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Modulation of the Effector Functions of a Human IgG1 through Engineering of Its Hinge Region

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We report here the engineering of a humanized anti-human EphA2 mAb (mAb 12G3H11) in an effort to explore the relationship between the hinge of a human IgG1 and its effector functions. mAb 12G3H11, used here as a model, is directed against the human receptor tyrosine kinase EphA2, which is an actively investigated target for cancer therapy due to its up-regulation in many cancer cells. Various rational modifications were introduced into the hinge region of mAb 12G3H11. These mutations were predicted to modulate the hinge's length, flexibility, and/or biochemical properties. We show that the upper and middle hinge both play important, although functionally distinct roles. In particular, middle hinge modifications predicted to decrease its rigidity or length as well as eliminating either one of its two cysteine residues had a strong negative impact on C1q binding and complement-dependent cytotoxicity. Disruption of covalent bonds between both H chains may account in part for these effects. We also describe middle hinge mutants with a significantly decreased ability to bind FcγRIIIA and trigger Ab-dependent cell-mediated cytotoxicity. Conversely, we also generated upper hinge mutants exhibiting an increase in C1q binding and complement-dependent cytotoxicity activity. Therefore, this approach represents a novel strategy to fine-tune the biological activity of a given human IgG1. We also define, for the first time in such a systematic fashion, the relationship between various characteristics of the middle and upper hinge and the corresponding effector functions. *The Journal of Immunology,* **2006, 177: 1129–1138.**

Conclusive evidence indicates that the effector functions of a given IgG are intricately linked to the presence and properties of its hinge region. Surprisingly, despite having been the object of study for over 20 years, the precise relationship between these parameters and complement activation is still unclear. For instance, conflicting results exist whether at least a portion of the hinge region is needed for complement-dependent cytotoxicity (CDC).[2] Although some studies have suggested that a "spacer" effect between the IgG's Fab and Fc regions is essential for binding of the Fc to the first component of complement activation C1q or to trigger complement mediated cytolysis (1–3), one report describing an IgG whose hinge was partially deleted has disputed this premise (4). Likewise, the exact structural requirements of the hinge region which determine the different aspects of IgG effector functions are still unclear. Here again, conflicting results have been reported. It has been argued that reducing the flexibility or length of the hinge region generally results in a decrease in complement fixation (5, 6). Conversely, some reports have suggested that a reduction in the flexibility or length of the hinge region directly correlates with an increase in complement activation (2, 7–10). Finally, other studies have indicated the lack of such a simple correlation (11–13). Likewise, the nature and properties of the hinge region also influence binding of the Fc to various FcγRs as well as Ab-dependent cell-mediated cytotoxicity (ADCC) activity. In particular, a reduction in the length of a human IgG3 hinge resulted in an increase in ADCC activity (14), whereas the suppression of the inter-H chain disulfide bonds in a human IgG1 had the reverse effect (15).

It is worth noting that all these elegant studies were conducted using IgGs from different species and various isotypes and whose disulfide bonding patterns were sometime unusual. Furthermore, they also often involved drastic and hard-to-interpret modifications, ranging from partial to complete elimination of the hinge. These inherent limitations make it difficult to draw general conclusions and may account for the seemingly confusing data. This is further compounded by the fact that different IgGs may use different molecular mechanisms to bind and activate complement, as is the case when comparing human and murine IgGs (16–18).

Therefore, to further clarify the relationship between the properties of the human IgG1 hinge and the molecule's effector functions, we manipulated this region in a more systematic fashion. More precisely, we generated 26 hinge variants by site-directed mutagenesis. The mutations were designed to have various effects on the properties of the upper and/or middle hinge, namely by increasing or decreasing their flexibility only, decreasing their length, increasing their length and increasing their flexibility, increasing their length and decreasing their flexibility, and/or modifying their local conformation. This in turn allowed us to explore in detail the nature of the relationship between these hinge characteristics and the IgG1 ability to bind C1q/FcγRIIIA and mediate CDC/ADCC. Our data also showed that it is possible to up or down-regulate the effector functions of a human IgG1 using rational design in its upper and middle hinge.

Materials and Methods

Reagents

All chemicals were of analytical grade. Restriction enzymes and DNA-modifying enzymes were purchased from New England Biolabs. Oligonucleotides were purchased from Invitrogen Life Technologies. Human

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[2] Abbreviations used in this paper: CDC, complement-dependent cytotoxicity; ADCC, Ab-dependent cell-mediated cytotoxicity; HEK, human embryonic kidney; BCA, bicinchoninic acid.

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EphA2-Fc fusion protein (consisting of human EphA2 fused with the Fc portion of a human IgG1) was expressed in human embryonic kidney (HEK) 293 cells and purified by protein G affinity chromatography using standard protocols.

General description of the test Ab and expression vectors

A humanized mAb (referred to thereafter as mAb 12G3H11) directed against the human receptor tyrosine kinase EphA2 was generated at MedImmune and used as the template for hinge modifications. The EphA2 receptor tyrosine kinase is a critical component in the control of cell growth and development (19). The variable light and variable heavy genes of mAb 12G3H11 were individually cloned into mammalian expression vectors encoding a human CMV major immediate early enhancer, promoter, and 5′-untranslated region (20). In this system, a human γ1 chain is secreted along with a human κ chain (21).

Generation of the various Ab constructs

Various mutations (listed in Table I) were introduced into the hinge region of the H chain of mAb 12G3H11. This was conducted by site-directed mutagenesis using PCR by overlap extension (22) followed by cloning into the 12G3H11 H chain-encoding mammalian expression vector described above. This resulted in the replacement of the H chain constant portion of 12G3H11 by its 26 hinge-mutated counterparts. The sequences were verified using an ABI 3100 sequencer (Applied Biosystems).

Expression, purification, and characterization of the various Ab variants

HEK 293 cells were transiently transfected with the various Ab constructs in 90-mm dishes using LipofectAMINE (Invitrogen Life Technologies) and standard protocols. Supernatants were typically harvested twice at 72 and 144 h posttransfection and pooled. The secreted, soluble human IgG1s were purified from the conditioned medium directly on 1 ml of HiTrap protein A or protein G columns according to the manufacturer's instructions (APBiotech). Purified human IgG1s (typically >90% homogeneity, as judged by SDS-polyacrylamide gels-PAGE) were dialyzed against PBS, flash frozen and stored at −70°C. Variants were further characterized on a 10% SDS-PAGE (Invitrogen Life Technologies) under reducing and nonreducing conditions to investigate their chain pairing.

Cloning, expression, and purification of FLAG-tagged human FcγRIIIA

The C-terminal end of the extracellular domain of human FcγRIIIA (F158 allotype) was fused with a FLAG tag. Cloning and expression were conducted as follows: briefly, the EA1/EA2 (see below) oligonucleotides combination was used to PCR amplify the extracellular domain of human FcγRIIIA using a cDNA library of human bone marrow (BD Clontech) as the template and standard protocols. The PCR product was then cloned as an *Xba*I/*Not*I fragment into the mammalian cell expression vector described earlier for IgG expression. HEK 293 cells were transiently transfected with this construct using LipofectAMINE and standard protocols. Supernatants were harvested thrice at 72, 144, and 216 h posttransfection and pooled. The secreted, soluble human FLAG-tagged FcγRIIIA was purified from the conditioned medium directly on an anti-FLAG M2 agarose column according to the manufacturer's instructions (Sigma-Aldrich). The FLAG-tagged FcγRIIIA was then dialyzed against PBS buffer and stored at −70°C: EA1. 5′-TCCACAGGTGTCCACTCCCGGACTGAAGAT CTCCCAAAG-3′; and EA2: 5′-GGGAGAATTCCGCGGCCGCTTAT TTGTCATCGTCATCTTTGTAGTCATGGTGATGGTGATGGTGTGC GCCTGCCAAACCTTGAGTGATGGT-3′.

Cloning, expression, and purification of a human FcγRIIIA-streptavidin fusion protein

The C-terminal end of the extra cellular domain of human FcγRIIIA (F158 allotype) was fused with streptavidin. Cloning and expression were conducted as follows: briefly, the SA1/SA2 and A1/A2 (see below) oligonucleotides combinations were used to PCR amplify the streptavidin and extracellular domains of human FcγRIIIA, respectively. A cDNA library

Table I. *Amino acid sequences of the upper and middle hinge of 12G3H11 and its variants*[a]

Name	Upper Hinge (216–225)	Middle Hinge (226–230)	C1q	FcγRIIIA
12G3H11	EPKSCDKTHT	CPPCP		
1	**GGGG**CDKTHT	CPPCP	0	0
2	EPKSC**GGGGG**	CPPCP	0	0
3[b]	EPKSCDKTHT	C**GG**CP	−	0
4	EPKSCDKTHT	CPPC**G**	−	0
5	EPKSCDKTHT	CPP**S**P	−	0
6[b]	EPKSCDKTHT	**S**PPCP	−	0
7	EPKSCDK**C**HT	CPPCP	0	0
8	EPKSCDKT**CC**	CPPCP	0	0
9	EPKSC**PPPPP**	CPPCP	0	0
10	**PPPP**CDKTHT	CPPCP	0	0
11[b]	EPKSCDKTHT	C−−CP	−	−
12	EPKSCDK−−T	CPPCP	0	0
13[b]	EPKSCDK−−T	C−−CP	−	−
14[b]	EPKSCDKTHT	CP**GGG**PCP	−	−
15	EPKSCD**GGG**KTHT	CPPCP	0	0
16	EPKSCD**PPP**KTHT	CPPCP	0	0
17	EPKSCDKTHT	CP**PPP**PCP	0	0
18[b]	EPKSCDKTHT	C**WW**CP	−	−
19	EPKSCD**WW**HT	CPPCP	+	0
20	EPK**CS**DKTHT	CPPCP	0	0
21	EPKS**DC**KTHT	CPPCP	+	0
22	EPKS**DCWW**HT	CPPCP	+	0
23	EPKSCD**FF**HT	CPPCP	0	0
24	EPKSCD**WWW**T	CPPCP	+	0
25	EPKSC**WW**THT	CPPCP	+	0
26	EP**WW**CDKTHT	CPPCP	0	0

[a] Sequences are given using the standard one letter code and numbered according to the EU index as reported (25). Bold typeface indicates changes in amino acid sequences when compared with 12G3H11.

[b] Variants exhibiting a disruption in their inter-H chain covalent assembly. Simultaneous disruption of both middle hinge covalent bonds in a given IgG sample occurred with various frequencies: low (<25% of molecules; variants 3 and 6), average (−50% of molecules; variants 11 and 13), or high (>75% of molecules; variants 14 and 18).

The disulfide bond between the H and L chains is formed by the lone upper hinge cysteine. + indicates increased binding to C1q, 0 indicates no significant change in C1q and/or FcγRIIIA binding, − indicates loss of binding to C1q and/or FcγRIIIA when compared with 12G3H11. −− indicates amino acid deletions.

of human bone marrow (BD Clontech) and the genomic DNA of *Streptomyces avidinii* (American Type Culture Collection) were used as the templates for the amplification of FcγRIIIA and streptavidin, respectively. An overlapping PCR using the A1/SA2 oligonucleotides combination was then used to assemble the FcγRIIIA-streptavidin fusion protein, which was subsequently cloned into the pET-28a expression vector (Novagen) as an *Nco*I/*Nhe*I fragment. The procedure used to express and refold the FcγRIIIA-streptavidin fusion protein was conducted essentially as described (23). The refolded material was then purified using an immunobiotin column according to manufacturer's instructions (Pierce), dialyzed against PBS buffer, and stored at −70°C. Due to the presence of a streptavidin moiety, the FcγRIIIA generated using this strategy is tetrameric. Because of the intrinsically low binding affinity of FcγRIIIA for human IgG, such a multimeric format is required to detect binding by ELISA (see below): A1, 5′-AAGCTTCGGTCCGCCACCATGGCAACTGAAGATC TCCCAAAG-3′, and A2, 5′-GTCTGCCGAACCGCTGCCTGCCAAAC CTTGAGTGATGGT-3′; and SA1, 5′-GGCAGCGGTTCGGCAGAC CCCTCCAAGGAC-3′, and SA2, 5′-CAGGGGCTAGCTTACTGCTGA ACGGCGTCGAGCGG-3′.

Analysis of human C1q binding to 12G3H11 and its hinge variants by ELISA

To characterize the binding of the different human IgG1 variants listed in Table I to human C1q, the following ELISA was conducted: briefly, individual wells of a 96-well Maxisorp Immunoplate were coated overnight at 4°C with 50 μl of 2-fold serially diluted samples (purified 12G3H11 or hinge variants) at concentrations typically ranging from 20 to 0.31 μg/ml and then blocked with 3% BSA/PBS for 2 h at 37°C. 12G3H11 (as wild-type control) was systematically coated on each individual assay plate. Plates were then successively incubated with 100 μl of 2 μg/ml human C1q (Quidel) for 1 h at 37°C and sheep anti-human C1q (1/1,000 dilution; BioDesign) for 1 h at 37°C. Incubation with a donkey anti-sheep IgG HRP conjugate (1/10,000 dilution; Serotec) for 1 h at room temperature then followed. HRP activity was detected with tetramethylbenzidine substrate (KPL) and the reaction quenched with 1% H_2SO_4. Plates were read at 450 nm. For each human IgG1 concentration, the ratio of the sample's average OD_{450} over the average OD_{450} exhibited by 12G3H11 on the same plate was calculated.

Analysis of human FcγRIIIA binding to 12G3H11 and its hinge variants by ELISA

To characterize the binding of the different human IgG1 variants listed in Table I to human FcγRIIIA (F158 allotype), the following ELISA was conducted: briefly, individual wells of a 96-well Maxisorp Immunoplate were coated overnight at 4°C with 25 ng of protein A/G (Pierce), blocked with 3% BSA/PBS for 2 h at 37°C, and incubated with 50 μl of 2-fold serially diluted samples (purified 12G3H11 or hinge variants) at concentrations typically ranging from 10 to 0.156 μg/ml for 1 h at 37°C. 12G3H11 (as wild-type control) was systematically loaded on each individual assay plate. Plates were then successively incubated with 100 μl of 2.5 μg/ml human FcγRIIIA-streptavidin and biotin HRP conjugate (1/1000 dilution; Pierce) for 1 h at 37°C. HRP activity was detected with tetramethylbenzidine substrate (KPL) and the reaction quenched with 1% H_2SO_4. Plates were read at 450 nm. For each human IgG1 concentration, the ratio of the sample's average OD_{450} over the average OD_{450} exhibited by 12G3H11 on the same plate was calculated.

Analysis of human C1q binding to 12G3H11 and its hinge variants by surface plasmon resonance

The interaction of soluble human C1q (Quidel) with immobilized 12G3H11 and variants 3, 13, 14, 18, 19, and 21 was monitored by surface plasmon resonance detection using a BIAcore 3000 instrument (Biacore International). The corresponding protein concentrations were calculated by the bicinchoninic acid (BCA) method. The different human IgG1s were coupled to the dextran matrix of a CM5 sensor chip (Pharmacia Biosensor) using an Amine Coupling kit as previously described (24) at a surface density of between 4830 and 9221 resonance units. Excess reactive esters were quenched by injection of 70 μl of 1.0 M ethanolamine hydrochloride (pH 8.5). Human C1q was buffer-exchanged against PBS buffer and used in equilibrium binding experiments at concentrations ranging from 750 to 12 nM at a flow rate of 5–10 μl/min. Dilutions and binding experiments were conducted in 50 mM HBS buffer containing 0.01 M HEPES (pH 7.4), 0.15 M NaCl, 3 mM EDTA, and 0.005% P-20. Data were collected for ~50 min, and two 1-min pulses of 1 M NaCl/50 mM NaOH were used to regenerate the surfaces. Human C1q was allowed to flow over an uncoated cell, and the sensorgrams from these blank runs subtracted from those obtained with IgG1-coupled chips. Dissociation constants (K_Ds) were determined by fitting the binding isotherms using GraphPad Prism (GraphPad Software) and are recorded in Table II.

Analysis of human FcγRIIIA binding to 12G3H11 and its hinge variants by surface plasmon resonance

The interaction of soluble human FcγRIIIA (F158 allotype) with immobilized 12G3H11, variants 3, 13, and 14, was monitored by surface plasmon resonance detection using a BIAcore 3000 instrument. Protein concentrations were calculated by the BCA method. The different human IgG1s were coupled to the dextran matrix of a CM5 sensor chip using an Amine Coupling kit as previously described (24) at a surface density of between 7765 and 8385 resonance units. Excess reactive esters were quenched by injection of 70 μl of 1.0 M ethanolamine hydrochloride (pH 8.5). Flag-tagged human FcγRIIIA was used in equilibrium binding experiments at concentrations ranging from 20,000 to 9.8 nM at a flow rate of 5–10 μl/min. Dilutions and binding experiments were conducted in 50 mM HBS buffer containing 0.01 M HEPES (pH 7.4), 0.15 M NaCl, 3 mM EDTA, and 0.005% P-20. Data were collected for ~50 min, and one 30-s pulse of 5 mM HCl was used to regenerate the surfaces. Flag-tagged human FcγRIIIA was allowed to flow over an uncoated cell, and the sensorgrams from these blank runs subtracted from those obtained with IgG1-coupled chips. K_Ds were determined by fitting the binding isotherms using GraphPad Prism and are recorded in Table II.

Analysis of human EphA2 binding to 12G3H11 and its hinge variants by KinExa

The interaction of immobilized human EphA2-Fc with soluble 12G3H11, variants 3, 4, 5, 6, 11, 13, 14, and 18, was monitored using a KinExA 3000 instrument (Sapidyne Instruments). Protein concentrations were calculated by the BCA method. Typically, human EphA2-Fc was coated onto Azlactone beads at a concentration of 100 μg/ml in 0.05 M $NaHCO_3$ (pH 9.0) for 1–2 days at 4°C according to the manufacturer's instructions (Sapidyne Instruments). Coated beads were then separated from unreacted EphA2-Fc using a gentle pulse spin and blocked for ~2 h at room temperature with 1 M Tris (pH 8.0) and 10 mg/ml BSA. Beads were then resuspended in 30 ml of run buffer (PBS (pH 7.4), 0.02% NaN_3) and packed into a column. Typically, human IgGs were prepared at concentrations of 10, 25, and/or 50, pM. Human EphA2-Fc was then titrated across these IgG solutions at concentrations ranging from 39 fM to 80 pM and 156 fM to 400 pM and incubated for 3–7 days at room temperature. The amount of free IgG in the samples was derived from the fluorescence signal obtained after the passing of Cy5-labeled goat anti-human IgG $F(ab')_2$ (typically 0.5–2 μg/ml; Jackson ImmunoResearch Laboratories) through the column. K_Ds were determined by fitting the individual equilibrium titration data to a 1:1 binding model using the KinExA Pro 1.0.3. software.

ADCC assay

Typically, the ADCC activity of 12G3H11 and of hinge variant 14 was assessed as follows: human blood samples were collected from several individual healthy volunteers using heparinized syringes, diluted with twice the volume of PBS buffer, layered onto a Lymphoprep gradient (ICN), and centrifuged at 400 × *g* for 30 min at room temperature. PBMCs were harvested from the interface, washed three times with PBS, and resuspended in RPMI 1640 medium with L-glutamine (Invitrogen Life Technologies) supplemented with 10% FBS. A total of 40 ng/ml recombinant

Table II. *Affinity measurements for the binding of different hinge-mutated human IgG1s to human C1q and human FcγRIIIA (F158 allotype)*[a]

Hinge Variant	C1q-K_D (nM)	FcγRIIIA-K_D (nM)
Wild type (12G3H11)	89 ± 21	6,050 ± 950
3	155 ± 36	6,400 ± 1,000
13	607 ± 141	12,300 ± 1,930
14	282 ± 66	42,400 ± 6,660
18	274 ± 64	ND
19	61 ± 14	ND
21	92 ± 21	ND

[a] K_Ds were determined by BIAcore using equilibrium measurements as described in *Materials and Methods.* Errors in the C1q and FcγRIIIA binding measurements were estimated from the SDs of three (12G3H11/C1q pair) and two (12G3H11/FcγRIIIA pair) independent determinations. ND, not determined.

human IL-2 (R&D Systems) was then added to the PBMCs. Overnight incubation at 37°C in T-175 flasks (BD Biosciences) then followed. Cultured A549 cells (human lung carcinoma) endogenously expressing human EphA2 were harvested the following day and resuspended in RPMI 1640 medium supplemented with 5% FBS (assay buffer) at a density of 2×10^5 cells/ml. These were then added to a 96-well round-bottom tissue culture plate (BD Biosciences) at 50 μl/well along with various concentrations of Ab at 50 μl/well in assay buffer (see above) and preincubated at 37°C for 30 min. PBMCs were then harvested from their overnight incubation and resuspended at 5×10^6 cells/ml (for an E:T ratio of 50:1) and 2.5×10^6/ml (for an E:T ratio of 25:1) in assay buffer (see above) and added at 100 μl/well to the assay plate. A total of 25 μl/well 9% Triton X-100 (Promega) was added as a control for complete lysis. The plates were centrifuged at $300 \times g$ for 3 min and incubation at 37°C was continued for 4 h. Plates were then centrifuged at $300 \times g$ for 10 min, and 50 μl of supernatant from each well was transferred to MaxiSorp 96-well plates (BD Biosciences). Fifty microliters of reconstituted substrate mix (CytoTox 96 Nonradioactive Cytotoxicity Assay kit; Promega) was then added to all wells and incubated in the dark at room temperature for 30 min. Fifty microliters of stop solution (Promega) was added to each well and lactate dehydrogenase release was quantified by measuring the absorbance at 490 nm. Percent cytotoxicity was calculated using the following equation: percent cytotoxicity = (experimental − effector spontaneous − target spontaneous)/(target maximum − target spontaneous) × 100, where "experimental" corresponds to the signal measured in one of the condition of interest described above, "effector spontaneous" corresponds to the signal measured in the presence of PBMCs alone, "target spontaneous" corresponds to the signal measured in the presence of A549 cells alone, and "target maximum" corresponds to the signal measured in the presence of detergent-lysed A549 cells.

CDC assay

Four different cell lines were used to assess the CDC activity of 12G3H11 and of different hinge variants: 1) KATO III (human gastric carcinoma) endogenously expressing human EphA2, 2) KATO III endogenously expressing human EphA2 and transiently transfected with full-length human EphA2 (MedImmune) using LipofectAMINE (Invitrogen Life Technologies) and standard protocols, 3) CT-26 (mouse colon carcinoma) stably transfected with full-length human EphA2 (gift from E. Bruckheimer, MedImmune), and 4) Chinese hamster ovary transiently transfected with full-length human EphA2 (MedImmune) using LipofectAMINE (Invitrogen Life Technologies) and standard protocols. Typically, these various cell types were individually harvested using enzyme-free cell dissociation buffer (Invitrogen Life Technologies), and plated at 50,000 cells/well in 50 μl/well assay buffer (RPMI 1640 medium/0.1% BSA/20 mM HEPES) in a 96-well plate. Cells were then incubated with 50 μl/well of the various concentrations of Ab for 1 h on ice. A total of 25 μl/well of 9% Triton X-100 (Promega) was added as a control for complete lysis. Normal human serum complement (Quidel) was diluted in assay buffer (see above) at 1/5 for transfected and nontransfected KATO III cells and at 1/3 for CT-26 cells. A total of 50 μl/well of diluted serum was added to the cells. Incubation for 2 h at 37°C then followed. Next, 50 μl/well Alamar Blue (BioSource International) was added, and the incubation was continued overnight. Fluorescence was read using a SpectraMax Fluorometer with the excitation and emissions wavelengths set at 530 and 590 nm, respectively. Percent cytotoxicity was calculated using a linear regression between 0 (cells, serum, no Ab) and 100% (cells, serum, no Ab, Triton X-100).

Results

Design of hinge mutations

Both the upper and middle hinge regions of 12G3H11 were targeted for mutagenesis (Table I). Specifically, the upper hinge encompasses residues 216–225 (EU numbering as reported in Ref. 25) and spans the region starting at the end of the C_H1 domain up to the first cysteine forming an inter-H chain disulfide bond. The middle hinge encompasses residues 226–230 and spans the region starting at the end of the upper hinge up to the beginning of the C_H2 exon. Modifications were introduced in these two regions in an effort to modify their general characteristics. Their effect was predicted to increase flexibility (substitution by, or introduction of glycine residues, elimination of existing disulfide bonds), decrease flexibility (substitution by, or introduction of proline residues to form potentially stabilizing secondary structure elements, substitution by cysteine residues to increase the potential to form additional disulfide bonds), modify length (addition or deletion of select residues), and/or change the local conformation (substitution by bulky residues, change in the placement of cysteine residue). When tested by BIAcore, all the hinge variants that exhibited a significant change in C1q/FcγRIIIA binding and CDC/ADCC activity (see below) had similar apparent binding affinity to their cognate Ag (human EphA2) as 12G3H11 (data not shown). Of these, all which showed a decrease in their effector functions were more accurately characterized using a KinExa instrument, as their apparent dissociations rates were near or over BIAcore's sensitivity limit ($\sim 5 \times 10^{-6}$ s^{-1}). The corresponding affinities to human EphA2 were very similar to 12G3H11 (Table III). This, along with the ability of these IgGs to be purified by protein A or G (*Materials and Methods*), indicated that these effects on effector functions were not caused by major structural changes.

Chain pairing

All hinge mutants described in Table I were further characterized by SDS-PAGE under reducing and nonreducing conditions. Under reducing conditions, all variants migrated as two main bands of ~60 and 30 kDa corresponding to their heavy and light polypeptide chains, respectively. Representative data for all variants exhibiting altered effector functions are shown in Fig. 1, *A* and *B*.

Under nonreducing conditions, six variants (3, 6, 11, 13, 14, and 18) consistently migrated as two main bands of ~200 and 100 kDa (Fig. 1*C*). The 200-kDa band corresponded to full-length IgG. The 100-kDa band, present at significantly higher levels when compared with 12G3H11, corresponded to an IgG half-structure consisting of covalently bound H and L chains. A range in the relative proportions of these two species was observed, varying from mostly half-IgG (variants 14 and 18) to similar amounts of full-length and half-IgG (variants 11 and 13) to mostly full length-IgG (variants 3 and 6). All other variants did not exhibit a significantly greater proportion of the 100-kDa species when compared with 12G3H11 and all showed a band corresponding to the full-length IgG at ~200 kDa. Representative data for all variants exhibiting altered effector functions are shown in Fig. 1, *C* and *D*.

Variants 3, 4, 6, and 14 were further characterized by size exclusion chromatography. These molecules did not show any evidence of H chain dissociation in solution and eluted similarly to 12G3H11 in a single peak (data not shown).

Table III. *Affinity measurements for the binding of different hinge-mutated human IgG1s to human EphA2*[a]

Hinge Variant	K_D (pM)
Wild type (12G3H11)	1.1 ± 0.9
3	2.8 ± 2.1
4	5.2 ± 3.5
5	0.8 ± 0.6
6	2.7 ± 2.0
11	≤1[b]
13	1.7 ± 0.8
14	7.2 ± 4.3
18	1.5 ± 0.1

[a] K_Ds were determined using a KinExa instrument as described in *Materials and Methods*. Errors in the binding measurements were estimated from the SDs of two independent determinations.

[b] The signal strength and KinExa's sensitivity limit precluded a more accurate determination. In all cases, the residual error between the fitted and theoretical curves was ≤6 %.

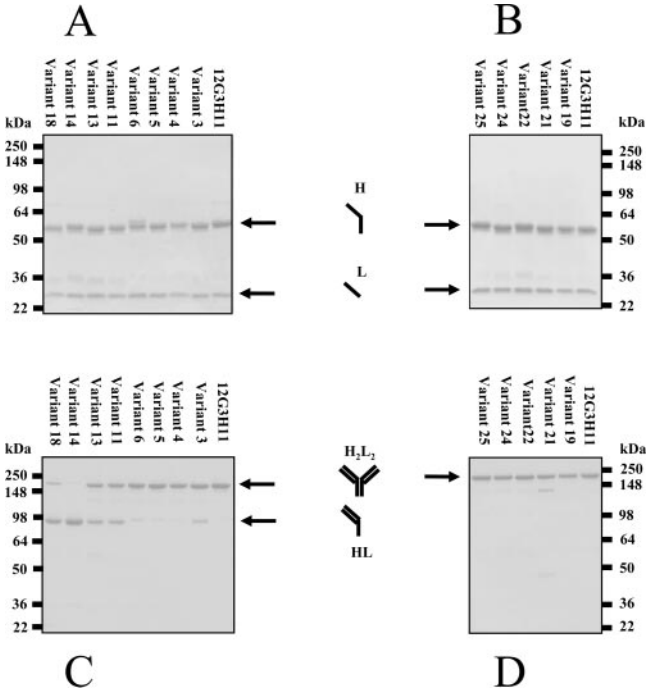

FIGURE 1. Ten-percent SDS-PAGE profile of purified 12G3H11 and select hinge variants under reducing (*A* and *B*) and nonreducing (*C* and *D*) conditions. Gels were stained with Brilliant Blue R Concentrate (Sigma-Aldrich). Molecular mass standards were SeeBlue Plus 2 (Invitrogen Life Technologies). H = H chain; L = L chain.

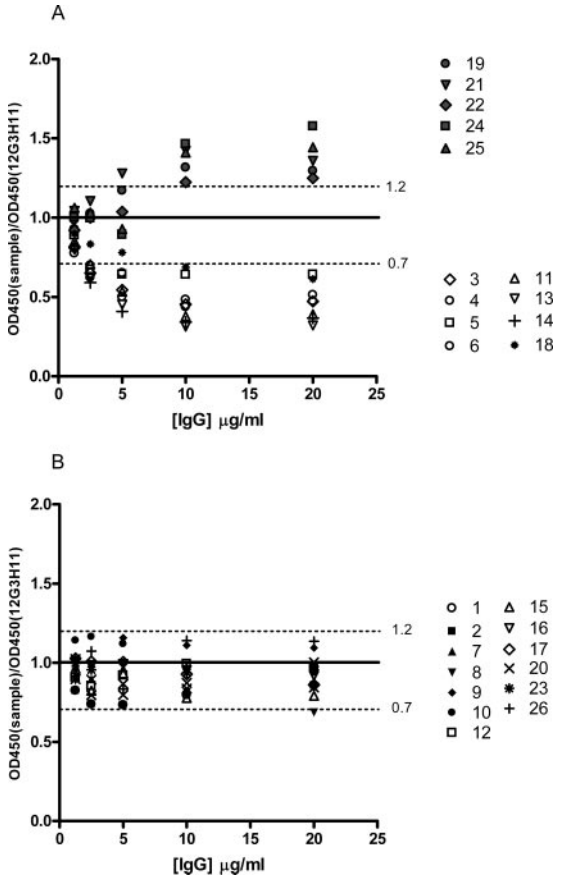

FIGURE 2. C1q binding of various hinge variants. For each IgG variant concentration, the ratio of the corresponding OD_{450} over the OD_{450} exhibited by the wild-type IgG (12G3H11) at equal concentration was reported. A signal ratio > 1 indicates enhanced binding to C1q when compared with wild type, whereas a signal ratio < 1 indicates a loss of binding. *A*, Hinge variants consistently exhibiting a >20% increase or a >30% decrease in C1q binding when compared with wild type. *B*, Hinge variants consistently exhibiting a <20% increase or a <30% decrease in C1q binding when compared with wild type. Data shown are representative of two independent series of experiments.

Complement fixation

Binding of the first component of complement C1q to the different upper and middle hinge variants was analyzed by ELISA as described in *Materials and Methods*. The sequences of the various hinge mutants are summarized in Table I, whereas representative binding results are shown in Fig. 2, *A* and *B*. For the purpose of this study, changes in C1q binding were considered to be significant when they showed either: 1) a >20% increase compared with wild type using at least two IgG concentrations or 2) a >30% decrease compared with wild type using at least two IgG concentrations. Using these thresholds, several trends are apparent. The consequences of increasing the flexibility of the middle hinge region (variants 3, 4, 5, and 6) were generally detrimental to C1q binding (Fig. 2*A*). However, increasing the flexibility of the upper hinge (variants 1 and 2) only had a minimal effect as shown in Fig. 2*B*. Likewise, decreasing the flexibility of the upper hinge (variants 7, 8, 9, and 10) also had a minimal effect on C1q binding (Fig. 2*B*). Increasing the length of the hinge region had a dramatic negative effect on C1q binding when in conjunction with an increase in the overall flexibility of its middle portion (variant 14) as shown in Fig. 2*A*. When the same additional flexibility component was grafted in the upper hinge (variant 15), the negative effect was significantly attenuated almost back to normal (Fig. 2*B*). An increase in length coupled with a decrease in flexibility of the hinge region did not have significant consequences whether the additional rigidity component was introduced in its middle (variant 17) or upper (variant 16) portion (Fig. 2*B*). Decreasing the length of the hinge region had different effects depending on where the deletion was made. More precisely, a 2-aa deletion in the middle part of the hinge (variant 11) resulted in knocking out binding to C1q to a large extent, whereas a 2-aa deletion in the upper part of the hinge (variant 12) did not have a significant effect on C1q binding (Fig. 2, *A* and *B*, respectively). A 2-aa deletion in both the upper and middle parts of the hinge (variant 13) had roughly the same effect on C1q binding as a 2-aa deletion in the middle part of the hinge alone (variant 11) as shown in Fig. 2*A*.

A 2- to 3-aa substitution by hydrophobic and bulky tryptophane residues in the lower portion of the upper hinge (variants 19, 24, and 25) resulted in an increase in C1q binding (Fig. 2*A*), whereas it had no significant effect when in the upper portion of the upper hinge (variant 26; Fig. 2*B*). Interestingly, a 2-aa substitution by the same residue in the middle hinge (variant 18) had an adverse effect on C1q binding (Fig. 2*A*). Finally, a 2-aa substitution by hydrophobic phenylalanine residues in the lower portion of the upper hinge (variant 23) did not significantly alter the ability to of the Fc region to bind C1q (Fig. 2*B*).

Changing the position of the upper hinge cysteine had various effects, ranging from increased binding to C1q (variant 21) to no significant effect (variant 20) as shown in Fig. 2, *A* and *B*, respectively. The combination of "enhanced" variants 19 and 21 (variant 22) did not yield additive or synergistic effects, resulting in a molecule exhibiting C1q binding properties similar to variants 19 and 21 (Fig. 2*A*).

Select representative variants (3, 13, 14, 18, 19, and 21) were chosen for further characterization of C1q binding using surface plasmon resonance (BIAcore). The K_Ds determined are reported in

Table II and agree reasonably well with the corresponding ELISA data (Fig. 2, *A* and *B*). Variant 13, whose loss of binding to C1q was one of the most profound as determined by ELISA, exhibited a ~7-fold reduction in binding affinity to C1q when compared with its wild-type counterpart (12G3H11). Variant 14, whose ELISA signal was comparable to variant 13, showed a loss in binding affinity to C1q of only ~3-fold. We do not know what accounts for this difference but think that it is likely attributable to the different format of the assays. Unlike what was seen by ELISA (Fig. 2*A*) and in the CDC assays (see below), "enhanced" variants 19 and 21 did not exhibit a significant increase in C1q binding when compared with 12G3H11 using BIAcore (Table II). Here again, this probably reflected a difference in the assay format and/or sensitivity.

CDC activity

Representative mutants (as based on their binding profile to C1q) were selected for further characterization by CDC. In terms of active concentration, variants 4, 13, and 14 exhibited a significantly reduced CDC activity on both human EphA2-transfected CT26 (Fig. 3*A*) and human EphA2-transfected KATO III (Fig. 3*B*) cells when compared with their wild-type counterpart (mAb 12G3H11). In particular, variant 13 consistently exhibited a >500-fold reduction in CDC activity on both cell types (Fig. 3, *A* and *B*). This is in good agreement with the corresponding binding data (Table II, Fig. 2*A*). Variants 19, 21, 22, 24, and 25 consistently exhibited a moderate, although significant enhancement in CDC activity on various cell types tested (Fig. 3, *C* and *D*) when compared with their wild-type counterpart (12G3H11). Here again, these data are in good agreement with the corresponding ELISA experiments (Fig. 2*A*).

Binding to human FcγRIIIA

Binding of human FcγRIIIA (F158 allotype) to the different hinge variants summarized in Table I was analyzed by ELISA as described in *Materials and Methods*. Representative results are shown in Fig. 4, *A* and *B*. For the purpose of this study, changes in FcγRIIIA binding were considered to be significant when they showed a >20% decrease compared with wild type using at least two IgG concentrations. Using this threshold, several conclusions can be made. The consequences of increasing flexibility in the upper (variants 1 and 2) or middle (variants 3, 4, 5, and 6) hinge were minimal (Fig. 4*B*). Likewise, decreasing the flexibility of the upper hinge (variants 7, 8, 9, and 10) also did not seem to have a significant effect on FcγRIIIA binding (Fig. 4*B*). Increasing the length of the hinge region had a dramatic "knockout" effect on FcγRIIIA binding only when it was in conjunction with an increase in its overall flexibility of its middle portion (mutant 14; Fig. 4*A*). When the additional flexibility component was grafted in the upper hinge (mutant 15), the negative effect on FcγRIIIA binding was not observed (Fig. 4*B*). An increase in length coupled with a decrease in flexibility did not have significant consequences for FcγRIIIA binding whether the additional rigidity component was

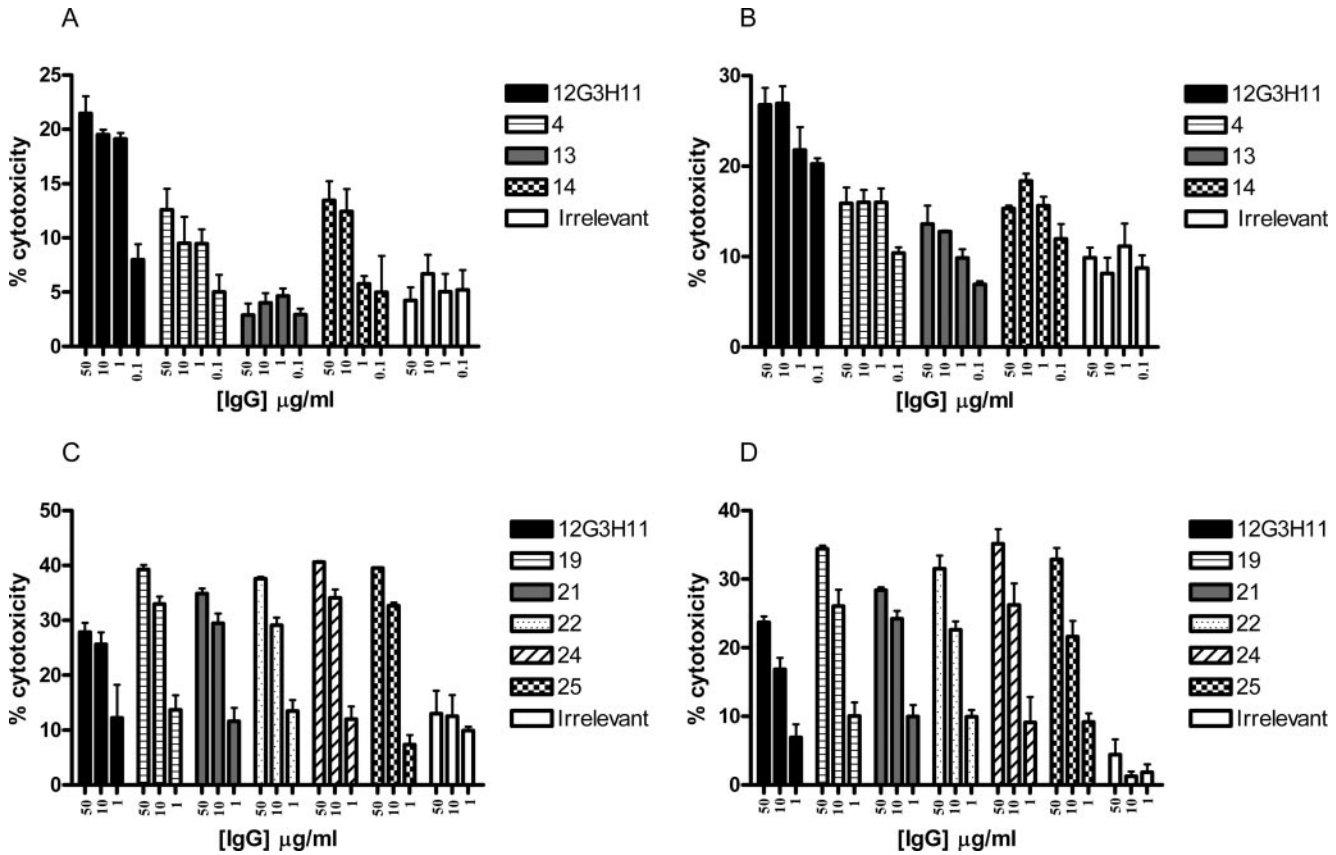

FIGURE 3. CDC activity of C1q "knockout" (*A* and *B*) and "enhanced" (*C* and *D*) hinge variants. All three "knockout" variants consistently exhibited a significantly decreased CDC activity when tested against (*A*) human EphA2-transfected CT 26 and (*B*) human EphA2-transfected KATO III cells when compared with their wild-type counterpart (mAb 12G3H11). All "enhanced" variants consistently exhibited a significantly increased CDC activity when tested against (*C*) KATO III and (*D*) human EphA2-transfected CT 26 cells when compared with 12G3H11. SEs are indicated by error bars and represent triplicate measurements within the same experiment. Data shown are representative of two independent series of experiments. When human EphA2-transfected KATO III and human EphA2-transfected Chinese hamster ovary cells were used, variants 19, 21, 22, 24, and 25 still consistently exhibited a significantly increased CDC activity when compared with wild type (data not shown).

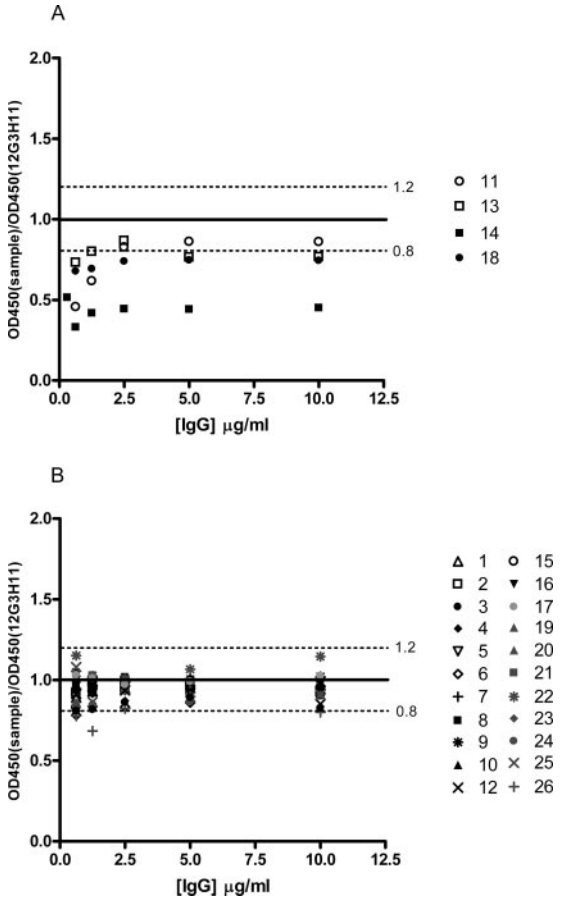

FIGURE 4. FcγRIIIA binding of various hinge variants. For each IgG variant concentration, the ratio of the corresponding OD_{450} over the OD_{450} exhibited by the wild-type IgG (12G3H11) at equal concentration was reported. A signal ratio > 1 indicates enhanced binding to FcγRIIIA when compared with wild type, whereas a signal ratio < 1 indicates a loss of binding. *A*, Hinge variants consistently exhibiting a >20% decrease in FcγRIIIA binding when compared with wild type. *B*, Hinge variants consistently exhibiting a <20% increase or a <20% decrease in FcγRIIIA binding when compared with wild type. Data shown are representative of two independent series of experiments.

introduced in the upper (variant 16) or middle (variant 17) hinge as shown in Fig. 4*B*. A 2-aa deletion in the upper hinge (variant 12) had no significant effect (Fig. 4*B*), whereas a 2-aa deletion made in the middle hinge (variant 11) had a small, albeit significant negative impact (Fig. 4*A*). A 2-aa deletion in both the upper and middle hinge (variant 13) had roughly the same effect as a 2-aa deletion in the middle part of the hinge alone (variant 11) as shown in Fig. 4*A*.

Double or triple substitutions by hydrophobic tryptophane or phenyalanine residues in the upper hinge (variants 19, 22, 23, 24, 25, and 26) minimally impacted FcγRIIIA binding (Fig. 4*B*), whereas a double substitution by tryptophane residues in the middle hinge (variant 18) slightly reduced binding to FcγRIIIA (Fig. 4*A*). No significant effect was seen by changing the position of the upper hinge cysteine normally involved in the covalent bond between the L and H chains (variants 20 and 21; Fig. 4*B*).

Select representative variants (3, 13, and 14) were then chosen for affinity characterization using surface plasmon resonance (BIAcore). The K_Ds determined are reported in Table II. These values agree well with the ELISA data (Fig. 4, *A* and *B*). Variant 14 exhibited the most dramatic reduction in binding to FcγRIIIA by ELISA, translating to an ~7-fold reduction in binding affinity

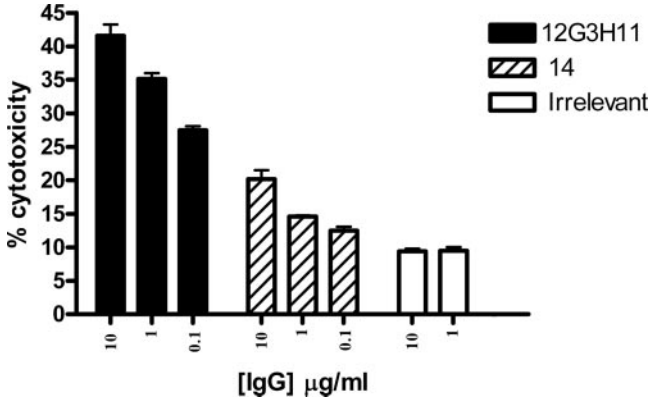

FIGURE 5. ADCC activity of one FcγRIIIA "knockout" hinge variant using PBMCs from a single donor and a E:T ratio of 25:1 against A549 cells. SEs are indicated by error bars and represent triplicate measurements within the same experiment. Data shown are representative of three independent series of experiments using PBMCs from different individual donors.

to FcγRIIIA when compared with its wild-type counterpart. Also as expected from the ELISA measurements, variant 13 exhibited a slight reduction in binding affinity to FcγRIIIA (~2-fold), whereas variant 3 did not see its binding properties significantly changed.

ADCC activity

One representative hinge modified molecule (variant 14) was selected for further characterization by ADCC as judged from its binding profile to human FcγRIIIA. In terms of active concentration, variant 14 exhibited a >100-fold reduction in ADCC activity on A549 cells when compared with its wild-type counterpart (12G3H11) as shown in Fig. 5. Although this is in excellent agreement with the corresponding binding data (Table II, Fig. 4*A*), it is important to note that the PBMCs of the various donors were not genotyped in terms of their FcγRIIIA allotypic diversity at 158 (V/F). Therefore, it is possible that the corresponding ADCC activities do not exactly reflect the binding data in which the F158 allotype was used.

Discussion

Effector functions are critical to many of IgG cell killing properties. In particular, ADCC and CDC are thought to play important roles in the efficacy of some therapeutic IgG molecules (26–28). ADCC and CDC can be initiated by the binding of FcγRIIIA and C1q, respectively, to IgG/Ag complexes. Therefore, being able to engineer and enhance the corresponding interactions could provide clear therapeutic benefits. Indeed, it is now well accepted that efficient effector functions are crucial to the ability of many therapeutic Abs to treat liquid and solid tumors. Among those are rituximab (an anti-CD20 chimeric Ab designed to treat non-Hodgkin's B cell lymphoma; Refs. 26 and 27) and L6 (a chimeric Ab directed against a tumor-associated cell surface Ag expressed in lung, colon, and breast cancer; Ref. 27). Conversely, the ability to down-regulate ADCC and CDC would have practical applications to decrease or eliminate toxicity in a therapeutic setting. Such is the case of the anti-CD3 mAb OKT3 whose complement activation properties are linked to the release of cytokines associated to an acute clinical syndrome (29, 30).

Several important sites on human IgG1 for binding C1q and FcγRIIIA have been described. More precisely, critical "hot spots" involved in binding C1q and/or mediate CDC activity have been shown to locate in various parts of the Fc region in both the C_H2

and C_H3 domains (17, 18, 31), whereas binding to FcγRIIIA has been mapped primarily to the lower hinge region on both H chains (32). However, more indirect mechanisms are likely to play additional roles in modulating effector functions. In particular, the hinge region is thought to be a significant contributor. The mechanisms by which the hinge could influence binding to C1q or FcγRIIIA include its inherent flexibility which allows the Fc to assume a favorable conformation or precludes the Fab arms from covering Fc interaction sites (5, 13, 33). Its role as a spacer preventing interference of the Fab arms with the Fc binding sites has also been suggested or discussed (1, 3, 13). However, as outlined in the introduction section, the exact nature of the relationship between the sequence and property of the hinge and the corresponding IgG effector functions is still to be determined. As part of the effort to elucidate this relationship and engineer safe and potent therapeutic IgGs, we have generated 26 hinge mutations in the upper (residues 216–225 according to EU numbering) and middle (residues 226–230) hinge of a human IgG1. 12G3H11, a humanized anti-human EphA2 mAb, was used as the prototypic human IgG1. The mutations were designed in an effort to generate variants exhibiting well-defined hinge characteristics. However, their predicted effect on the actual hinge characteristics remains hypothetical.

The flexibility and length of the upper and middle hinge were tuned using various amino acid substitutions, additions, or deletions. The flexibility of the upper hinge was decreased through substitution by cysteine residues to increase the potential to form additional inter-H chain disulfide bonds (variants 7 and 8; Table I). The rigidity of the upper hinge was also increased using substitution by proline residues (variants 9 and 10; Table I) to form potentially stabilizing polyproline helical structures (34, 35) as observed in the middle hinge of a human IgG1 (36). The middle hinge was not submitted to the above described modifications since their net effect would be unclear. Conversely, the rigidity of the upper and middle hinge was decreased using substitution by glycine residues to eliminate existing stabilizing polyproline helices in the middle hinge (Ref. 36; variants 3 and 4; Table I) and/or remove potentially stabilizing side chain contacts in the upper hinge (variants 1 and 2; Table I). The flexibility of the middle hinge was also increased through substitution of cysteine by serine residues to remove existing inter-H chain disulfide bonds (variants 5 and 6; Table I). This last type of modification was not conducted in the upper hinge so as not to directly impact the pairing of the L and H chains and negatively affect the overall stability of the whole IgG molecule. Combinations of increases in length and decreases in flexibility of either the upper or middle hinge was addressed by introducing additional proline residues (variants 16 and 17; Table I). In a similar fashion, increases in both length and flexibility of either the upper or middle hinge were conducted by introducing additional glycine residues (variants 15 and 14; Table I). To further investigate the impact of hinge length, 2- or 4-aa deletions were introduced in the upper and/or middle hinge (variants 11, 12, and 13; Table I).

Taken together, our data show that the inherent rigidity of the middle hinge is predictive of the Fc ability to fix C1q, whereas the flexibility characteristics of the upper hinge is not. Indeed, increasing or decreasing the flexibility of the upper hinge (variants 1, 2, 7, 8, 9, and 10) does not seem to have a significant effect on C1q binding. Conversely, increasing the flexibility of the middle hinge negatively affects binding to C1q (variants 3, 4, 5, and 6). The important role played by each of the two middle hinge covalent bonds (variant 5 and 6) is in agreement with previous results suggesting that the opening of the bridge formed by the middle hinge upper cysteine results in a dramatic loss of complement binding (37, 38). Thus, we show here that the role played by the middle hinge lower cysteine is also a determinant one. We also demonstrate that an increase in length in the upper or middle hinge does not inherently impact C1q binding (variants 15, 16, and 17) except, as noted above, when the rigidity of the middle hinge is decreased (variant 14). The central role played by the middle hinge is further demonstrated by the fact that a 2-aa deletion in this region (variant 11) decreases binding to C1q to a large extent, whereas a similar deletion in the upper hinge (variant 12) has no significant effect. It is important to note that variants 3, 6, 11, 13, and 14 exhibited a significantly increased proportion of noncovalently bound H chains. Presumably, this phenomenon is linked to the disruption of both middle hinge covalent bonds by the corresponding mutations. Thus, it is possible that all or part of the effect seen on C1q binding and/or CDC activity for these mutants is the direct consequence of differential interactions between both H chains. Although size exclusion chromatography of such variants did not reveal any significant dissociation in solution (data not shown), it seems likely that incomplete inter-H chain covalent assembly could alter the rigidity of the Fc domain and thus preclude the optimal positioning of the various sites making contact with C1q. Alternatively, long-range effects caused by a defective assembly could conceivably induce conformational changes in the C1q binding sites of the constant regions. Therefore, we conclude that: 1) the rigidity of the middle hinge may play a determinant role in modulating the Fc/C1q interaction, 2) the flexibility level of the upper hinge may have no significant effect on C1q binding, 3) each of the disulfide bond formed by the middle hinge two cysteine residues is individually important to fix complement, 4) in its native form, the length of the middle hinge is near the acceptable minimum requirement for efficient complement fixation and further increases in length have no impact per se, 5) the length of the upper hinge does not affect C1q binding within the range tested (from -2 to $+3$ residues), and 6) the effects mediated by some middle hinge modifications may be at least partly due to a disruption of both inter-H chain covalent interactions.

An enhancing effect in terms of complement fixation was seen for mutations located in the upper hinge region (variants 19, 21, 22, 24, and 25), indicating that this region also plays an influential role. Interestingly, similar substitutions in the middle hinge (variant 18) result in an opposite effect (i.e., a decrease in complement fixation). This, along with the previously discussed flexibility and length-related data, indicates the existence of a clear functional difference between these two hinge regions. It is unclear what the structural consequences of the tryptophane (W) substitutions in variants 18, 19, 22, 24, and 25 may be. However, it is worth noting that the effects of the "two W" substitutions in variant 19 appear to be side chain-specific since variant 23, otherwise identical to variant 19 but for a "two F" instead of a "two W" substitution, does not see its C1q binding ability significantly altered. Conceivably, such substitutions may result in differential steric effects. The impaired ability of variant 18 to bind C1q, along with its propensity to form noncovalently bound H chains further illustrate the importance of the bonds mediated by the middle hinge cysteines. The characteristics of the covalent bond formed by the upper hinge cysteine between the H and L chains appear to be important since modification in the placement of this residue (variant 21) resulted in increased complement fixation. This effect strongly depends on the specific position of the cysteine (compare variants 20 and 21).

Defining hinge requirements for the binding to human FcγRIIIA seems to be more challenging since most of the mutations tested do not have a significant effect. However, it appears that the integrity of the middle hinge in terms of length/flexibility (variants 11 and 14) and content (variant 18) is a crucial factor. In light of the

proximity of the FcγRIIIA binding site with the middle hinge (32), it is perhaps not surprising that major changes in this region would affect binding to this receptor. As observed and discussed above, the formation of noncovalently bound H chains may also account for a significant portion of the effects seen. Importantly, the relative arrangement between both C_H2 domains is critical to maintain the FcγRIII binding site in a favorable conformation (32). Thus, a disruption of the natural hinge-mediated tethering between these domains could possibly alter IgG binding to FcγRIII. Disruption of both middle hinge disulfide bonds in variants 11, 14, and 18 agrees well with previous results showing that the presence of both middle hinge cysteine residues in human IgG1 are crucial for ADCC activity (15). However, it is also apparent that retention of only one disulfide bond in the middle hinge is enough for efficient binding to human FcγRIIIA (variants 5 and 6). Alternatively, it is also possible that clearer or different hinge requirements would appear if other FcγRIIIA allotypes were tested (for instance FcγRIIIA/V158 which exhibits a higher binding affinity to human IgGs than FcγRIIIA/F158 (39)).

It is worth noting that all the hinge mutants that we tested for binding to human EphA2 exhibited very similar affinities (see Table III). This indicated that in our case, the hinge length/flexibility characteristics do not play a major role in Ag binding for a human IgG1.

In summary, we have dissected out the individual contribution of the upper and middle hinge of a human IgG1 on its effector functions. To our knowledge, this is the first systematic study of the functional consequences of well-defined modifications in an Ab upper and middle hinge regions. The lower hinge of a human IgG1 (part of the C_H2 exon and encompassing residues 231–238 according to the EU numbering) had already been shown to modulate C1q binding and CDC (40). This, along with our data, suggests that the entire (upper, middle, and lower) hinge in human IgG1 plays a significant role in regulating effector functions. It is likely that this conclusion could be further extended to other isotypes as a mutation in the lower hinge of a primatized IgG4 was shown to modulate ADCC (41). We have shown that the hinge constitutes an attractive target to fine tune the effector functions of a human IgG1. The "knockout" and enhanced variants which we have generated exhibit desirable characteristics for therapeutic use. It will be interesting to know if our approach can be used in conjunction with a C_H2 engineering approach previously described (28) to further increase CDC activity. Further elucidation of the molecular mechanisms by which our hinge modifications modulate these functions will require a more detailed analysis, such as segmental flexibility measurements to experimentally verify the flexibility properties of the variants (5, 42), testing of alternate FcγRIIIA allotypes, generation of additional mutants and x-ray crystallography of IgG/C1q and IgG/FcγRIIIA complexes.

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Disclosures

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References

1. Klein, M., N. Haeffner-Cavaillon, D. E. Isenman, C. Rivat, M. A. Navia, D. R. Davies, and K. J. Dorrington. 1981. Expression of biological effector functions by immunoglobulin G molecules lacking the hinge region. *Proc. Natl. Acad. Sci. USA* 78: 524–528.
2. Michaelsen, T. E., A. Aase, C. Westby, and I. Sandlie. 1990. Enhancement of complement activation and cytolysis of human IgG3 by deletion of hinge exons. *Scand. J. Immunol.* 32: 517–528.
3. Coloma, M. J., K. R. Trinh, L. A. Wims, and S. L. Morrison. 1997. The hinge as a spacer contributes to covalent assembly and is required for function of IgG. *J. Immunol.* 158: 733–740.
4. Brekke, O. H., T. E. Michaelsen, R. Sandin, and I. Sandlie. 1993. Activation of complement by an IgG molecule without a genetic hinge. [Published erratum appears in *1996 Nature 383: 103.*] *Nature* 363: 628–630.
5. Oi, V. T., T. M. Vuong, R. Hardy, J. Reidler, J. Dangle, L. A. Herzenberg, and L. Stryer. 1984. Correlation between segmental flexibility and effector function of antibodies. *Nature* 307: 136–140.
6. Dangl, J. L., T. G. Wensel, S. L. Morrison, L. Stryer, L. A. Herzenberg, and V. T. Oi. 1988. Segmental flexibility and complement fixation of genetically engineered chimeric human, rabbit and mouse antibody. *EMBO J.* 7: 1989–1994.
7. Sandlie, I., A. Aase, C. Westby, and T. E. Michaelsen. 1989. C1q binding to chimeric monoclonal IgG3 antibodies consisting of mouse variable regions and human constant regions with shortened hinge containing 15 to 47 amino acids. *Eur. J. Immunol.* 19: 1599–1603.
8. Norderhaug, L., O. H. Brekke, B. Bremnes, R. Sandin, A. Aase, T. E. Michaelsen, and I. Sandlie. 1991. Chimeric mouse human IgG3 antibodies with an IgG4-like hinge region induce complement mediated lysis more efficiently than IgG3 with normal hinge. *Eur. J. Immunol.* 21: 2379–3284.
9. Redpath, S., and P. D. Sun. 1998. Activation of complement by human IgG1 and human IgG3 antibodies against the human leucocyte antigen and CD52. *Immunology* 93: 595–600.
10. Bastida-Corcuera, F. D., J. E. Butler, S. Yahiro, and L. B. Corbeil. 1999. Differential complement activation by bovine IgG2 allotypes. *Vet. Immunol. Immunopathol.* 71: 115–123.
11. Tan, L. K., R. J. Shopes, V. T. Oi, and S. L. Morrison. 1990. Influence of the hinge region on complement activation, C1q binding, and segmental flexibility in chimeric human immunoglobulins. [Published erratum appears in *1991 Proc. Natl. Acad. Sci. USA 88: 5066.*] *Proc. Natl. Acad. Sci. USA* 87: 162–166.
12. Shopes, B. 1993. A genetically engineered human IgG with limited flexibility fully initiates cytolysis via complement. *Mol. Immunol.* 30: 603–609.
13. Brekke, O. H., T. E. Michaelsen, and I. Sandlie. 1995. The structural requirements for complement activation by IgG: does it hinge on the hinge? *Immunol. Today* 16: 85–90.
14. Redpath, S., T. E. Michaelsen, I. Sandlie, and M. R. Clark. 1998. The influence of the hinge region length in binding of human IgG to human Fcγ receptors. *Hum. Immunol.* 59: 720–727.
15. Gillies, S. D., and J. S. Wesolowsi. 1990. Antigen binding and biological activities of engineered mutant chimeric antibodies with human tumor specificities. *Hum. Antibod. Hybridomas* 1: 47–54.
16. Duncan, A. R., and G. Winter. 1988. The binding site for C1q on IgG. *Nature* 21: 332: 738–740.
17. Morgan, A., N. D. Jones, A. M. Nesbitt, L. Chaplin, M. W. Bodmer, and J. S. Emtage. 1995. The N-terminal end of the CH2 domain of chimeric human IgG1 anti-HLA-DR is necessary for C1q, FcγRI and FcγRIII binding. *Immunology* 86: 319–324.
18. Idusogie, E. E., L. G. Presta, H. Gazzano-Santoro, K. Totpal, P. Y. Wong, M. Ultsch, Y. G. Meng, and M. G. Mulkerrin. 2000. Mapping of the C1q binding site on rituxan, a chimeric antibody with a human IgG1 Fc. *J. Immunol.* 164: 4178–4184.
19. Kinch, M. S., and K. Carles-Kinch. 2003. Over expression and functional alterations of the EphA2 tyrosine kinase in cancer. *Clin. Exp. Metastasis* 20: 59–68.
20. Boshart, M., F. Weber, G. Jahn, K. Dorsch-Hasler, B. Fleckenstein, and W. Schaffner. 1985. A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. *Cell* 41: 521–530.
21. Johnson, S., C. Oliver, G. A. Prince, V. G. Hemming, D. S. Pfarr, S. C. Wang, M. Dormitzer, J. O'Grady, S. Koenig, J. K. Tamura, et al. 1997. Development of a humanized monoclonal antibody (MEDI-493) with potent in vitro and in vivo activity against respiratory syncytial virus. *J. Infect. Dis.* 176: 1215–1224.
22. Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 77: 51–59.
23. Gao, C., C. H. Lin, C. H. Lo, S. Mao, P. Wirsching, R. A. Lerner, and K. D. Janda. 1997. Making chemistry selectable by linking it to infectivity. *Proc. Natl. Acad. Sci. USA* 94: 11777–11782.
24. Johnsson, B., S. Lofas, and G. Lindquist. 1991. Immobilization of proteins to a carboxymethyldextran-modified gold surface for biospecific interaction analysis in surface plasmon resonance sensors. *Anal. Biochem.* 198: 268–277.
25. Kabat, E. A., T. T. Wu, H. M. Perry, K. S. Gottesman, and C. Foeller. 1991. In *Sequences of Proteins of Immunological Interest*, U.S. Public Health Service, National Institutes of Health, Washington, DC.
26. Anderson, D. R., A. Grillo-Lopez, C. Varns, K. S. Chambers, and N. Hanna. 1997. Targeted anti-cancer therapy using rituximab, a chimaeric anti-CD20 antibody (IDEC-C2B8) in the treatment of non-Hodgkin's B-cell lymphoma. *Biochem. Soc. Trans.* 25: 705–708.
27. Green, M. C., J. L. Murray, and G. N. Hortobagyi. 2000. Monoclonal antibody therapy for solid tumors. *Cancer Treat. Rev.* 26: 269–286.
28. Idusogie, E. E., P. Y. Wong, L. G. Presta, H. Gazzano-Santoro, K. Totpal, M. Ultsch, and M. G. Mulkerrin. 2001. Engineered antibodies with increased activity to recruit complement. *J. Immunol.* 166: 2571–2575.

29. Raasveld, M. H., F. J. Bemelman, P. T. Schellekens, F. N. van Diepen, A. van Dongen, E. A. van Royen, C. E. Hack, and I. J. ten Berge. 1993. Complement activation during OKT3 treatment: a possible explanation for respiratory side effects. *Kidney Int.* 43: 1140–1149.
30. Vallhonrat, H., W. W. Williams, A. B. Cosimi, N. Tolkoff-Rubin, L. C. Ginns, J. C. Wain, F. Preffer, I. Olszak, S. Wee, F. L. Delmonico, and M. Pascual. 1999. In vivo generation of C4d, Bb, iC3b, and SC5b9 after OKT3 administration in kidney and lung transplant recipients. *Transplantation* 67: 253–258.
31. Tao, M. H., R. I. Smith, and S. L. Morrison. 1993. Structural features of human immunoglobulin G that determine isotype-specific differences in complement activation. *J. Exp. Med.* 178: 661–667.
32. Radaev, S., and P. Sun. 2001. Recognition of immunoglobulins by Fc γ receptors. *Mol. Immunol.* 38: 1073–1083.
33. Burton, D. R. 1985. Immunoglobulin G: functional sites. *Mol. Immunol.* 22: 161–206.
34. Adzhubei, A. A., and M. J. Sternberg. 1993. Left handed polyproline helices commonly occur in globular proteins. *J. Mol. Biol.* 229: 472–493.
35. Adzhubei, A. A., and M. J. Sternberg. 1994. Conservation of polyproline helices in homologous proteins. *Protein Sci.* 3: 2395–2410.
36. Ito, W., and Y. Arata. 1985. Proton nuclear magnetic resonance study on the dynamics of the conformation of the hinge segment of human G1 immunoglobulin. *Biochemistry* 24: 6467–6474.
37. Schauenstein, E., F. Dachs, M. Reiter, H. Gombotz, and W. List. 1986. Labile disulfide bonds and free thiol groups in human IgG. I. Assignment to IgG1 and IgG2 subclasses. *Int. Arch. Allergy Appl. Immunol.* 80: 174–179.
38. Schauenstein, E., F. Dachs, M. Reiter, H. Gombotz, and W. List. 1996. Reactive disulfide bonds in immunoglobulin G: a unique feature in serum proteins of different species. *Biochem. Mol. Biol. Int.* 40: 433–446.
39. Koene, H. R., M. Kleijer, J. Algra, D. Roos, A. E. von dem Borne, and M. de Haas. 1997. FcγRIIIa-158V/F polymorphism influences the binding of IgG by natural killer cell FcγRIIIa, independently of the FcγRIIIa-48L/R/H phenotype. *Blood* 90: 1109–1114.
40. Hezareh, M., A. J. Hessell, R. C. Jensen, J. G. van de Winkel, and P. W. Parren. 2001. Effector function activities of a panel of mutants of a broadly neutralizing antibody against human immunodeficiency virus type 1. *J. Virol.* 75: 12161–12168.
41. Reddy, M. P., C. A. Kinney, M. A. Chaikin, A. Payne, J. Fishman-Lobell, P. Tsui, P. R. Dal Monte, M. L. Doyle, M. R. Brigham-Burke, D. Anderson, et al. 2000. Elimination of Fc receptor-dependent effector functions of a modified IgG4 monoclonal antibody to human CD4. *J. Immunol.* 164: 1925–1933.
42. Reidler, J., V. T. Oi, W. Carlsen, T. M. Vuong, I. Pecht, L. A. Herzenberg, and L. Stryer. 1982. Rotational dynamics of monoclonal anti-dansyl immunoglobulins. *J. Mol. Biol.* 158: 739–746.