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The Differential Ability of Human IgG1 and IgG4 to Activate Complement Is Determined by the COOH-terminal Sequence of the C_H2 Domain

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Summary

Using domain switch chimeric antibodies, we confirm the important role of C_H2 in complement activation. In addition, we demonstrate that the structures responsible for the differential ability of human IgG1 and IgG4 to activate complement are located at the COOH-terminal part (from residue 292 to 340) of the C_H2 domain. The amino acids in C_H2 that might be involved in complement interaction are discussed. While C_H3 contributes to efficient complement activation, C_H3 from IgG2 and C_H3 IgG3 are equally effective.

The ability to activate the complement cascade and thereby remove pathogenic agents is one of the most important effector functions of the antibody molecule. IgM and IgG are the only isotypes that activate the classical complement pathway. The four human IgG isotypes have very similar amino acid sequences, but differ markedly in their ability to activate complement. IgG3 and IgG1 are effective in activating complement, IgG2 fixes complement poorly, and IgG4 appears completely deficient in the ability to activate complement (1, 2). An unresolved question is what amino acid differences determine this isotype-specific complement activation.

The hinge region shows the most sequence variation among the IgG isotypes (2, 3). The hinge serves to both covalently link the two heavy chains and to endow the antibody molecule with segmental flexibility. We and others have shown that a hinge region is essential for Clq binding and complement activation (3, 4). However, the flexibility of the hinge does not directly correlate with proficiency in complement activation, and replacing the rigid hinge of IgG4 with the flexible hinge of IgG3 did not result in an IgG4 molecule that can activate complement (3). Therefore, while the hinge region may modulate the ability of the IgG molecule to activate complement, it does not appear to determine the isotype-specific differences.

Studies using antibody fragments suggested that the C_H2 domain plays an important role in complement activation. Facb (IgG depleted of C_H3) and C_H2 fragments bind C1 and activate complement, while Fab and C_H3 fragments did not show any activity (5–7). Moreover, aglycosylated IgG lacking carbohydrate in C_H2 is unable to activate complement (8, 9). While these experiments indicate that C_H2 is primarily responsible for C1 binding and complement activation, C_H2

is not the only structure required. IgG that lacks C_H3 is only 50% as efficient in its ability to activate complement as intact IgG (5, 7). Moreover, while Glu 318, Lys 320, and Lys 322 in C_H2 have been shown by *in vitro* mutagenesis to be important for complement activation (10), these residues are conserved among isotypes that do and do not activate complement, and thus could not determine the isotype-specific differences.

In this study, we have used domain exchange molecules to further investigate the residues responsible for the isotype-specific variation in complement activation. We confirm that C_H2 appears to be the domain important for complement activation. Furthermore, we provide evidence that the COOH-terminal part (residues 292–340) of the C_H2 domain contains the residues responsible for the isotype-specific differences in complement activation.

Materials and Methods

Construction of Chimeric IgG Molecules. The expressed V_κ and V_λ genes from the mouse antidansyl (DNS) hybridoma 27–44 were joined to human C_κ in the pSV2ΔHneo expression vector and to human IgG C_H in the pSV2ΔHgpt vector, respectively (11). As shown in Fig. 1, a novel PvuI site was generated between exons in the IgG2 and IgG3 constant region genes such that each gene contains one PvuI site. The C_H1 and C_H3 domain switch constant region genes were produced using clones with the appropriate PvuI sites. A PvuI site located between the hinge and C_H2 exons and a SacII site between the C_H2 and C_H3 exons were used to generate the C_H2 domain switch genes. IgG1 and IgG4 domain switch genes were generated using a SacII site located within the C_H2 exon. The domain switch heavy chain genes were cloned into the pSV2ΔHgpt expression vector and transfected with their specific

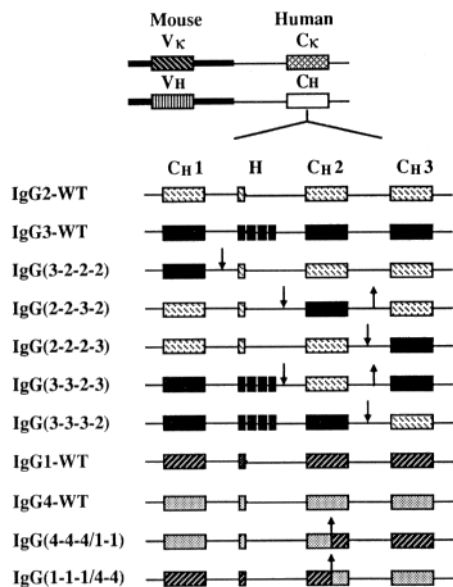


Figure 1. Schematic diagram of the domain switch chimeric antibodies. Up arrows (↑) represent *Sac*II sites and down arrows (↓) represent *Pvu*II sites, which were introduced by using oligonucleotide linkers. All heavy chain constant regions were joined to the murine antidiarrheal variable region. Mutants with exon exchanges are given a four-digit name: the first digit refers to the subclass of the $C_{\kappa 1}$ domains, and the second, third, and fourth digits indicate the subclasses of the hinge, $C_{\kappa 2}$, and $C_{\kappa 3}$ domains, respectively. WT indicates the wild-type gene.

light chain gene into the Ig nonproducing cell line, P3X63 Ag8.653, by either protoplast fusion or electroporation (12). The clones were selected in medium containing the antibiotic G418 and screened for antibody production by ELISA. Chimeric antidiarrheal antibodies were purified by affinity chromatography.

Complement-mediated Hemolysis. SRBC were coated with DNS/BSA and incubated with ^{51}Cr -sodium chromate (Amersham Corp., Arlington Heights, IL). The free ^{51}Cr -sodium chromate was removed by washing the cells three times in 10 ml of fresh Gel-HBS buffer (0.01 M HEPES, 0.15 M NaCl, 0.15 mM CaCl_2 , 0.5 mM MgCl_2 , 0.1% gelatin, pH 7.4). Twofold serial dilutions of chimeric antibodies in Gel-HBS buffer were added to the round-bottomed, 96-well plate (Corning Glass Works, Corning, NY) in a volume of 50 μl . 50 μl of 2% ^{51}Cr -loaded SRBC and 25 μl of 2 U CH_{50} of guinea pig complement (Colorado Serum Co., Denver, CO) were added to each well sequentially. The plates were

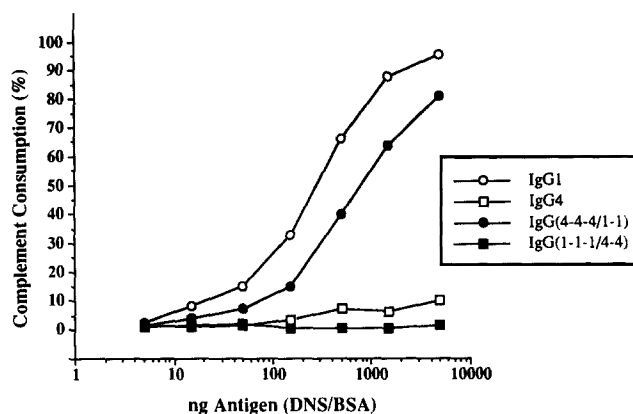


Figure 3. Complement consumption by IgG1/IgG4 domain switch antibodies. 8 μg of antibodies was incubated with the indicated amount of DNA/BSA in the presence of guinea pig complement at 37°C for 45 min. ^{51}Cr -loaded, hemolysin-sensitized SRBC were added, incubated for 45 min, and the ^{51}Cr released was used to determine the percentage of complement consumption.

incubated at 37°C for 45 min, unlysed SRBC were pelleted by centrifugation of the plate, and 50 μl supernatant was counted in a gamma counter. The percentage of cell lysis was determined.

Complement Consumption Assay. To assay complement consumption, 8 μg of chimeric antibody was incubated with increasing amounts of DNS/BSA and 2 U CH_{50} of complement at 37°C for 45 min. ^{51}Cr -loaded hemolysin-sensitized SRBC were then added and incubated for another 45 min. After centrifugation, the amount of released ^{51}Cr was determined. Antibody only, antigen only, buffer, and water controls were included in the experiments. The percentage of complement consumption was calculated as: $100 \times [(1 - \text{cpm of Ag} + \text{Ab complement}) / (\text{cpm of Ab} + \text{complement})]$.

Results

A set of mouse-human chimeric antibodies having identical antigen-combining sites and light chain but with constant region domains switched between IgG2 and IgG3 or IgG1 and IgG4 were generated as described above. Fig. 1 is a schematic diagram of the genetic structures of the antibody constant regions.

To locate the domain responsible for complement activation, domains were changed between IgG2 and IgG3, and

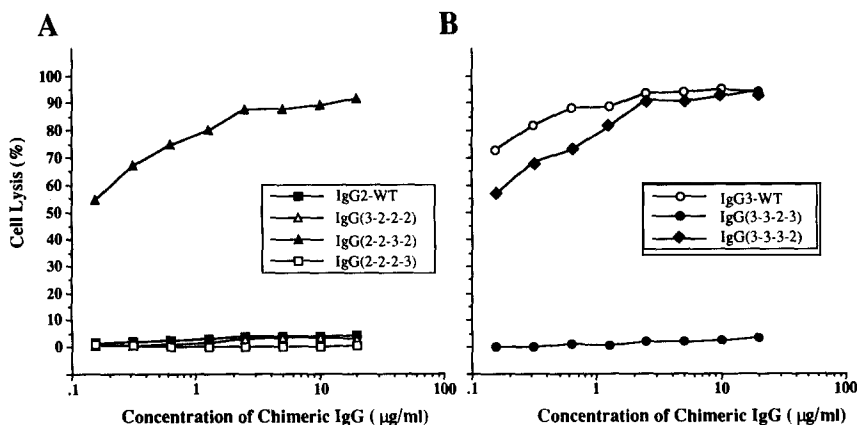


Figure 2. Complement-mediated hemolysis of IgG2 and IgG3 domain switch antibodies. For clarity of presentation, the data are shown in two panels. ^{51}Cr -loaded, DNS/BSA-coated SRBC were lysed by various concentrations of antibody in the presence of guinea pig complement, and the percentages of cell lysis was calculated. The structure of the chimeric antibodies is shown in Fig. 1.

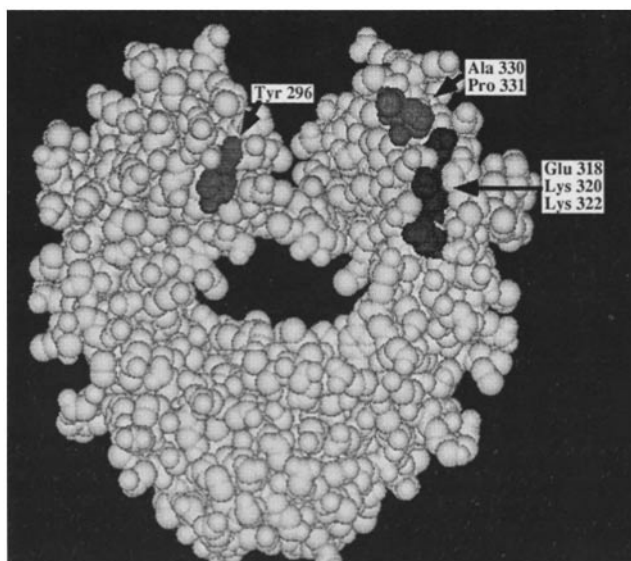


Figure 4. Three-dimensional structure of human IgG1 Fc. Amino acids that are discussed in the text are indicated as solid balls. Ala 327 is buried in the molecule. This graphic is made using the MacIcmdad program and is based on the coordinates from Deisenhofer (14). The two C_{H2} domains face in opposite directions. Therefore, Glu 318, Lys 320, Lys 322, Ala 330, and Pro 331 are invisible on the other side of the left domain, and Tyr 296 is similarly hidden on the other side of the C_{H2} domain on the right.

the ability of the resulting antibodies to mediate complement-dependent cell lysis of SRBC coated with the hapten dansyl was measured. As shown in Fig. 2 A, replacement of the C_{H1} and C_{H3} domains of IgG2 with the corresponding domains from IgG3, IgG(3-2-2-2), and IgG(2-2-2-3), did not increase the complement binding activity. However, IgG(2-2-3-2) containing the C_{H2} domain from IgG3 performs complement-mediated cell lysis almost as well as wild-type IgG3. Likewise, the replacement of the C_{H2} domain of IgG3 with the corresponding domain from IgG2, IgG(3-3-2-3), completely abolished the capacity to fix complement (Fig. 2 B). These results clearly demonstrate that the structures that determine the differing ability of IgG2 and IgG3 to activate complement are largely located in the C_{H2} domain.

To further localize the region of the C_{H2} domain that determines its ability to activate complement, a SacII site in the C_{H2} exon of the heavy chain genes of IgG1 and IgG4 was used to generate intra-domain switch antibodies between IgG1 and IgG4 (Fig. 1). The SacII site is located at residue 292 in C_{H2} ; thus, IgG(1-1-1/4-4) has the COOH-terminal part (from residue 292-340) of the C_{H2} domain and the C_{H3} domains from IgG4. The same region in IgG(4-4-4/1-1) is from IgG1. The more sensitive complement consumption assay was used to measure the effect of domain switch between IgG1 and IgG4 in order to detect small amounts of residual activity. It was found that IgG(1-1-1/4-4) completely

lost its ability to activate complement; in contrast, IgG(4-4-4/1-1) showed significant activity compared with wild-type IgG4 (Fig. 3). These results indicate that residues 292-340 in C_{H2} contain amino acids determining the isotype-specific differences in complement activation.

Discussion

The human IgG molecules differ markedly in their ability to activate complement in spite of having very similar amino acid sequences. The important role of the C_{H2} domain of IgG in complement activation was supported by earlier antibody fragmentation studies (5-7). The contribution by C_{H3} was also indicated with the finding that C_{H3} could stabilize the conformation of C1 and protect it from attack by the C1 inhibitor (13). The studies reported here show that it is the C_{H2} but not C_{H3} that determines the human IgG isotype-specific differences in complement activation. Moreover, our studies now locate the important residues to the COOH terminus of the C_{H2} domain between residues 292 and 340. Within that region, IgG1 differs from IgG4 at only four residues: 296 (Tyr vs. Phe), 327 (Ala vs. Gly), 330 (Ala vs. Ser), and 331 (Pro vs. Ser). The locations of these amino acids in the three-dimensional structure of IgG1 Fc are shown in Fig. 4. Using the nomenclature of Beale and Feinstein (15), residues 330 and 331 are located on the Fy3 β strand and fold into proximity with the previously identified 318-320-322 residues (10). Therefore, these residues together may provide the binding site for C1q. The side chain of residue 327 is mostly buried inside the molecule and is probably not directly involved in C1q binding. However, the greater flexibility afforded by the Gly residue in IgG4 may change the conformation of the nearby C1q binding site and thus affect complement activation. Residue 296 is located on the surface of X face. It seems unlikely that a C1q molecule can bind the proposed binding site on Y face and residue 296 simultaneously. However, residue 296 in the other C_{H2} is quite accessible, as shown in Fig. 4, and might contribute to the C1q binding site. The fact that both C_{H2} domains are required for C1 activation give indirect support for this possibility (5).

Noteworthy is the fact that the IgG(4-4-4/1-1) molecule is slightly deficient in its ability to activate complement relative to wild-type IgG1. A significant difference between the two molecules is in the hinge region. It is quite possible that the rigid hinge of IgG4 impairs access to the C1q binding site thus decreasing the effectiveness of the molecule. We have observed a similar modulating effect of the hinge region on Fc γ RI binding (Canfield, S. M., and S. L. Morrison, manuscript submitted for publication). Alternatively, additional amino acid variation between the two isotypes within the C_{H2} NH₂ terminal to the exchange point may further influence complement activation.

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