhydramine prior to the second dose of GMA161 and each dose thereafter.

The first dose of GMA161 was administered without incident. However, upon repeat dosing, several animals succumbed to severe hypersensitivity-like reactions within 15–30 min following dosing. These reactions occurred despite prophylactic administration of up to 30 mg/kg diphenhydramine. Due to the severity of the reactions, animals were sacrificed following the second administration of GMA161 and evaluated for cytokines, immunogenicity, hematology/clinical chemistry, and histopathological analysis.

Modest increases in interleukin (IL)-6, IL-12, and other cytokines were noted 1 h after dosing, but not to levels that would be associated with a significant cytokine release syndrome (Fig. 5A). A subset of mice was also analyzed for circulating antibodies against GMA161 prior to and 14 days following the second administration of GMA161. There was a clear dose-dependent immune response against GMA161 with increased titers between the first and the second doses (Fig. 5B, titers after the first dose are shown). Hematology analysis confirmed the pharmacodynamic effect of GMA161 by showing effective neutrophil depletion at all dose levels 1 h after the second dose of GMA161 (Fig. 5C), indicating that even in the face of significant immunogenicity after one dose, GMA161 retained pharmacologic activity. Clinical chemistry analysis found no significant changes. Liver and spleen weights were significantly increased in animals that were sacrificed 1 h following a second dose of GMA161. Extramedullary

![Figure 5](https://academic.oup.com/toxsci/article-abstract/125/1/299/1670261)
hematopoiesis was notable in the spleen, possibly accounting for the increase in weight, likely secondary to the neutrophil and monocyte depletion induced by GMA161. Finally, minimal to mild hypercellularity was observed in several bone marrow smears of these mice.

Contributions of A and B Isoforms of CD16 to Hypersensitivity Observations

Several studies were conducted to further evaluate the severe hypersensitivity-like responses observed following repeated administration of GMA161 in the hCD16 Tg mouse. Cynomolgus monkeys only have the CD16A isoform, which is expressed on monocytes and macrophages. These monkeys were dosed at 1 or 10 mg/kg by iv infusion every 14 days for 6 weeks. Although binding to monocytes and NK cells are observed in this species (Rogers et al., 2006), monocyte numbers were not significantly changed, whereas NK cells were modestly decreased. There were no test article–associated changes observed in neutrophil counts, which would be expected, as the CD16B isoform is not present in this species. Immunogenicity against GMA161 was not observed after a single dose but was observed in one animal per dosing group after the third dose—titers in each of these animals were substantially lower than what was seen in mice. None of the animals in this study experienced any adverse reactions.

Because repeat dosing in the cynomolgus monkey, which only has the CD16A isoform, was well tolerated, questions were raised about the role of the CD16B isoform in the hypersensitivity reactions, although this type of hypersensitivity reaction has only been noted in mice. To evaluate this, transgenic mice with only the CD16A, only the CD16B, or both CD16A and B isoforms were administered test article. However, instead of the humanized GMA161 antibody, this experiment utilized the murine parent antibody (aglycosyl-3G8) to evaluate if the humanized nature of GMA161 contributed to the immunogenicity observed in the studies described above in the double-transgenic mice. Mice were dosed with 10 mg/kg aglycosyl-3G8 every 14 days for up to seven doses and assessed for clinical signs of hypersensitivity throughout the study. SV129 mice, which do not possess either hCD16 isoform but are the partial genetic background on which the hCD16Tg mice are created, were unaffected over the course of the study. Both single transgenic hCD16A and hCD16B mice slowly developed mild hypersensitivity reactions throughout the study; however, the presence of both the hCD16A and the hCD16B transgenes led to significant reactions that were more pronounced in females (Fig. 6A). A stronger and more rapid antibody response against aglycosyl-3G8 was developed in the double-transgenic hCD16A/hCD16B mice compared with the single transgenic animals, and no significant antibody titer was observed in the SV129 control mice (Fig. 6B). This set of data illuminated several discoveries relating to these repeat dosing reactions: (1) a human target must be present in order to develop any reaction or antibodies against aglycosyl-3G8; (2) both isoforms are needed to elicit the full reaction; (3) use of the murine parent of GMA161 did not avoid immunogenicity or hypersensitivity issues in mice, although it appeared to be less severe than the humanized GMA161; and (4) when both targets are present, females exhibit a stronger clinical response to repeat aglycosyl-3G8 dosing compared with males.

Hypersensitivity Pathways in the hCD16Tg Mouse

The clinical observations of mice and the immunogenicity results after repeat dosing of GMA161 clearly suggested a Type I hypersensitivity reaction, not unlike what has been observed nonclinically and clinically for other protein therapeutics (Finkelman et al., 2005; Strait et al., 2002). However, a standard remedy for this issue, the antihistamine diphenhydramine, did not appear to resolve the effects seen in the first repeat dosing study. A review of the literature surrounding hypersensitivity reactions in mice suggests that such events can proceed either through IgE, IgζRI on mast cells, and subsequent histamine release pathway or via an IgG, FcγRIII/CD16 on macrophages, and subsequent release of PAF pathway (Chao and Olson, 1993). Because blocking the histamine pathway did not alleviate the hypersensitivity reactions and because GMA161 targets a receptor known to be involved in PAF release, it was proposed that blocking PAF activity would relieve repeat dosing hypersensitivity-like events. Therefore, hCD16Tg mice, with both the A and B isoforms, were pretreated with 5 mg/kg diphenhydramine, 3 mg/kg CV-6209 (a PAF receptor antagonist), or left untreated prior to dosing with vehicle or 10 mg/kg GMA161 every 14 days for up to seven doses. Animals with no pretreatment succumbed to hypersensitivity reactions upon repeat dosing of GMA161 similar in frequency and gender specificity as in previous experiments. Interestingly, animals pretreated with diphenhydramine fared substantially worse when compared with un-pretreated animals, suggesting that blockage of the histamine pathway is actually detrimental in this situation. Animals pretreated with CV-6209 exhibited no clinical signs of hypersensitivity throughout the course of the study (Figs. 7A and 7B), and all animals survived until the completion of dosing. Subsequent studies evaluating a different PAF receptor antagonist, CV-3988, resulted in identical protection. These PAF antagonists did not interfere with GMA161 activity, denoted by neutrophil depletion, or with immune responses against GMA161 (Figs. 7C and 7D). Thus, PAF appears to be a driver of hypersensitivity reactions against GMA161, unique among protein therapeutics delivered to preclinical animal models in the literature to date.

DISCUSSION

Protein therapeutics, such as mAbs, receptor decoys, and enzyme replacements are typically studied in animal models to evaluate efficacy, pharmacokinetics, and safety. For safety assessment, mAbs have recently often required the use of
nonhuman primates because of the target specificity of the antibody being studied—species below primate often possess a target sequence that evolutionarily diverges from the human target and, as such, cannot bind the therapeutic. However, some antibodies are so specific for their target that binding can only occur in humans or in primates such as the chimpanzee, which are not feasible for safety evaluation. As such, either surrogate antibodies binding to rodent targets or mice transgenic for the human target are developed.

Here, we describe our experience in evaluating GMA161 in a transgenic mouse expressing both isoforms of human CD16. The model proved to be responsive pharmacologically, as disease mimicking ITP was reversed in the presence of GMA161. The antibody also appears to deplete neutrophils, expressing the decoy receptor CD16B, with which GMA161 also reacts. This is an unintended consequence of GMA161 dosing; however, the extent of neutropenia was greatly reduced by deglycosylation of the Fc region of GMA161. This result also shows that we have effectively presented CD16 on a cell type where it is not normally expressed in species below chimpanzee; thus, the hCD16Tg mouse model appears to be reproducing the human target distribution and activity. A downside of the hCD16Tg model is that the evolutionary difference between mice and humans could likely lead to significant immunogenicity of GMA161 when dosed into mice. This is not something unexpected when dosing large foreign proteins into rodents, as previous experience with other large molecules has led to Type I hypersensitivity responses largely mediated by histamine release. Comedication with antihistamines such as diphenhydramine has mostly proven successful in mitigating this response. Immune responses on occasion can also lead to enhanced clearance of test article from the circulation—in this case, repeat dosing may not be feasible because the animal is no longer exposed to the agent long enough to have an effect.

The hypersensitivity responses observed with GMA161 were unusual in that they were not alleviated by diphenhydramine dosing and, in fact, were exacerbated by that treatment. However, noting the substantial increase in antibodies of the IgG isotype, we learned that these reactions were mediated almost entirely by PAF release. Some publications have taken

**FIG. 6.** Repeat dosing of aglycosyl-3G8 into single transgenic hCD16 mice. The indicated mouse strains were dosed with 10 mg/kg aglycosyl-3G8 and monitored for clinical symptoms with mean clinical scores presented in (A). Mice were also bled for serum after doses 2 and 4 for evaluation of antibodies against aglycosyl-3G8 with mean titers presented in (B).
note that, particularly in mice, hypersensitivity reactions mediated by antibodies can take either an IgE-histamine or an IgG-PAF pathway. Ovalbumin has been used in some experiments to induce the PAF-mediated reactions, which can be reversed by co-dosing with PAF antagonists like the ones described here. Recent data in humans have also shown that severe anaphylactic reactions such as peanut allergies may be caused more by PAF than through other mediators (Vadas et al., 2008). Although we recognize that the PAF pathway is well described in mice and is just being understood in humans, the relevance of the target for GMA161 cannot be understated.

CD16 is the prime activating receptor for IgG-containing immune complexes. It is precisely through this receptor that IgG complexes signal on macrophages that leads to PAF release. Thus, it is possible that this is not a "hypersensitivity" response at all but a "target-mediated" response through GMA161 binding to CD16. One argument against this is that GMA161 does not induce a clinically observable hypersensitivity-like response on initial dosing. However, in this case, GMA161 would be binding CD16 as a monomer and CD16, in contrast to binding to Fcγ receptor I (CD64), responds best to immune complexes. PAF release in this instance may have occurred, but at levels suboptimal for systemic reactions. Maximum reactions to GMA161 occur after multiple doses, once substantial levels of IgG antibodies have been generated against GMA161. GMA161 still may be binding to CD16 but now will exist in a large complex of other IgGs and would likely yield ample activation of CD16 resulting in PAF release from macrophages. Also noted in these studies were the levels of antibodies generated against the murine anti-CD16, aglycosyl-3G8, or its humanized form, GMA161. It is initially surprising that a murine antibody would be so immunogenic in a murine host. However, CD16 is a receptor present on cell types that would be ideal antigen-presenting cells, such as macrophages and dendritic cells. High concentrations of any protein on such cells may lead to presentation as a foreign protein. It is notable that immunogenicity of GMA161 or aglycosyl-3G8 was dependent on the presence of human CD16, so this does not appear to be a typical immune response toward a xenogeneic protein.

FIG. 7. Effects of co-dosing with PAF inhibitors with GMA161. GMA161 was dosed every other week for up to 7 doses into hCD16Tg mice at 10 mg/kg after predosing with 5 mg/kg diphenhydramine (DPH), 3.3 mg/kg of a PAF inhibitor (CV-6209), or no pretreatment. Animals were observed for survival (A). They were also scored for clinical signs of hypersensitivity (B) and evaluated to determine possible gender differences with dotted lines representing females and solid lines representing males. Mice were bled for whole blood 3 and 24 h following the first GMA161 dose to confirm that neutrophil depletion was not inhibited by the coadministration of a PAF inhibitor (C). Finally, serum was collected from all mice to confirm that coadministration of a PAF inhibitor did not alter the antidrug antibody response (D).
In conclusion, GMA161, a humanized antibody designed from a murine antibody that was previously observed to be very effective in patients in reversing autoimmune disorders mediated through autoantibodies, causes severe reactions in mice upon repeat dosing. These reactions appeared to be mediated through PAF, an atypical pathway observed when dosing immunogens, and can be inhibited by PAF inhibitors. Development of future therapies targeting this pathway should take into consideration the nature of this receptor and the pathways it activates.

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REFERENCES


