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Engineered Antibodies with Increased Activity to Recruit Complement

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This manuscript describes two sites in a human IgG1 that, when mutated individually or in combination, result in a dramatic increase in C1q binding and complement-dependent cytotoxicity activity. These two residues, K326 and E333, are located at the extreme ends of the C1q binding epicenter in the C_H2 domain of a human IgG. A mutation to tryptophan at K326 debilitates Ab-dependent cell-mediated cytotoxicity activity. In addition, substitutions of the residues E333 with serine and of K326 with tryptophan in a human IgG2 confer biological activity in the complement-dependent cytotoxicity assay in which the wild-type IgG2 is inactive. This study reveals that the residues K326 and E333 play a significant role in the control of the biological activity of an IgG molecule and can rescue the activity of an inactive IgG isotype. ***The Journal of Immunology*****, 2001, 166: 2571–2575.**

Recruitment of the complement pathway is one mechanism by which Abs carry out their cell-killing function. It is initiated by the binding of C1q, a constituent of the first component of complement, to the C_H2 domain of Igs, IgG and IgM, which are in complex with Ag (1, 2). The C1q binding epicenter in the C_H2 domain of human IgG1 has recently been mapped by site-directed mutagenesis (3). The human IgG complement-binding epicenter centered around residues D270, K322, P329, and P331 (3) was found to differ from that of the mouse, which was previously identified by Duncan and Winter as comprising residues E318, K320, and K322 (4). A second significant mechanism by which Abs promote cell lysis is the Ab-dependent cell-mediated cytotoxicity (ADCC)[2] pathway. ADCC is mediated by interaction of the Fc region of IgGs with Fcγ receptors expressed on a wide variety of cells (5, 6). The Fc receptor-bearing cells are activated upon binding to Ab-Ag complexes, resulting in lysis of the targeted cell (5, 6). The study described here is focused primarily on the C1q-IgG interaction, the initial step in the recruitment of complement-mediated cell killing, using Rituximab as the model Ab.

Rituximab is a therapeutic mAb with human IgG1 constant domains used for the treatment of non-Hodgkins B cell lymphoma (7). This Ab targets the CD20 Ag expressed on the surface of malignant and normal B cells. The precise mechanism of action recruited by Rituximab has not yet been defined; however, the cell-killing effector functions, complement-dependent cytotoxicity (CDC) and ADCC, have been implicated (8). To improve the cytotoxic effect of therapeutic IgGs, it is important to first understand the cell-killing mechanism recruited by Abs. We have used site-directed mutagenesis (3) to find sites that would ablate or improve the CDC activity of Rituximab. In this report we describe two sites, K326 and E333, in human IgG1 that can be substituted to improve the CDC activity of Rituximab. We also show that mutations at K326 may alter ADCC activity. In addition, we were able to rescue the biological activity of an inactive IgG2 isotype by amino acid substitutions at these two sites. Our results show that residues K326 and E333 play an important role in the biological activity of an IgG.

Materials and Methods

Construction of Rituximab mutants

Rituximab and variants were obtained as previously described (3). The chimeric light and heavy chains of Rituximab (IDEC Pharmaceuticals, San Diego, CA) (7) subcloned separately into previously described pRK vectors (9) were used. By site-directed mutagenesis (10), amino acid substitutions in the C_H2 domain of the heavy chain were constructed. The Abs were expressed in the adenovirus-transformed human embryonic kidney 293 cell line (American Type Culture Collection, Manassas, VA) (11) and purified using protein A-Sepharose CL-4B (Pharmacia, Piscataway, NJ). The concentration of the purified Ab was determined using total Ig-binding ELISA. The results reported here were consistent in two separate transfections and preparations of Ab. Also, Rituximab and mutants had the same the binding efficiency to the 96-well plates used in each assay.

C1q binding assay

The binding of human C1q to IgG Ab was assessed by a method previously described (3). High binding Costar 96-well plates (Cambridge, MA) were coated overnight at 4°C with various concentrations of Ab diluted in coating buffer (0.05 M sodium carbonate buffer, pH 9), and 2 μg/ml of human C1q was added. The binding of C1q to the Ab was detected using a 1/1000 dilution of sheep anti-human C1q peroxidase-conjugated Ab (Biodesign, Kennebunk, ME). The plates were developed with *O*-phenylenediamine dihydrochloride (Sigma, St. Louis, MO)] in a PBS/0.012% H_2O_2 solution. The reaction was stopped with 4.5 N H_2SO_4, and the absorbance at 492 nm was measured. To correct for background, the absorbance at 405 nm was subtracted from the absorbance at 492 nm. The mean results from two independent experiments are reported here. The binding efficiency of each mutant to the 96-well plate was examined using an anti-human IgG Fc peroxidase-conjugated Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) as the probe. Rituximab and mutants had the same binding efficiency to the 96-well plates used in each assay.

CDC assay

The CDC assay was performed by a method previously described (3). A CD20-expressing B lymphoblastoid cell line, WIL2-S (American Type Culture Collection), was used as the target cell, and human complement

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[2] Abbreviations used in this paper: ADCC, Ab-dependent cell-mediated cytotoxicity; CDC, complement-dependent cytotoxicity; RFU, relative fluorescence units.

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was used as the probe. The results are expressed in relative fluorescence units (RFU) (2), which are proportional to the number of viable cells. The ability of the mutants to confer complement-dependent lysis was examined by a plot of the percent lysis against the log of the Ab concentration using a four-parameter curve-fitting program (Kaleidagraph, Synergy Software, Reading, PA). The mean results from two independent experiments are reported here.

The percent lysis was calculated as: % complememt-dependent lysis = [(RFU test − background)/(RFU at total cell lysis − RFU background)] × 100.

ADCC assay

The ability of Rituximab and mutants to mediate ADCC was assessed by a method previously described (3). WIL2-S cells (10^4 cells/well) and varying concentrations of Ab samples were added to round-bottom 96-well plates. The mixture was preincubated for 30 min at 37°C. The effector cells, PBMC (2.5×10^5), were then dispensed into the wells. After incubation for 4 h at 37°C, the plates were centrifuged at 250 × *g* for 10 min, and the supernatants were harvested. The supernatants were tested for lactate dehydrogenase activity using a cytotoxic detection (lactate dehydrogenase) kit (Roche, Indianapolis, IN) and the manufacturer's protocol. The average absorbance of triplicate determinations was used to calculate the percentage of cytotoxicity.

The percentage of cytotoxicity was calculated as: % cytotoxicity = (experimental − effector − target spontaneous)/(target maximum − target spontaneous) × 100.

CD20 binding potency of the Rituximab mutants

The binding of Rituximab and mutants to the CD20 Ag was assessed by a method previously described (7, 12). This assay is a cell-based FACS assay in which WIL2-S cells are used as the CD20-expressing cell line. A goat anti-human IgG- FITC Ab (American Qualex, San Clemente, CA) was used to detect binding of Rituximab and mutants to the CD20 receptor.

Results and Discussion

As shown in Fig. 1, K326 and E333 are located in the C_H2 domain at the edges of the C1q binding epicenter of a human IgG1. Alanine substitutions at these two sites in the C_H2 domain of Rituximab resulted in mutants with about a 50% increase in binding to C1q and a similar increase in CDC activity compared with the wild-type Ab, Rituximab (Fig. 2). Based on this initial result it was apparent that K326 and E333 play an important role in the interaction of the Ab with C1q and are potential sites for improving the efficacy of Rituximab or any human IgG1 Ab by way of the CDC pathway.

To obtain an IgG1 molecule with greater increase in CDC activity, other single-point mutations at K326 and E333 of Rituximab were constructed and assessed for their ability to bind C1q and activate complement. All the mutants constructed bound normally to the CD20 Ag (data not shown). With respect to position 326, lysine was substituted with arginine, asparagine, aspartic acid, glycine, glutamine, glutamic acid, histidine, methionine, phenylalanine, tryptophan, tyrosine, and valine. Each of these substitutions increased C1q binding, and some increased CDC activity (Fig. 2). There appeared to be no consistent correlation between the side chain characteristics at position 326 and the C1q binding affinity. Substitutions with both charged and uncharged residues resulted in mutants with increased binding to C1q. The K326W substitution gave the highest increase in C1q binding (3-fold) and CDC activity (2-fold; Fig. 2). Since tryptophan has a large, aromatic, and weakly polar side chain (13), there is a possibility that a combination of side chain properties at this site (size, rigidity, and polarity) influences the C1q binding affinity, or the observed effect may be due to conformational alterations of structure. Two of the K326 mutants, K326Y and K326G, had higher affinity for C1q, but slightly lower CDC activity compared with the wild-type Ab (Fig. 2). These mutations most likely affect downstream events in the CDC pathway, as observed by Sensel et al. for other Ab variants (14),

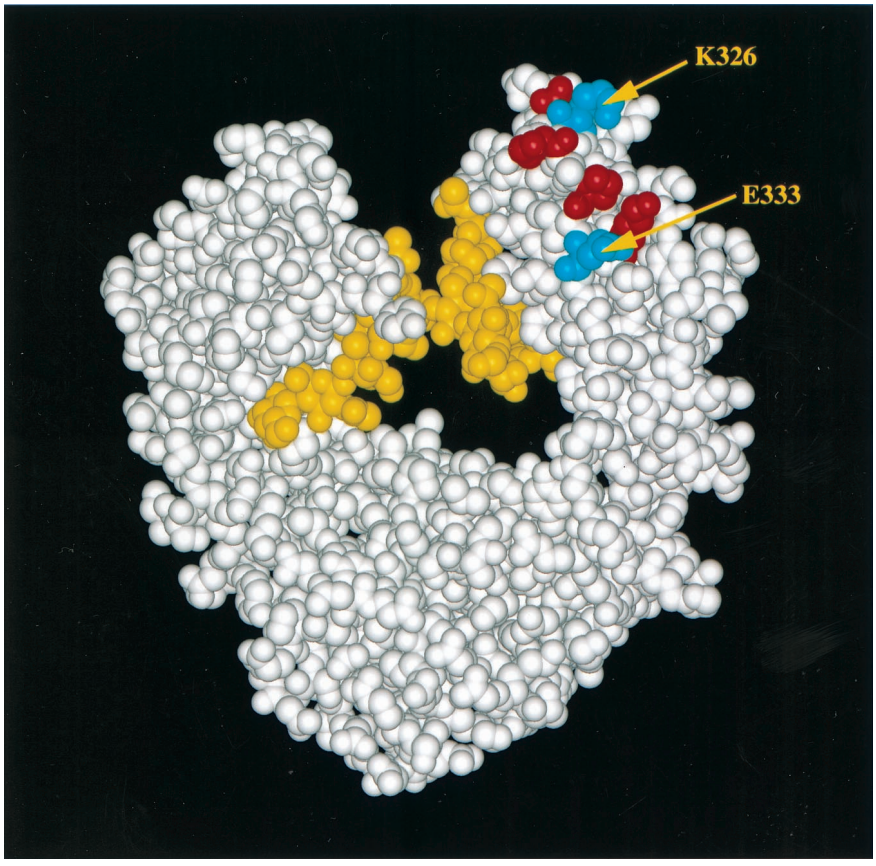

FIGURE 1. The three-dimensional structure of the Rituximab Fc (M. Ultsch et al., manuscript in preparation). The Eu nomenclature is used (20). The six residues in the C_H2 domain that constitute the C1q binding epicenter of human IgG1 are highlighted. In red are the residues D270, K322, P329, and P331, which typically result in decreased binding to C1q when mutated (3). The residues K326 and E333 are indicated in blue. The oligosaccharides are indicated in yellow.

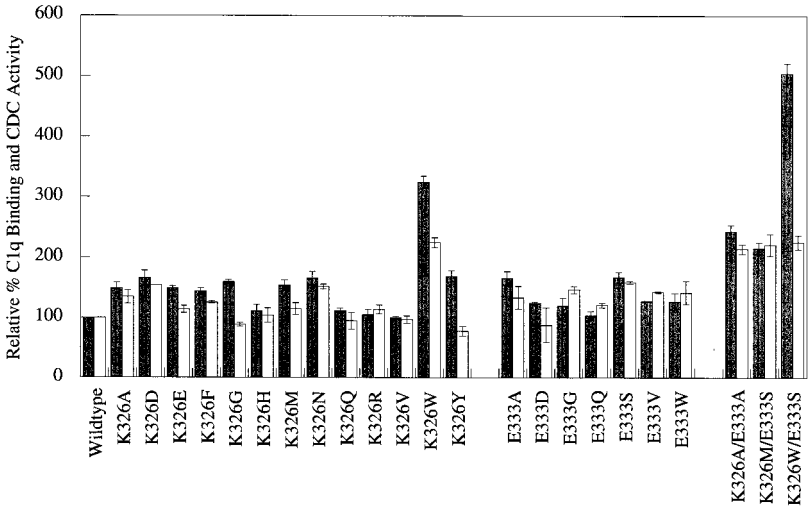

FIGURE 2. The K326 and E333 Rituximab mutants were assessed for the ability to bind C1q and promote complement-dependent lysis using a 1/12 dilution of human complement as a probe. For comparison purposes, the data presented for the mutants are relative to the wild-type Ab and are derived by comparing the EC_{50} values (effective Ab concentration at 50% of the maximum response) of the mutants to the EC_{50} value of wild-type Rituximab, which is taken as 100%. Shown are the relative percentages for C1q binding (■) and CDC activity (□) of the K326 and E333 mutants. The bars represent the mean ± SD of two independent experiments.

although at concentrations of complement approaching physiological levels, the CDC activity of these slightly deficient mutants may be comparable to that of wild-type Ab (3). In any case, by a single amino acid substitution at K326, a mutant Ab molecule, K326W, with a significant increase in C1q binding affinity and CDC activity was constructed.

At position 333, glutamic acid was substituted with aspartic acid, glycine, glutamine, serine, valine, and tryptophan. All these substitutions increased C1q binding, and most increased CDC activity (Fig. 2). Also, at this site there was no correlation between the amino acid side chain characteristics and the C1q binding affinity. The E333S substitution resulted in the highest increase in C1q binding affinity (2-fold) and CDC activity (1.6-fold; Fig. 2). Serine is a small and polar residue, but it is difficult to discern from the results whether these characteristics at position 333 are important for increasing the affinity of the Ab for C1q. Considering the data for both the K326 and E333 mutants, we can only conclude that the wild-type residues are detrimental to the C1q binding affinity and the CDC activity.

To maximize the increase in the C1q binding affinity of the Ab, mutations at positions E333 and K326 in Rituximab were combined to form double mutants. These double mutants, K326A/E333A, K326W/E333S, and K326 M/E333S, were then assessed for the ability to bind C1q and mediate CDC. As shown in Fig. 2, an additive effect on C1q binding was observed by combining mutations at the two sites. For example, the K326W/E333S double mutant showed about a 5-fold increase in binding to C1q, which is about equal to the sum of the individual mutants, increases of 1.7- and 3.3-fold for E333S and K326W, respectively (Fig. 2). An additive effect observed by combining mutations at two or more sites is indicative of sites that are functionally independent (15). Thus, the additivity of the double mutations, K326A/E333A, K326W/E333S, and K326 M/E333S, suggests that K326 and E333 interact with C1q independently. Two of the double mutants, K326A/E333A and K326 M/E333S, also showed an increase in CDC activity compared with the individual mutants. However, there appeared to be no difference in CDC activity between the K326W single mutant and the K326W/E333S double mutant (Fig. 2). Both the K326W and K326W/E333S mutants showed a 2.3-fold increase in CDC activity compared with Rituximab, and this was the maximum increase in CDC activity achieved under the experimental conditions. Thus, no further increase in CDC activity was observed by combining the K326W and E333S mutations. The apparent breakdown in additivity may be attributed to several factors, such as the limitations of the CDC assay or the effect of the mutation on subsequent steps in the complement cascade. It is also conceivable that there are rate-limiting steps in the complement cascade that are not overcome by increasing the affinity of the Ab for C1q. Noteworthy, increasing the complement concentration in the assay did not change the relative percent CDC activity of these higher affinity mutants (data not shown).

To determine whether the increase in CDC activity had any effect on ADCC activity, the double mutants were assessed for their ability to mediate ADCC. Both the K326M/E333S and the K326A/E333A mutants were able to recruit ADCC to essentially the same extent as Rituximab (Fig. 3). However, the K326W/E333S mutant appeared to have very little ADCC activity. In fact, the activity of this mutant was comparable to that of the Rituximab IgG2 isotype (Rituximab-IgG2; Fig. 3), a poor mediator of cell killing (3). The deficiency was caused by the K326W mutation (Fig. 3), suggesting that K326 is also important for ADCC via interaction with Fcγ receptors. Thus, position 326 may be used individually to ablate or in combination to increase the binding of human IgG to Fcγ receptors. In addition, since both the K326W

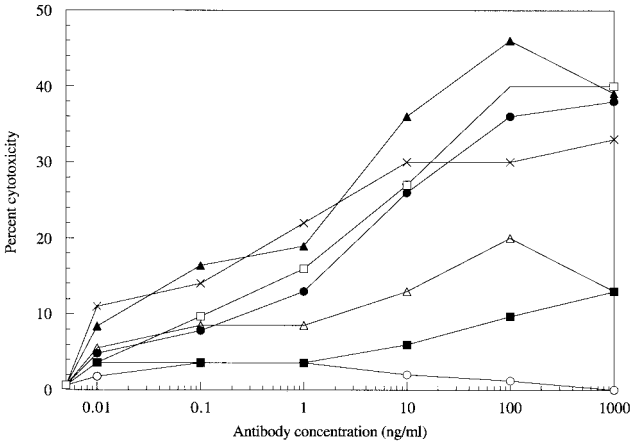

FIGURE 3. Rituximab and mutants were assessed for the ability to recruit ADCC. Shown are the ADCC activities of wild-type Ab (●) and the mutants, K326W (△), E333S (□), K326A/E333A (▲), K326 M/E333S (X), and K326W/E333S (■), using an E:T cell ratio of 25:1. An IgG2 (○) construct of Rituximab was used as a negative control. Shown is a plot of the percent cytotoxicity vs the log of Ab concentration in nanograms per milliliter.

and K326W/E333S mutants are deficient in ADCC, they may serve as diagnostic tools to select specific effector function pathways.

Since glycosylation has been shown to be important for Ab effector functions (16), all the mutants were examined for carbohydrate characteristics. Carbohydrate analyses by matrix-assisted laser desorption/ionization time-of-flight mass spectrometric analysis (17) revealed that the glycosylation patterns of the mutants and the wild-type were comparable (data not shown), with slight differences in the proportions of the various glycoforms (18). No correlation between bioactivity and glycosylation was observed, indicating that the change in activity of the mutants compared with that of the wild-type Ab was not due to modifications in oligosaccharide structure. It is also important to note that K326 and E333 do not interact with oligosaccharide residues (19) (M. Ultsch et al., manuscript in preparation).

Both K326 and E333 are conserved in the human IgG isotypes IgG2 and IgG4 (20), which bind poorly to C1q and are severely deficient in complement activation (reviewed in Ref. 21). To determine whether the IgG2 isotype can be engineered to recruit complement-mediated cell killing, the Rituximab-IgG2 was constructed with substitutions at K326 and E333. Shown in Fig. 4 are the data for C1q binding and CDC activity of the single and double Rituximab-IgG2 mutants. With respect to the C1q binding data, the K326W/E333S double mutation in IgG2 resulted in a molecule with higher affinity for C1q than Rituximab (Fig. 4*A*). Since complement at a high concentration is able to rescue the activity of low affinity C1q binding mutants (3), the CDC assay for the IgG2 mutants was performed using a concentration of complement approaching physiological levels (1/3 dilution of human complement) to maximize the cytotoxic effect. Consistent with the C1q binding data, the K326W/E333S mutation in IgG2 also increased CDC activity significantly (Fig. 4*B*). At a concentration of 50 μg/ml, the IgG2 K326W/E333S mutant was able to confer 100% lysis, whereas <20% lysis was observed for the IgG2 isotype at the same concentration (Fig. 4*B*). Thus, by amino acid substitutions at positions K326 and E333, the ability of an Ab to recruit complement may be augmented severalfold. There was one discrepancy in the results of the IgG2 mutants. The IgG2 K326W/E333S mutant showed approximately 20% higher C1q binding affinity (Fig. 4*A*), but 25-fold lower CDC activity, than Rituximab even when probed with a concentration of complement approaching physiological levels (Fig. 4*B*). The reason for this discrepancy is not known. Perhaps overcoming the C1q binding deficiency by constructing the K326W/E333S mutations in IgG2 was not sufficient to completely restore CDC activity due to deficiencies in other events of the complement cascade that either persisted or were created by these mutations. The results also indicate that IgG1 and IgG2 have different amino acid requirements for C1q interaction. These two isotypes show different effects on C1q binding and CDC activity when point mutations at K326 and E333 are constructed. For example, a mutation at position 326 in human IgG1 has a greater effect on C1q binding than a mutation at position 333 (Fig. 2). In human IgG2, the reverse effect was observed. In fact, a single mutation at position 333 in IgG2 was sufficient to rescue the C1q binding capability of the Ab, and a mutation at position 326 had little effect on C1q binding (Fig. 4, *A* and *B*). The precise role of K326 and E333 in the interaction with C1q is not clear, but the results indicate that there are isotypic differences in the interaction of C1q with human IgG and/or isotype-dependent changes in structure upon mutation. Nevertheless, mutations at positions 333 and 326 in human IgGs enhance binding to C1q and rescue the C1q binding capability of an inactive isotype.

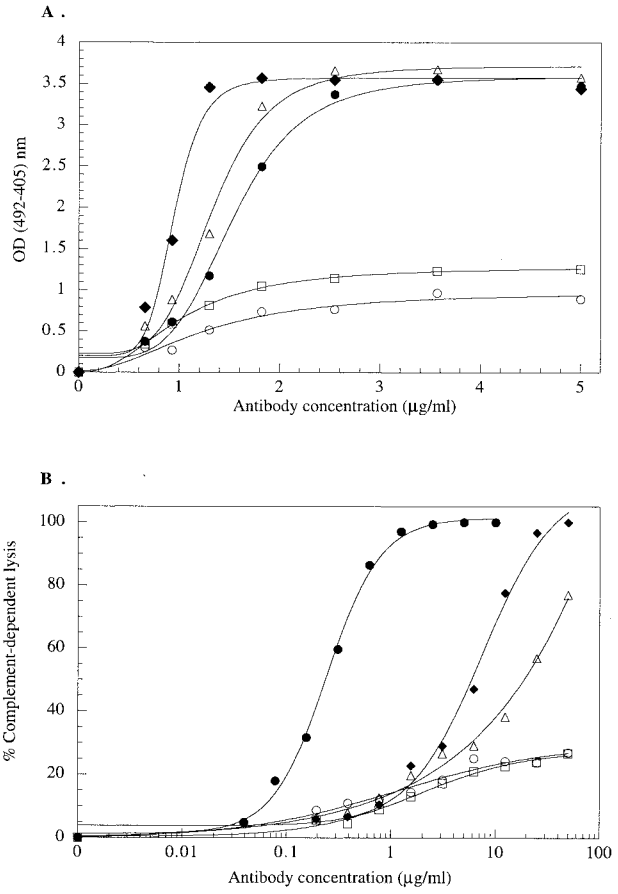

FIGURE 4. Rituximab and variants were assessed for their ability to bind C1q and confer complement-dependent lysis. Shown are the results for C1q binding and CDC activity of wild-type Ab (●) and variants, Rituximab-IgG2 (○), IgG2 E333S (△), IgG2 K326W (□), and IgG2 K326W/E333S (◆). C1q binding (*A*) and CDC activity (*B*), using a 1/3 dilution of human complement as a probe, are shown.

Mutant IgGs derived by amino acid substitutions at positions 326 and 333 have several therapeutic applications. Ab molecules with higher affinity for C1q and higher CDC activity than wild-type IgG1 have the potential to enhance the clearance of immune complexes in several disease conditions, such as systemic lupus erythematosus (22). They may be used to inhibit complement-mediated immune cytotoxicity in certain clinical situations (e.g., xenogenic hyperacute rejection) (23, 24). In addition, IgGs with increased CDC activity have been implicated in the treatment of some bacterial infections (25). The two mutants, K326W and K326W/E333S, which are exclusively deficient in their ability to mediate ADCC, may be effective in inhibiting complement-dependent immune cytotoxicity without interference from ADCC mechanisms.

Conclusion

We have identified two sites in a human IgG, K326 and E333, which, when substituted, have a significant effect on the biological activity of the Ab molecule. Amino acid substitutions at these two sites in a human IgG increased or rescued C1q binding and CDC activity. An E333S substitution in an inactive IgG2 isotype rescued the C1q binding capability and CDC activity of the molecule. It is also evident that position 326 in a human IgG impacts ADCC activity through binding to Fcγ receptors. As demonstrated here, isotype variants can be designed to recruit specific effector functions (i.e., CDC only) by amino acid substitutions at K326 and E333. The detailed mechanism of binding to C1q is not clear.

However, due to the isotypic differences observed in the interaction of C1q with the IgG mutants, it is possible that positions 326 and 333 play a structural role in the C1q-IgG interaction. Additional studies would be required to confirm this hypothesis and to determine the specific nature of the interaction between K326 and the Fcγ receptors. Nevertheless, variant Ab molecules derived by point mutations at K326 and E333 have tremendous potential in the design of Abs used in therapy and may provide for a more efficacious therapeutic human IgG molecule.

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