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# Mapping of the C1q Binding Site on Rituxan, a Chimeric Antibody with a Human IgG1 Fc

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**Rituxan (Rituximab) is a chimeric mAb with human IgG1 constant domains used in the therapy of non-Hodgkin's B cell lymphomas. This Ab targets B cells by binding to the cell-surface receptor, CD20. In our investigation of the mechanism of B cell depletion mediated by Rituximab, we first constructed mutants of Rituximab defective in complement activation but with all other effector functions intact. Our results demonstrate that the previously described C1q binding motif in murine IgG2b constituting residues E318, K320, and K322 is not applicable to a human IgG1 when challenged with either human, rabbit, or guinea pig complement. Alanine substitution at positions E318 and K320 in Rituximab had little or no effect on C1q binding and complement activation, whereas alanine substitution at positions D270, K322, P329, and P331 significantly reduced the ability of Rituximab to bind C1q and activate complement. We have also observed that concentrations of complement approaching physiological levels are able to rescue >60% of the activity of these mutant Abs with low affinity for C1q. These data localize the C1q binding epicenter on human IgG1 and suggest that there are species-specific differences in the C1q binding site of Igs.** *The Journal of Immunology,* **2000, 164: 4178–4184.**

Rituximab is a genetically engineered chimeric mAb used for the treatment of non-Hodgkin's B cell lymphomas (1). It has murine V regions and human IgG1 C regions. Rituximab functions by binding to the CD20 Ag expressed on the surface of normal and malignant B cells and then lysing the cells. There are two known mechanisms by which Abs recruit host cell killing functions: 1) the complement-dependent cytotoxicity (CDC)[2] pathway and 2) the Ab-dependent cell-mediated cytotoxicity (ADCC) pathway. More recently, studies have indicated that anti-tumor Abs such as Rituximab may also mediate cell killing by altering signal transduction events, thereby inducing apoptosis (2). The focus of this study is to gain a more comprehensive understanding of the CDC pathway.

Complement activation occurs by the binding of C1q to the Fc domain of Igs, IgG or IgM, complexed with Ags (3). C1q is a large structurally complex glycoprotein of ~410 kDa present in human serum at a concentration of ~70 $\mu$g/ml (4). Together with two serine proteases, C1r and C1s, C1q forms the complex C1, the first component of complement. At least two of the N-terminal globular heads of C1q must be bound to the Fc of Igs for C1 activation, hence for initiation of the complement cascade (4).

Various investigations with a focus on the C1q binding region of IgG have led to some understanding of the recruitment of complement by Abs (5). By assessment of solvent-accessible amino acid residues, sequence comparison, chemical modification studies, domain swapping, and site-directed mutagenesis experiments, the $C_H2$ domain has been shown to be required for C1q binding (6–10). More specifically, by focusing on conserved residues capable of polar interactions, Duncan and Winter revealed that N297, E318, K320, and K322 in murine IgG2b are important for C1q binding and proposed E318, K320, and K322 as the core of the C1q binding site (7). The presence of an oligosaccharide at position N297 has been shown to be important for most Ab effector functions (11–14). In their study, Duncan and Winter demonstrated that alanine substitution at E318, K320, and K322 in murine IgG2b resulted in mutants that were nonlytic and at least 30-fold lower in binding to guinea pig C1q than the wild-type Ab. Because these three residues, E318, K320, and K322, are conserved in human IgG and IgGs of several other species, they have been designated as the C1q binding motif (7). However, several studies implicate that the contact sites for C1q in murine IgG2b may be different from that of human IgG1 (9, 10, 13, 15, 16). In one of these studies, a K320A mutation in a human IgG1 Ab was shown to have little or no effect on C1q binding or CDC activity (10). Also, a few residues, L235, D265 and P331, which are important for C1q binding and CDC activity in a human IgG background (9, 10, 13, 15, 16), are of minimal importance for complement activation in a murine IgG2b background (7). Substitution of P331 with serine in human IgG1 resulted in a 60% decrease in binding to C1q and complete loss of complement activity (15). Vice versa, substitution of S331 to proline in IgG4 resulted in a molecule that could bind C1q and activate complement (15, 16). Substitution of D265, an oligosaccharide interaction site, with alanine in human IgG3 was shown to have impaired CDC and ADCC activity (13). Likewise, a leucine to glutamic acid substitution at position 235 in human IgG1 resulted in a mutant with impaired CDC and ADCC activity (10). Together, these data suggest that the contact site for C1q on murine IgG2b is perhaps different from that on human IgG1.

In this study, our aim was to identify mutations in the $C_H2$ domain of Rituximab that ablate binding to C1q without altering

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



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ADCC or binding to the CD20 and FcRn receptors, thereby designing a molecule that is deficient in complement activation exclusively. It is important to retain optimal binding to the neonatal Fc receptor, FcRn, because the ability of an IgG to bind FcRn determines its in vivo half-life (17, 18). By alanine substitutions, we demonstrate that two mutants of human IgG1, D270A and P329A, are deficient in complement activation and C1q binding. We confirm that the amino acid residues, D265 and P331, are important for C1q binding and complement activation in a human IgG1 background. In accordance with Morgan et al. (10), we show alanine substitution at position 320 in Rituximab had no effect on complement activation. Also, substitution of E318 to alanine in Rituximab had no effect on complement-mediated lysis. In addition, some of these mutants with low affinity for C1q recruit ADCC in a manner similar to the wild-type Ab and bind normally to the CD20 Ag and the FcRn receptor. Our results demonstrate that there are species differences in complement activation and reiterate the core C1q binding site in a human IgG1 is different from that of murine IgG2b.

## Materials and Methods

### *Construction of Rituximab mutants*

The chimeric L and H chains of Rituximab (IDEC Pharmaceuticals, San Diego, CA) (1) subcloned separately into previously described pRK vectors (19) were used. By site-directed mutagenesis (20), alanine variants of the $C_H2$ domain of the H chain were constructed. The H and L chain plasmids were cotransfected into the adenovirus-transformed human embryonic kidney 293 cell line (American Type Culture Collection, Manassas, VA) as previously described (21). The media was changed to serum-free 24 h after transfection, and the secreted Ab was harvested after 5 days. The Abs were purified using protein A-Sepharose CL-4B (Pharmacia, Piscataway, NJ), buffer exchanged with PBS and concentrated to 0.5 ml using a Centricon-30 (Amicon, Beverly, MA), and stored at 4°C. The concentration of the 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 (Quidel, San Diego, CA) to Rituximab and mutants was assessed by an ELISA binding assay. High binding Costar 96-well plates (Corning, NY) were coated overnight at 4°C with varying concentrations of Rituximab in coating buffer (0.05 M sodium carbonate buffer, pH 9). The plates were washed after each incubation step with PBS/0.05% Tween 20, pH 7.4, and incubations after coating were performed at room temperature. After coating, the plates were blocked with 200 $\mu$l of ELISA diluent (0.1 M $NaPO_4$/0.1 M NaCl/0.1% gelatin/0.05% Tween 20/0.05% ProClin300) for 1 h, and incubated for 2 h with 100 $\mu$l of 2 $\mu$g/ml human C1q in ELISA diluent. Then, 100 $\mu$l of a 1:1000 dilution of sheep anti-human C1q peroxidase-conjugated Ab (Biodesign, Kennebunkport, ME) in ELISA diluent was added and incubated for 1 h. The plates were developed with 100 $\mu$l of substrate buffer (PBS/0.012% $H_2O_2$) containing *o*-phenylenediamine dihydrochloride (Sigma, St. Louis, MO). The reaction was stopped by the addition of 100 $\mu$l of 4.5 N $H_2SO_4$, and the OD was measured at 492 nm using a microplate reader Spectra MAX 250 (Molecular Devices, Sunnyvale, CA). To correct for background, the OD at 405 nm was subtracted from the OD at 492 nm. The binding efficiency of each mutant to the plate was examined using an anti-human IgG Fc peroxidase-conjugated Ab as the probe (Jackson ImmunoResearch, West Grove, PA).

### *CDC assay*

The ability of Rituximab and mutants to promote cell killing of a CD20-expressing B lymphoblastoid cell line, WIL2-S (American Type Culture Collection), was assessed by a method previously described (22). Serum complement from human (Quidel, San Diego, CA), rabbit (ICN, Costa Mesa, CA), or guinea pig (Life Technologies, Grand Island, NY) was used. Rituximab (0.08–100 $\mu$g/ml) was diluted with RHB buffer (RPMI 1640 (Life Technologies), 20 mM HEPES, pH 7.2, 2 mM glutamine, 0.1% BSA, 100 $\mu$g/ml gentamicin). WIL2-S cells were washed in RHB buffer and resuspended at a density of $10^6$ cells/ml. In a typical assay, 50 $\mu$l of Rituximab, 50 $\mu$l of diluted complement, and 50 $\mu$l of a cell suspension (50,000 cells/well) were added to a flat-bottom tissue culture 96-well plate. The mixture was incubated for 2 h at 37°C in a 5% $CO_2$ incubator to facilitate complement-mediated cell lysis. Then, 50 $\mu$l of Alamar Blue (Accumed International, Westlake, OH) was added to each well and incubated overnight at 37°C. Fluorescence was read using a 96-well fluorometer with excitation at 530 nm and emission at 590 nm. As previously described, the results are expressed in relative fluoresence units (RFU) that are proportional to the number of viable cells. The activity of the various mutants was examined by plotting the percent CDC activity against the log of Ab concentration (final concentration before the addition of Alamar Blue) using a four-parameter curve fitting program (Kaleidagraph).

The percent CDC activity was calculated as follows: % CDC activity = (RFU test − RFU background) × 100 (RFU at total cell lysis − RFU background).

### *ADCC assay*

Blood from normal volunteers was drawn into heparinized syringes, mixed with an equal volume of HBSS without $Ca^{2+}/Mg^{2+}$ (Life Technologies), layered onto a Lymphoprep gradient (Life Technologies), and centrifuged at 800 × *g* for 20 min. PBMC at the interface were harvested and washed in HEPES-buffered saline and resuspended in assay medium (RPMI 1640 media (Life Technologies) containing 1% heat-inactivated FBS (HyClone Laboratories, Logan, UT), 2 nM L-glutamine, 10 mM HEPES, and 50 $\mu$g/ml gentamicin). WIL2-S cells ($10^4$ cells/well) in 50 $\mu$l of assay buffer, and varying concentrations of Ab samples in 50 $\mu$l of assay buffer were added into round-bottomed 96-well plates. The mixture was preincubated for 30 min at 37°C. Then, 50 $\mu$l of the effector cells ($2.5 \times 10^5$) were dispensed into the wells and incubation was continued for 4 h at 37°C. An E:T ratio of 25:1 was used. The plates were centrifuged at 250 × *g* for 10 min, and the supernatants were harvested. The activity of lactate dehydrogenase in the supernatants were determined by using a Cytotoxic Detection kit (Boehringer Mannheim, Indianapolis, IN) and the manufacturer's protocol. The average absorbance of triplicates was used to calculate the percentage of cytotoxicity.

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

### *CD20 binding potency of the Rituximab mutants*

The binding of Rituximab and mutants to the CD20 Ag was assessed by a method previously described (1, 22). This assay is a cell-based FACS assay in which WIL2-S cells are used as the CD20-expressing cell line. The binding of Rituximab and mutants to WIL2-S cells was detected by a goat anti-human IgG-FITC Ab (American Qualex, San Clemente, CA). The results expressed as RFU are proportional to the amount of FITC-labeled dectection Ab bound to the cell.

### *FcRn binding ELISA*

The $\alpha$ and $\beta_2$-microglobulin clones of the human FcRn were obtained (Research Genetics, I.M.A.G.E. Consortium, Huntsville, AL). Each polypeptide was subcloned separately into previously described pRK vectors (19) and cotransfected into human embryonic kidney 293 cells. The human FcRn was expressed as a $His_6$-tagged extracellular domain, purified by Ni-NTA column (Qiagen, Valencia, CA) chromatography, and buffer exchanged into PBS. The concentration was measured by absorbance at 280 nm using an extinction coefficient (0.1%, 1 cm, 280 nm) of 1.9. Maxisorb Nunc 96-well immunoplates (Nalge Nunc International, Naperville, IL) were coated with 100 $\mu$l of 2 $\mu$g/ml of streptavidin (Zymed Laboratories, South San Francisco, CA) in coating buffer (0.05 M carbonate/bicarbonate, pH 9.6) at 4°C overnight. The plates were washed after each incubation step with PBS/0.05% Tween 20, pH 7.2, and incubations were performed at room temperature. After coating, the plates were blocked with PBS/0.5% BSA, pH 7.2, for 1 h, incubated with 100 $\mu$l of 1–2 $\mu$g/ml biotinylated FcRn for 1 h, and then 100 $\mu$l containing various concentrations of Rituximab in dilution buffer (PBS/0.5%BSA/0.05% Tween 20, pH 6.0) was added to each well. After 2 h incubation, 100 $\mu$l of goat $F(ab')_2$ anti-human IgG $F(ab')_2$ HRP conjugate (Jackson ImmunoResearch) in dilution buffer was added and incubated for 1 h. The plates were developed using a 3,3′,5,5′-tetramethylbenzidine substrate detection method (Kirkegaard & Perry Laboratories, Gaithersburg, MD), and OD values were measured at 450 nm using a $V_{max}$ microplate reader (Molecular Devices, Sunnyvale, CA). To correct for background, the OD at 650 nm was substracted from the OD at 450 nm.

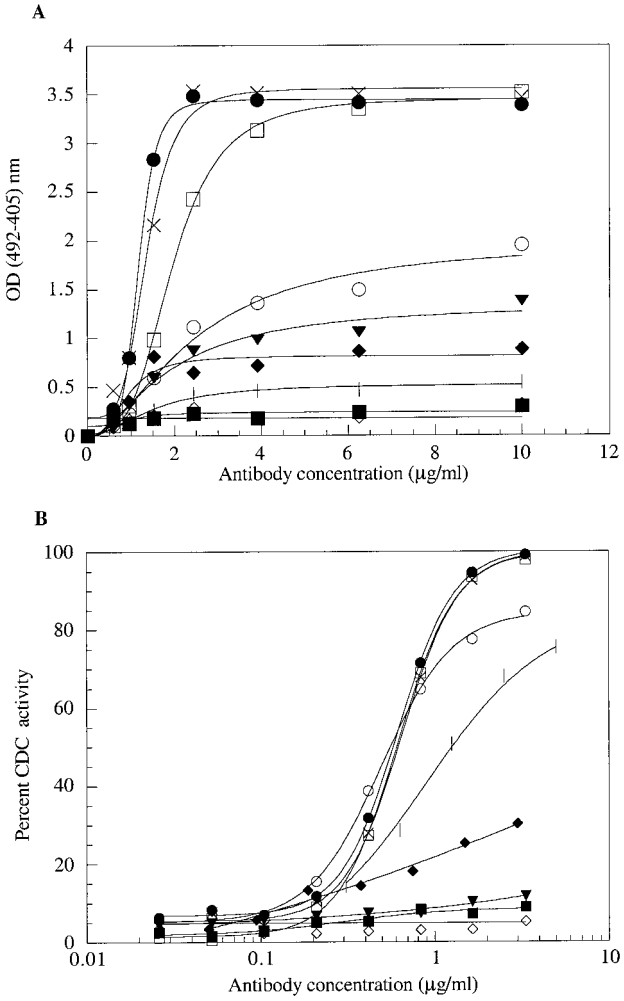


**FIGURE 1.** C1q binding and CDC activity of wild type (×) and the mutants, D265A (|), D270A (♦), E318A (□), K320A (●), K322A (■), P329A (◇), and P331A (○). An IgG2 (▼) construct of Rituximab was used as a negative control. C1q binding (*A*) and CDC activity (*B*) using a 1/12 dilution of human complement as a probe.

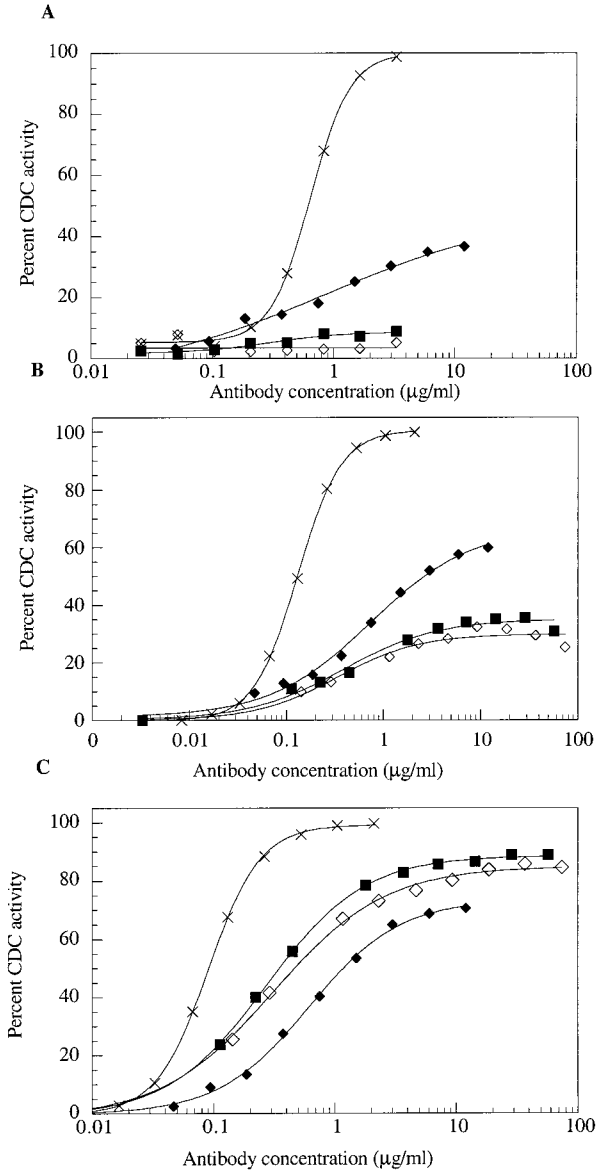


**FIGURE 2.** Dependence of CDC activity on complement concentration. The CDC activity of wild type (×) and the mutants, D270A (♦), K322A (■), and P329A (◇), using (*A*) 1/12 dilution of human complement as the probe, (*B*) a 1/6 dilution of human complement as the probe, and (*C*) a 1/3 dilution of human complement as the probe.

### *Determination of C3 and C4 levels in complement*

C3 and C4 levels in complement from the three different species used in this study, human, rabbit, and guinea pig, were measured by radial immunodiffusion (National Jewish Center Complement Laboratory, Denver, CO.).

## Results

Our aim was to obtain an Ab molecule that is exclusively incapable of binding C1q and therefore ineffective in mediating complement lysis, while retaining Fcγ receptors and FcRn binding. The alanine mutants, E318A, K320A, and K322A, having been found to ablate binding to murine IgG2b (7), were initially constructed and assessed for their ability to bind C1q and activate complement. When compared with Rituximab, there appeared to be little difference in the binding of E318A and K320A to C1q (Fig. 1*A*). The IgG2 negative control appears to have a much lower affinity for C1q than the E318A and K320A mutants. Also, the ability of E318A and K320A to activate complement was essentially identical with the wild-type Ab (Fig. 1*B*). These results demonstrate that the effect of the E318A and the K320A substitution in a human Fc on complement activation and C1q binding was minimal. Conversely, we found that the K322A substitution had a significant effect on both complement activity and C1q binding. The K322A mutant displayed no CDC activity when a 1/12 dilution of human complement was used and was significantly lower in binding to C1q when compared with wild-type (Fig. 1, *A* and *B*). Under these conditions, the IgG2 construct did not promote cell killing (Fig. 1*B*).

As demonstrated above, K322 was the only residue of the proposed C1q binding sites (7) that appeared to be critical for C1q binding and complement activation in our system. Because our aim was to completely ablate C1q binding and CDC activity, we further probed the $C_H2$ domain of Rituximab for additional C1q binding sites. As assessed from the three-dimensional structure of the Rituximab Fc (M. Ultsch et al., manuscript in preparation), several point mutations in the vicinity of K322 were constructed. These mutants, D270A, E272A, K274A, N276A, Y278A, E283A, S324A, P329A, P331A, K334A, and T335A, were assessed for their ability to bind C1q and to activate complement. With the

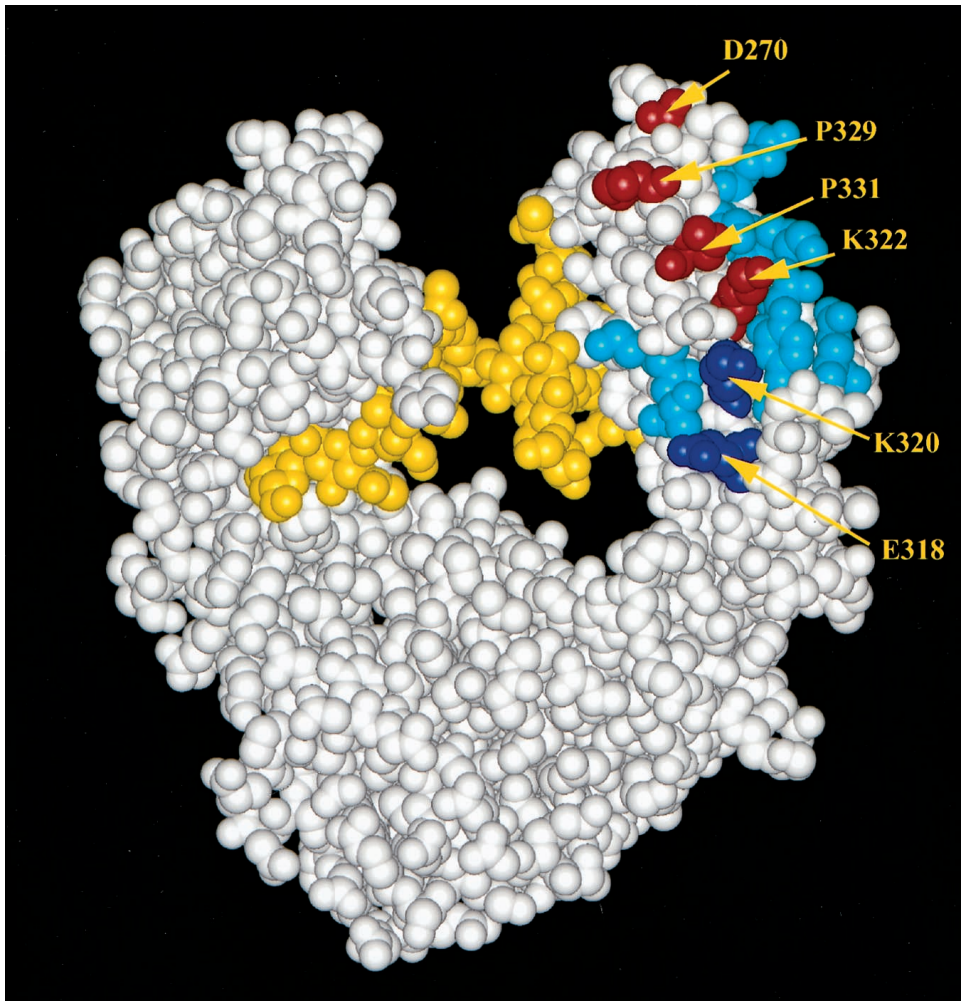


**FIGURE 3.** The three-dimensional structure of the Rituximab Fc (M. Ultsch et al., manuscript in preparation). The EU nomenclature is used. Highlighted in red are the four residues (D270, K322, P329, and P331) that constitute the C1q binding epicenter. The residues E318 and K320 are indicated. The oligosaccharides are indicated in yellow, and the blue region corresponds to other residues on the surface of the $C_H2$ domain that had no significant effect on C1q binding or complement activation.

exception of D270A, P329A, and P331A, none of these substitutions significantly decreased C1q binding or complement activation (data not shown). The D270A and P329A mutants showed no binding to C1q (Fig. 1*A*) in our standard assay and no CDC activity (Fig. 1*B*) when a 1/12 dilution of human complement was used. Although considerably impaired in its ability to bind C1q (Fig. 1*A*), only a slight decrease in CDC activity (Fig. 1*B*) was observed for the P331A mutant when a 1/12 dilution of human complement was used. An additional mutant, D265A, was also constructed to determine whether D265 was important for complement activation in a human IgG1 background. As shown in Fig. 1, the D265A substitution in Rituximab severely impaired C1q binding and decreased CDC activity.

We observed that the activity of these low-affinity mutants, D270A, K322A, and P329A, could be rescued by increasing the complement concentration from a 1/12 to a 1/3 dilution, which approaches physiological levels of complement (Fig. 2, *A–C*). At a 1/6 dilution of human complement, some activity was observed for D270A, K332A, and P329A, and at a 1/3 dilution of human complement, although decreased in bioactivity when compared with Rituximab, these mutants were able to confer >60% lysis (Fig. 2, *B* and *C*). To ensure that the activity of the low-affinity C1q binding mutants at high concentrations of complement was not due to activation of the alternative pathway, the CDC assays were also performed in the presence of $Mg^{2+}$ and EGTA. The addition of $Mg^{2+}$-EGTA at either a 1/12, 1/6, or 1/3 dilution of human complement reduced the CDC activity of Rituximab and mutants to background (data not shown). In the presence of $Mg^{2+}$-EGTA, the CDC activity observed for the mutants as well as wild type ranged from 2 to 6% when probed with a 1/3 dilution of human complement. This experiment was performed at a fixed concentration of Ab (0.6 $\mu$g/ml). Under the same experimental conditions but without $Mg^{2+}$-EGTA, the wild type showed about 65% activity and the activity of the mutants was between 30 and 40%. In addition, in the presence of $Mg^{2+}$-EGTA the CDC activity of the mutants as well as the wild type did not change with an increase in complement concentration from a 1/12 to a 1/3 dilution. This indicates the observed CDC activity of the low-affinity binding mutants at high concentration of complement is not due to activation of the alternative pathway. Nonetheless, our results demonstrate that the human C1q binding epicenter of human IgG1 is centered around these four spatially close residues, D270, K322, P329, and P331 (Fig. 3). For the most part, single point mutations at these sites, D270, K322, P329, and P331, do not alter any other Ab effector functions. All four mutants, D270A, K322A, P329A, and P331A, bound normally to the CD20 and FcRn receptors (data not shown). However, unlike the D270A, K322A, and P331A mutants, which were able to recruit ADCC to an appreciable extent, the P329A mutant was markedly impaired in its ability to recruit ADCC (Fig. 4).

To understand the nature of the interaction between the core C1q binding sites (D270, K322, P329, and P331) and C1q, we constructed several point mutations at these sites. At position 322, lysine was substituted with alanine, leucine, methionine, asparagine, glutamic acid, and arginine. With the exception of K322R, the ability of these mutants to bind C1q was greatly reduced (Fig. 5*A*). The binding of the K322R mutant to C1q was comparable to

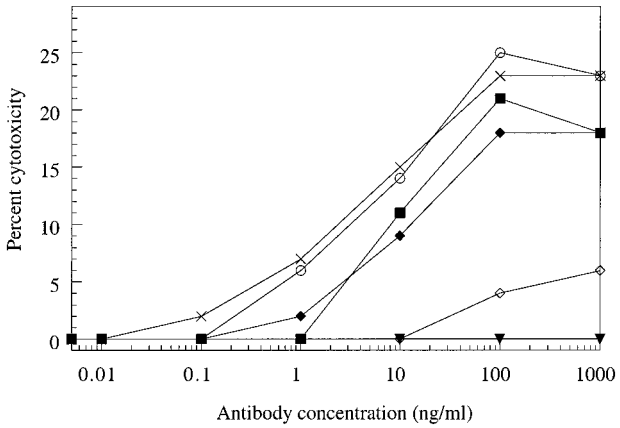


**FIGURE 4.** ADCC activity of wild type (×) and the mutants, D270A (♦), K322A (■), P329A (◇), and P331A (○), using an E:T ratio of 25:1. An IgG2 (▼) construct of Rituximab was used as a negative control. Shown is a plot of percent cytotoxicity vs the log of Ab concentration in ng/ml.

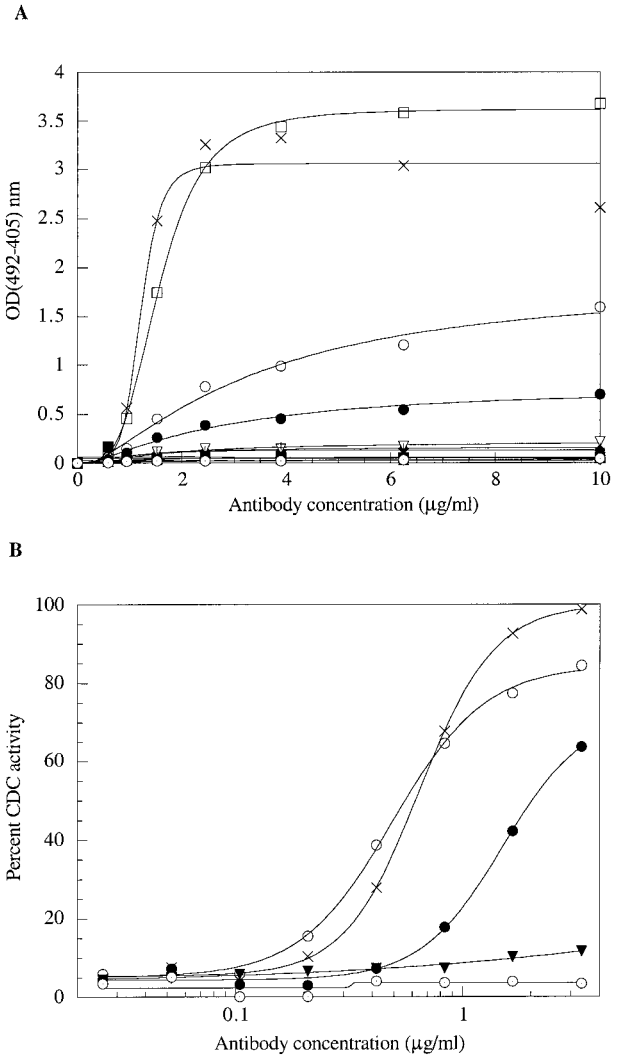


**FIGURE 5.** *A*, C1q binding of wild type (×) and the mutants, K322A (■), K322 M (♦), K322L (▽), K322R (□), K322E (△), K322N (◇), P331A (○), P331G (⊙), P331S (●), D270A (+), D270K (–), and D270V (▲). *B*, CDC activity of wild type (×) and the mutants, P331A (○), P331G (⊙), and P331S (●). An IgG2 (▼) construct of Rituximab was used as a negative control.

that of the wild-type Ab, indicating that the K322R mutation did not alter C1q binding. The lack of binding of the K322M mutant suggests that a positive charge is required at this position. Substitution of D270 with alanine, valine, or lysine resulted in mutants that were severely deficient in C1q binding (Fig. 5*A*). At P329 and P331, any amino acid substitution resulted in mutants that were deficient in C1q binding and CDC activity (data not shown). Due to the unique restriction on backbone conformation imposed by proline, it is possible that point mutations at these sites, 329 and 331, cause local perturbations in structure. Among the P331 mutants constructed were P331S and P331G. Although the binding of the P331S mutant was substantially decreased when compared with the wild-type Ab and the P331A mutant, the P331S mutant was able to bind C1q and promote cell killing in our system (Fig. 5, *A* and *B*). In contrast, the P331G mutant showed no binding to C1q and no CDC activity when a 1/12 dilution of human complement was used (Fig. 5, *A* and *B*).

To determine whether the source of complement was responsible for our results differing from those of Duncan and Winter (7), we challenged our mutants with complement from different species. Fig. 6 shows the E318A and K320A mutants were as efficient as Rituximab in complement-mediated lysis when probed with either human (Fig. 6*A*), rabbit (Fig. 6*B*), or guinea pig (Fig. 6*C*) complement. Likewise, D270A, K322A, P329A, and P331A showed a deficiency in CDC when tested with the different sources of complement (Fig. 6, *A–C*). However, there were a few differences in CDC activity with the different sources of complement. For example, Rituximab and the mutants appeared to be less effective in promoting cell lysis when probed with guinea pig complement. At the same dilution (1/9) of human and rabbit complement, at least 100-fold higher concentration of Rituximab was required for 50% cell lysis when guinea pig complement was used as the probe (Fig. 6, *A–C*). The mutant D270A appeared to be much more active when challenged with rabbit (Fig. 6*B*) than with human complement (Fig. 6*A*) and showed no activity with guinea pig complement (Fig. 6*C*). At a concentration of 1 μg/ml of D270A, 80% cell lysis was observed with rabbit complement, and 20% cell lysis was observed with human complement. In contrast, the CDC activity of the wild-type Ab (~100% cell lysis at 1 μg/ml of Ab) was essentially the same with either rabbit or human complement. Also, the P331A mutant showed only a slight deficiency in the ability to recruit complement-mediated cell lysis when probed with a 1/9 dilution of rabbit and human complement, but appeared to be completely inactive when probed with the same dilution of guinea pig complement. To determine whether the disparity between the three complement sources was to some extent a reflection of concentration differences, the levels of complement components, C3 and C4, in the complement from the three different species was determined. Table I shows the levels of C3 and C4 in the human, rabbit, and guinea pig sera were comparable and within the reference range for human sera (P. C. Giclas, unpublished observations). Also, C1q is present in similar concentrations (~70 μg/ml) in human (4) and guinea pig (23) sera. These data indicate the concentration of complement components in sera of human, rabbit, and guinea pig complement is similar and thus the discrepancy in CDC activity between the three sources of complement is probably not due to concentration differences.

## Discussion

With Rituximab as the model Ab, we have demonstrated that the C1q binding site in human IgG1 is centered about D270, K322, P329, and P331. Two of the previously proposed residues com-

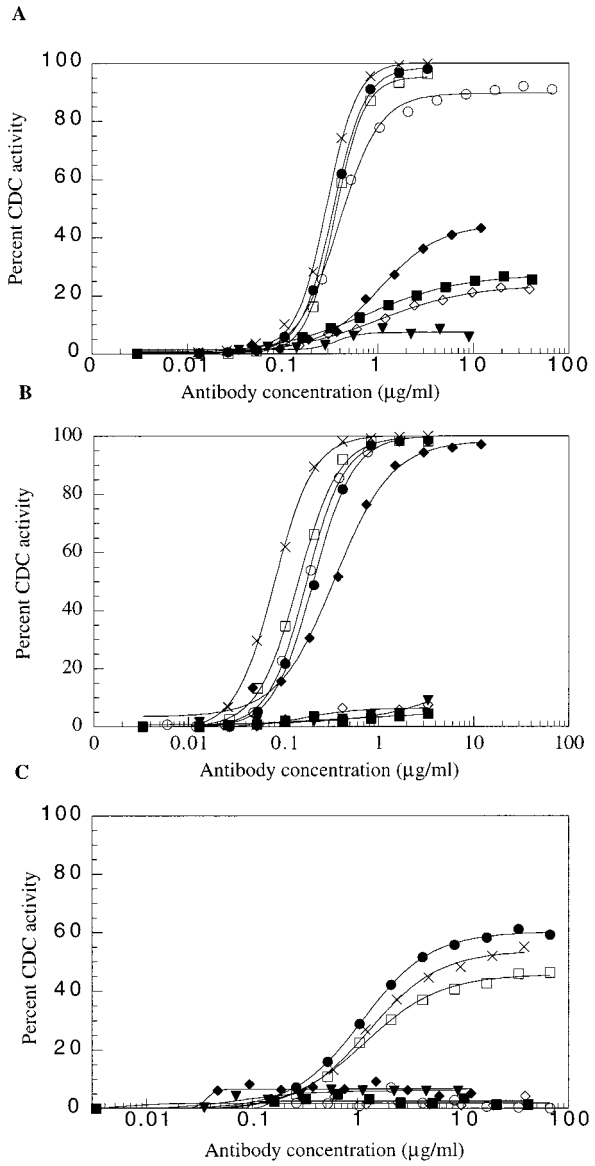


**FIGURE 6.** CDC activity of wild type (×) and the mutants, D270A (♦), E318A (□), K320A (●), K322A (■), P329A (◇), and P331A (○), using a 1/9 dilution of complement from different species. An IgG2 (▼) construct of Rituximab was used as a negative control. Human complement (*A*), rabbit complement (*B*), and guinea pig complement (*C*) were used as probes.

prising the C1q binding site, E318 and K320 (7), are of minimal importance for C1q binding and complement-mediated lysis in a human IgG1 system, as shown by only a slight decrease in C1q binding observed for the E318A and K320A mutants (Fig. 1*A*). This is consistent with the previous finding of Morgan et al. that a K320A mutation in human IgG1 had no effect on complement lysis (10). Also, P331 in human IgG1 has been shown by other investigators to be important for C1q binding (15, 16). We attribute the incongruity between our results and those obtained by Duncan and Winter to the Ab background used. The results reported here, on the C1q binding sites of human IgG1, were consistent with three different sources of complement (Fig. 6, *A–C*). With either guinea pig, rabbit, or human complement as the probe, the D270A, K322A, P329A, and P331A mutants were deficient in CDC activity and the ability of the E318A and K320A mutants to activate complement was comparable to Ritiuximab (Fig. 6). The Duncan

Table I. *Levels of C3 and C4 in complement from three different species*

| Serum | C3 | C4 |
|---|---|---|
| Human | 114 mg/dl | 25.6 mg/dl |
| Rabbit[a] (equivalent to human) | 126.2% of NRS[b] (145 mg/dl) | 103.8% of NRS (26 mg/dl) |
| Guinea pig[a] (equivalent to human) | 83.6% of NGPS[c] (96 mg/dl) | 97.3% of NGPS (24 mg/dl) |
| Reference range for human serum | 80–150 mg/dl | 13–37 mg/dl |

[a] Rabbit and guinea pig results are reported as a percentage of the protein in the serum from a normal animal, because purified components from these animals are not available for standardization of the assay.
[b] NRS, Normal rabbit serum.
[c] NGPS, Normal guinea pig serum.

and Winter study was performed using murine IgG2b with guinea pig complement as the probe (7). Comparing the guinea pig data, the major difference between our study and that of Duncan and Winter is the Ab background used, human IgG1 vs murine IgG2b. Our data and that of other investigators reveal several differences in the C1q binding site between murine IgG2b and human IgG1. For example, E318 and K320 play only a minor role in the C1q binding site on human IgG1 but play a major role in murine IgG2b (Refs. 7 and 10 and this work). The residues, D265, D270, and P331, which are important for CDC in human IgG1, do not effect a change in CDC activity when altered in a murine IgG2b background (Refs. 7, 9, 10, 13, 15, and 16 and this work). In addition, a L235E substitution had no effect on CDC when constructed in murine IgG2b (7), but abolished the ability of human IgG1 to activate complement (10). These findings strongly suggest the C1q binding sites on murine IgG2b are different from human IgG1. Notably, however, extremely dilute guinea pig complement (1/40 dilution) was used to assess the activity of the murine IgG2b mutants (7), and this may have affected the results to some extent.

The core C1q binding site in human IgG1 was, for the most part, independent of the source of complement (Fig. 6). However, there are species differences in complement activation. One important difference was at P331. Tao et al. (15) and Xu et al. (16) demonstrated that a P331S mutation in human IgG1 resulted in a molecule that was unable to promote cell killing; their studies were performed using guinea pig complement as the probe. Consistent with their results, we show in this study that a mutation at P331 (P331A) in human IgG1 resulted in a molecule that was unable to confer complement lysis when guinea pig complement was used as the probe (Fig. 6*C*). However, with either rabbit or human complement, a substantial amount of CDC activity was observed for the P331A mutant (Fig. 6, *A* and *B*). Also, a P331S mutation resulted in a molecule, although impaired, was still able to promote cell killing when challenged with human complement (Fig. 5*B*). The mutant D270A was much more active with rabbit complement than with human or guinea pig complement. In addition, Rituximab appeared to be less effective in conferring lysis when probed with guinea pig complement than with human or rabbit complement. These results demonstrate that there are species-specific differences in complement, although complement concentration may also play a role in some of the observed discrepancies.

As demonstrated here, and by other investigators (24, 25), complement concentration is an important factor in complement-mediated cytotoxicity. By increasing the human complement concentration, we were able to recruit the activity of the D270A, K322A, and P329A mutants, which appeared to be completely inactive at

lower concentrations (Fig. 2, *A–C*). The results at higher concentrations of complement indicate that impaired C1q binding by single point mutations at D270, K322, and P329 will not lead to complete ablation of CDC activity.

In terms of the interaction between C1q and the core C1q binding site in Rituximab, our data indicates that lysine at position 322 may be involved in an electrostatic interaction with C1q (only the K322R mutant retained binding; loss of a positive charge, e.g., methionine, reduced binding). At position D270, a hydrophobic or positively charged residue does not promote C1q binding (Fig. 5). Besides possible hydrophobic interactions with C1q, the two surface-exposed prolines (P329 and P331) may play an important structural role; the fact that the P329A mutant was also severely deficient in ADCC (Fig. 4) would support this hypothesis.

In conclusion, our results demonstrate the concentration and source of complement are important considerations in these types of studies. We have also shown that for human IgG1, four spatially close sites on the surface of the Ab (Fig. 3), D270, K322, P329, and P331, constitute the C1q binding epicenter (Fig. 3). Because most of these sites are conserved in human IgG isotypes that are deficient in C1q binding and complement activation (5), it is evident that there are other factors that influence complement activation. Also, other amino acid residues in human IgG1 have been shown to be important for C1q binding and complement activation (9, 10, 15). Perhaps, the differences in function between these IgG isotypes is due to the conformation of the Ab modulated by a few residues, the hinge (26), and/or carbohydrate (27). With respect to our goal, which is to completely ablate the ability of Rituximab to mediate complement lysis, continued efforts are being made to construct a mutant molecule with no detectable CDC activity.

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