Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 162–166, January 1990
Immunology

Influence of the hinge region on complement activation, C1q binding, and segmental flexibility in chimeric human immunoglobulins

LEE K. TAN*, ROBERT J. SHOPES†‡, VERNON T. OI†, AND SHERIE L. MORRISON*¶

*Department of Microbiology, Columbia University College of Physicians and Surgeons, 701 West 168th Street, New York, NY 10032; †Becton Dickinson Monoclonal Center, 2375 Garcia Avenue, Mountain View, CA 94043; and ‡Department of Cell Biology, Stanford University Medical Center, Stanford, CA 94305

Communicated by Elvin A. Kabat, August 22, 1989

ABSTRACT **We have characterized a series of genetically engineered chimeric human IgG3 and IgG4 anti-dansyl (DNS) antibodies with identical antibody-combining sites but different hinge region amino acid compositions to determine how the hinge region influences Fab fragment segmental flexibility, C1q binding, and complement activation. Our data support the correlation between "upper hinge" length and Fab segmental flexibility; moreover, we confirm that a hinge region is essential for C1q binding and complement activation. However, the hinge length by itself is not sufficient for complement activity in IgG molecules. We have demonstrated that the IgG4 hinge, which imparts restricted segmental flexibility, reduces the ability of IgG3 molecules to activate complement. We also find that the IgG3 hinge region, which imparts greater segmental motion, is not sufficient to create complement activation activity in IgG4 anti-DNS antibodies. Finally, we conclude that (*i*) segmental motion is correlated with "upper hinge" length, (*ii*) hinge length and segmental flexibility is not enough to alter complement binding and activation, and (*iii*) segmental flexibility does not correlate with proficiency to activate the complement cascade.**

The constant (C) region domains of the four human IgG subclasses have virtually identical amino acid sequences, yet each subclass has different biological effector functions—e.g., IgG3 and IgG1 and not IgG2 and IgG4 activate the classical complement cascade (1, 2) in the presence of human complement; chimeric IgG2 will fix rabbit complement (2). Although the first component of complement, C1q, is believed to bind to the heavy chain constant region 2 (C_H2) domain of all IgG subclasses, neither a specific C1q binding site nor a mechanism by which complement is activated is known (3–5). Recently, Duncan and Winter (6) have come closest to defining the exact C1q binding site by characterizing the complement activation and C1q binding properties of variant antibody molecules, which they generated by genetically engineering mutations in selected residues in the mouse IgG2b C_H2 domain. Duncan and Winter discovered that only three residues, Glu-318, Lys-320, and Lys-322, when altered, affected complement-mediated hemolytic activity and C1q binding. Interestingly, these residues are conserved in all of the human IgGs whether or not they are able to activate complement, suggesting that there must be additional structural requirements for C1 binding and activation.

Isenman *et al.* (7) showed that although intact human IgG4 does not bind C1, its Fc fragment does; furthermore IgG4 Fc aggregates activate complement. They concluded that the structural features required for C1 binding are present on the IgG4 Fc but that access to this binding site is hindered sterically by the IgG4 Fab "arms" (7, 8). This model provided a structural basis for the differing effectiveness of different IgG subclasses in activating complement despite the high degree of amino acid identity between the subclasses. Beale and Feinstein (9) proposed that the hinge serves as a spacer, separating the antibody's Fab arms from the Fc, and that hinge length determines Fab segmental flexibility and may affect biological effector functions. They too suggested that the relatively short hinge regions in human IgG2 and IgG4 molecules might place the Fab arms of these antibodies in close proximity to their C_H2 domains and hinder C1 binding and activation. The different biological effector functions exhibited by the different immunoglobulin isotypes may be due to the structural variation in the hinge regions. Two variant human IgG1 myeloma proteins, Dob and Lec, have no hinge regions and do not bind C1 (8) or the monocyte Fc receptor (10).

The human IgG hinge regions in contrast to the C region domains are remarkably diverse (11). They vary in amino acid sequence and composition as well as length. IgG1, IgG2, and IgG4 have hinge regions consisting of 12–15 amino acids. IgG3 has an extended hinge region, consisting of 62 amino acids, including 21 prolines and 11 cysteines. The IgG3 hinge region can be divided into four segments: one resembles the IgG1 hinge and the other three are identical and unique to IgG3. The "functional" hinge region, deduced from crystallographic studies, extends from amino acid residues 216–237 of the IgG1 H chain (EU numbering; ref. 12) and includes a small segment of the N terminus of the C_H2 domain. The hinge can be divided into three regions; the "upper hinge," the "core," and the "lower hinge" (12, 13). The upper hinge includes the amino acids from the end of C_H1 to the first residue in the hinge that restricts motion (generally the first cysteine that forms an inter-heavy-chain bridge). The core hinge contains the inter-H-chain disulfide bridges and a high content of proline. The lower hinge connects to the C_H2 domain and includes amino acids in the C_H2 domain. Recent studies have demonstrated that upper hinge length correlates with segmental flexibility (2). In addition, Schneider *et al.* (14) demonstrated that interactions between structural loops proximal to the hinge region in the C_H1 domain and the hinge affect segmental flexibility; however, their data did not distinguish between the contribution of domain–domain interactions and hinge length to Fab segmental flexibility.

In this study, we characterize a number of chimeric human IgG3 and IgG4 molecules with different hinge lengths and amino acid composition. All of these antibodies have iden-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: BSA, bovine serum albumin; H, heavy chain; L, light chain; C_H, heavy-chain constant region; DNS, 5-(dimethylamino)-1-naphthalenesulfonyl (dansyl) chloride; V_H, heavy chain variable region.

¶Present address: Department of Microbiology, Molecular Biology Institute, University of California at Los Angeles, Los Angeles, CA 90024.

tical murine anti-dansyl (DNS) hapten-binding sites. Analysis of this set of antibodies demonstrates that segmental flexibility correlates with the length of the upper hinge, but does not correlate with C1q binding or complement activation.

MATERIALS AND METHODS

Construction of Hinge-Modified Anti-DNS Antibodies. The V_κ and V_H [V_κ, κ light (L)-chain variable region; V_H, H-chain variable region] genes from the mouse anti-DNS hybridoma 27-44 were joined to human C_κ in the pSV184ΔHneo expression vector (15) and to human IgG3 and IgG4 C_H in the pSV2ΔHgpt expression vector (2), respectively; *Pvu* I linkers were inserted at the *Stu* I sites between exons in the IgG3 and IgG4 C region genes such that each gene has a single *Stu* I site converted to a *Pvu* I site (16). C region genes containing the desired hinge modification were engineered by using clones with the appropriate *Pvu* I sites (see Fig. 1). The modified IgG3 and IgG4 C_H genes were ligated to the DNS V_H gene in the pSV2ΔHgpt vector. The H- and L-chain genes were transfected simultaneously by protoplast fusion into a mouse myeloma cell line (15). Stable transfectant cell lines were selected by using only G418 (GIBCO) at 1 mg/ml. The level of secretion was between 1 and 5 μg per 10^6 cells per 24 hr. Chimeric anti-DNS antibodies were purified by affinity chromatography as described by Scheider *et al.* (17).

Cell Culture. Cell lines were maintained in Iscove's modified Dulbecco's medium (GIBCO) supplemented with 5% fetal calf serum (HyClone), penicillin/streptomycin (10 units of penicillin per ml, 10 μg of streptomycin per ml) (GIBCO), 44.6 mM sodium bicarbonate, and 50 μM 2-mercaptoethanol.

Complement Depletion Assay. This assay was done as described by Hardy (18) with DNS coupled to bovine serum albumin (BSA). Sheep erythrocytes were obtained from Pocono Rabbit Farm (Canadensis, PA). Guinea pig complement and anti-sheep hemolysin were purchased from Colorado Serum (Denver). ^{51}Cr was from Amersham. Complement activity of each antibody was measured in three to five experiments done at different times.

Radioiodination. Anti-human κ monoclonal antibody was radiolabeled with $Na^{125}I$ (Amersham) using Iodo-Gen (Pierce). Human C1q was radiolabeled with Enzymobeads (Bio-Rad). Aliquots of radiolabeled C1q were stored at −70°C.

C1q Binding Assay. C1q binding was determined by a solid-phase assay in which increasing amounts of ^{125}I-labeled human C1q were added to fixed amounts of antigen–antibody complexes coated onto microtiter plates (S. Friend and V.T.O., unpublished data). Microtiter wells were coated with DNS_{38}/BSA [50 mg/ml in phosphate-buffered saline (PBS)] overnight at 4°C. Plates were washed three times with PBS/0.05% Tween-20 and blocked with 3% BSA in PBS. Saturating amounts of anti-DNS antibodies were incubated in the DNS_{38}/BSA-coated wells for 2 hr at room temperature or overnight at 4°C. Excess antibody was removed, and serial dilutions of ^{125}I-labeled C1q were added starting with 0.2 μg in 25 μl and were incubated at room temperature for 30 min. Unbound C1q was removed and the amount of C1q bound was determined with a γ counter. A chimeric human IgG4 anti-DNS antibody, which does not bind C1q, was used as a negative control.

Fluorescence Anisotropy. Segmental flexibility of the anti-DNS antibodies was determined by measuring the fluorescence depolarization kinetics as described (14, 19). The mean rotational correlation time, $\langle\phi\rangle$, was calculated as described (14).

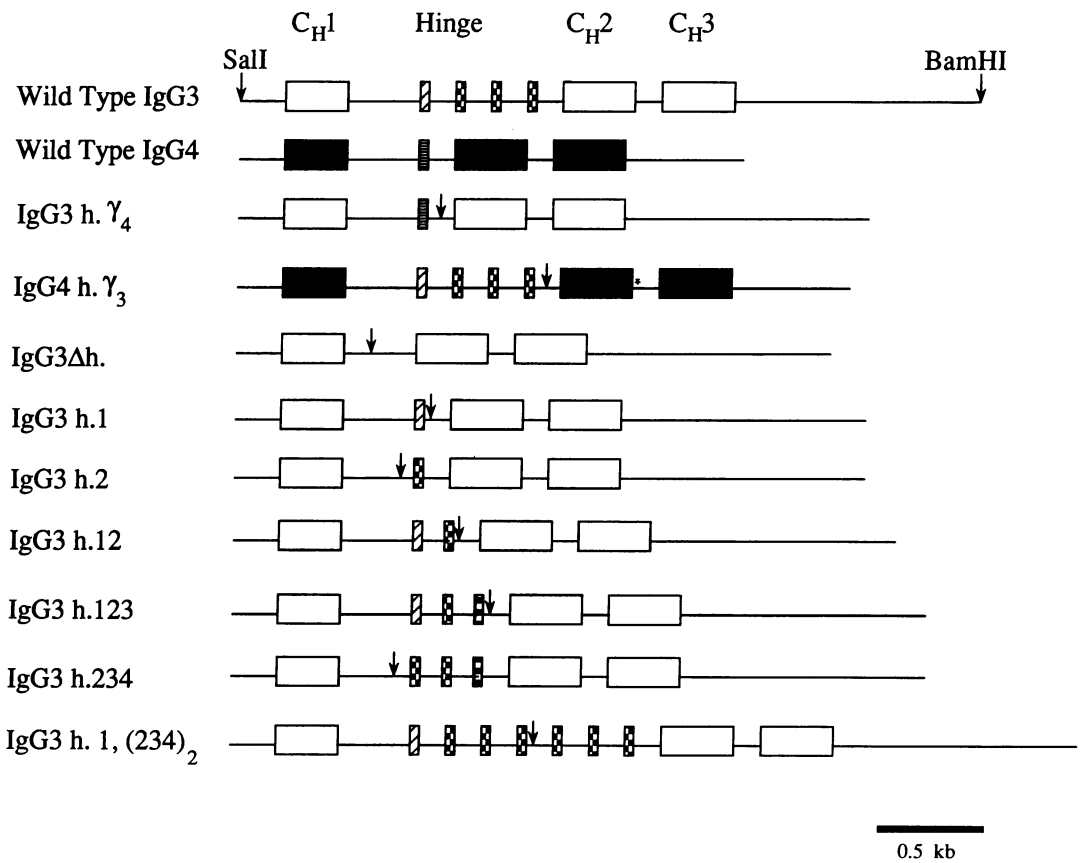

FIG. 1. Schematic diagram of the IgG3 and IgG4 wild-type and hinge-modified C regions. □, C_H1, C_H2, and C_H3 exons of IgG3; ■, C_H1, C_H2, and C_H3 exons of IgG4. In IgG3, hinge exon 1 (diagonal lines) is unique and exons 2–4 (checkered) are identical. Small arrows indicate *Pvu* I sites, which were introduced by using oligonucleotide linkers and were used to make the hinge modified genes. kb, Kilobase.

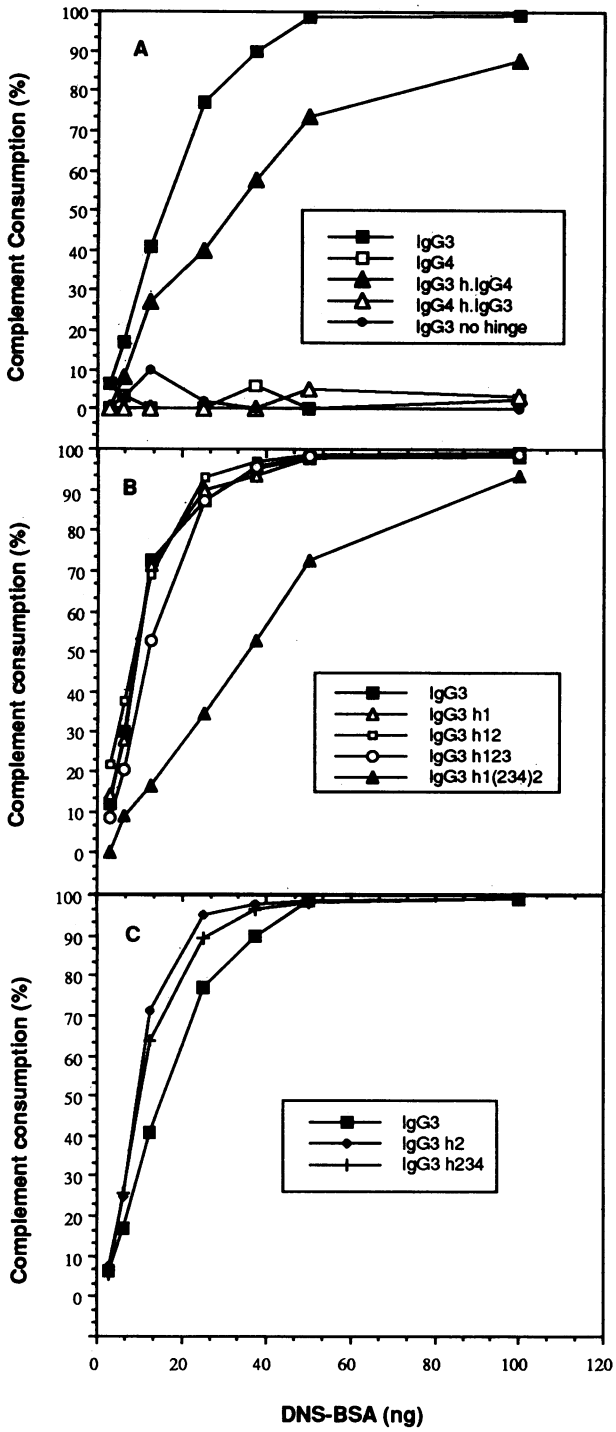

FIG. 2. Complement consumption by anti-DNS antibodies. Increasing amounts of DNS_{38}/BSA were mixed with a fixed amount of antibody (8 μg) and two C_H50 units of complement and the ^{51}Cr release from lysis of presensitized sheep erythrocytes was monitored. Complete lysis (100%) was equivalent to the ^{51}Cr release from erythrocytes incubated with distilled water. h, Hinge.

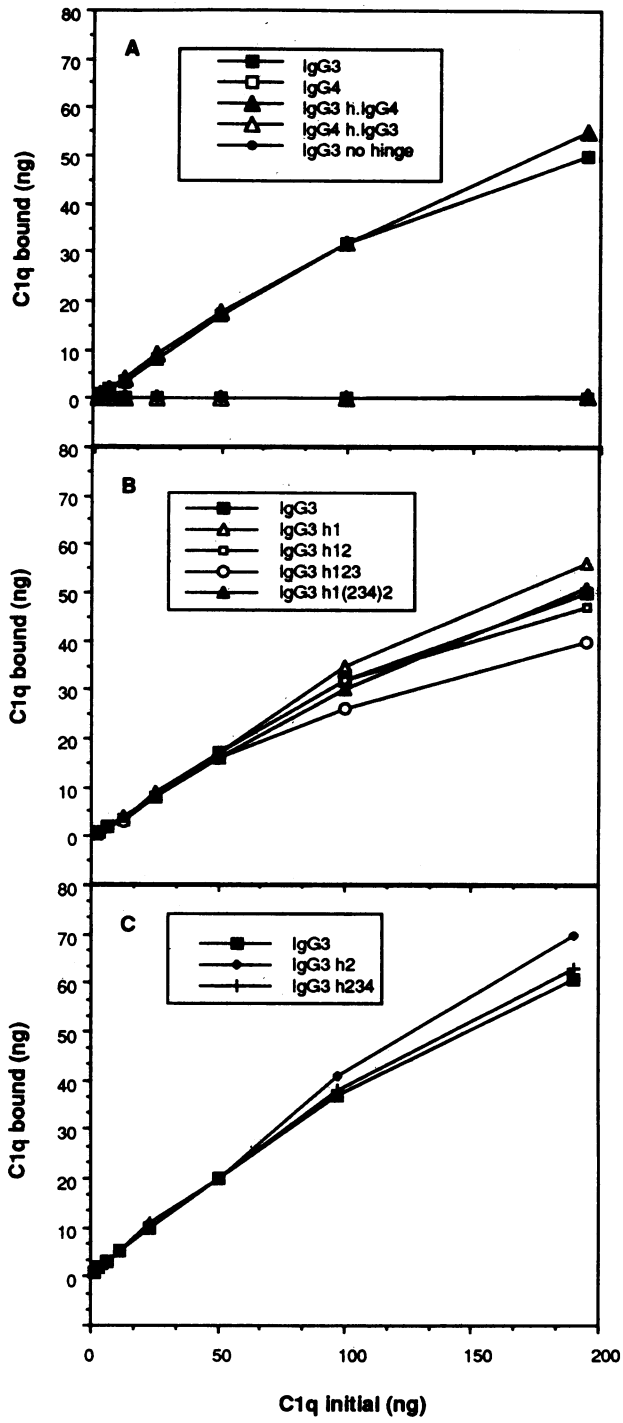

FIG. 3. C1q binding by anti-DNS antibodies. Increasing amounts of ^{125}I-labeled human C1q were added to a fixed quantity of chimeric anti-DNS antibody–antigen complexes coated on microtiter plates and the amount of ^{125}I-labeled C1q bound was quantitated. h, Hinge.

RESULTS

Transfectomas synthesizing wild-type and hinge-modified IgG3 and IgG4 antibodies were established. Fig. 1 is a schematic diagram of the genetic structures of the antibody C regions. Antibodies were purified from culture supernates by affinity chromatography and were then reduced with 2-mercaptoethanol and analyzed by SDS/PAGE to determine their chain composition and purity. The antibody molecules with altered hinge regions had gel mobilities consistent with their expected molecular weights (data not shown). The wild type IgG1, IgG2, and IgG3 molecules were found to be secreted as H_2L_2 tetramers, as expected; the IgG4 molecule, however, was secreted as both H_2L_2 tetramers and HL half molecules in a 2:1 ratio (data not shown). Interestingly, the IgG3 with the hinge of IgG4 was secreted as tetramers and half-molecules, while IgG4 with the hinge of IgG3 was secreted only as tetramers. The hinge region appears to determine the H- and L-chain assembly pattern. Gel filtration analyses revealed that the wild-type IgG4 molecule, the IgG3 molecule with the IgG4 hinge, and the hinge deleted IgG3

molecule migrate as a single tetramer peak (results not shown), indicating that the HL half-molecules are assembled into tetramers by noncovalent interactions.

Complement Fixation. Purified antibodies used in complement assays were >95% pure and free of detectable antibody aggregates when analyzed by SDS/PAGE and gel filtration. Complement consumption by the anti-DNS antibodies was titered with various concentrations of DNS_{38}/BSA (Fig. 2). The hinge-modified anti-DNS antibodies fall into three groups: those that have a high, intermediate, or negligible ability to activate the classical complement cascade. There are six molecules that effectively activate complement: the three IgG3 molecules with hinge regions shortened by one, two, and three reiterated IgG3 hinge exons; and the IgG3 antibody molecules with IgG3 hinge exon 2 and hinge exons 2, 3, and 4. The IgG3 antibody molecules with seven IgG3 hinge exons and the IgG4 hinge exhibit intermediate complement activation activity. If noncovalent tetramers vis-a-vis the hingeless myeloma proteins do not bind or activate complement, the fraction of IgG3 antibody molecules with the IgG4 hinge, which are secreted as noncovalent tetramers, may not bind and activate complement at all. This may account for the reduced proficiency with which this antibody molecule activates complement. The other three anti-DNS antibodies—the IgG3 with no hinge region, wild-type IgG4, and the IgG4 with the IgG3 hinge—have little or no detectable complement activity. Table 1 summarizes these results.

C1q Binding. A solid-phase C1q binding assay was performed to determine the effect of the hinge modifications (Fig. 3). The IgG3 molecule without a hinge, wild-type IgG4, and the IgG4 with the IgG3 hinge do not bind human C1q. The others bind C1q as does wild-type IgG3. The variation in the binding curves of this group of antibodies may be significant, but since they also could be due to small variations in purity and antibody concentration, we have not attempted to interpret these differences.

Segmental Flexibility. The fluorescence emission anisotropy kinetics of DNS-lysine bound to these engineered antibodies was measured to determine the effect of hinge region structures on Fab segmental flexibility. These results are summarized in Table 1. All of the antibodies can be grouped into two classes with respect to their segmental flexibility: molecules with average $\langle\phi\rangle$ values similar to wild-type IgG3, including all antibodies with IgG3 hinge exon 1 juxtaposed to the C_H1 domain, as well as the IgG4 molecule with the IgG3 hinge; molecules with a greater $\langle\phi\rangle$ value, indicating molecules having more rigidity, including the IgG3 molecule with no hinge, the IgG3 with the IgG4 hinge, and the IgG3 molecules with one or three of the reiterated hinge exons. Despite the range of $\langle\phi\rangle$ values of this second group of antibodies, we have classified these molecules into a single class, because of the relatively large variance in the data.

DISCUSSION

In this study, we have confirmed the correlation between upper hinge length and the segmental flexibility of the antibody molecule. Since the human IgG3 and IgG4 molecules both have the disulfide bridge between H and L chains crossing from the carboxyl end of the L chain to a cysteine in the homologous position in either the IgG3 or IgG4 C_H1 domain, the position of this disulfide is not affected by either hinge shuffling or C_H1 exchange. Thus, the interaction between the C_H1 domain and the hinge regions should be similar in all the molecules. We found that whenever the unique IgG3 hinge domain (exon 1), which has an upper hinge length of 12 amino acids, is proximal to the C_H1 domain, antibodies exhibit segmental flexibility similar to wild-type IgG3 molecules. This correlation is true regardless of total hinge length (17–107 amino acids) or whether the other C region domains are IgG3 or IgG4. When a reiterated IgG3 hinge domain (exon 2, 3 or 4) is proximal to the C_H1 domain, the upper hinge length is shortened to 4 amino acids and the antibody becomes more rigid. Similarly, when the IgG4 hinge with an upper hinge length of 7 amino acids replaces the IgG3 hinge region, the antibody becomes more rigid. The effect of hinge length on the "waggle" of the Fc portion of the antibody molecule is still unknown.

Previous studies (2, 21) indicated that hinge length and Fab segmental flexibility correlate with complement consumption. This correlation is disputed in our current analyses. Two variant IgG3 molecules, as rigid as IgG4, activate complement effectively (see Table 1). In contrast, IgG4 with the IgG3 hinge is as flexible as wild-type IgG3 but is unable to activate complement. The inability of wild-type IgG4 antibodies to activate the complement pathway was thought to be due to its restricted hinge region, since aggregated IgG4 Fc fragments activate the complement cascade (7). However, when we replaced the IgG4 hinge region with the IgG3 hinge, thereby increasing the segmental flexibility and spatial separation between the IgG4 Fab arms and its Fc, the IgG4 antibody was still unable to activate complement. Therefore a long flexible hinge is not sufficient to reveal cryptic complement activation activity in the IgG4 C region domains.

Notably, the IgG3 molecule with the IgG4 hinge is less effective at activating complement than the wild-type IgG3 molecule (see Fig. 2); however, this result must be interpreted carefully, because preparations of this antibody con-

Table 1. Summary of mean correlation times (in ns), complement fixation, and C1q binding with hinge (h) sequence

Antibody	Hinge sequences			$\langle\phi\rangle$,* ns	Complement fixation	C1q binding
	Upper hinge	Core hinge	Lower hinge			
	216		238			
IgG3 h.1	ELKTPLGDTTHT	CPRCP	APELLGGP	56 ± 6	High	++
IgG3 h.12	ELKTPLGDTTHT	CPRCPEPKSCDTPPPCPRCP	APELLGGP	52 ± 6	High	++
IgG3 h.123	ELKTPLGDTTHT	CPRCP(EPKSCDTPPPCPRCP)$_2$	APELLGGP	54 ± 3	High	++
IgG3	ELKTPLGDTTHT	CPRCP(EPKSCDTPPPCPRCP)$_3$	APELLGGP	51 ± 2	High	++
IgG3 h.1(234)$_2$	ELKTPLGDTTHT	CPRCP(EPKSCDTPPPCPRCP)$_7$	APELLGGP	51 ± 4	Intermediate	++
IgG4 h. of IgG3	ELKTPLGDTTHT	CPRCP(EPKSCDTPPPCPRCP)$_3$	APEFLGGP	52 ± 4	Negligible	0
IgG3 h. of IgG4	ESKYGPP	CPS	APELLGGP	82 ± 4	Intermediate	++
IgG3 h.2		(EPKSCDTPPPCPRCP)	APELLGGP	94 ± 8	High	++
IgG3 h.234		(EPKSCDTPPPCPRCP)$_3$	APELLGGP	85 ± 9	High	++
IgG4	ESKYGPP	CPS	APEFLGGP	93 ± 7	Negligible	0
IgG3 h. no hinge	—	—	APELLGGP	77 ± 5	Negligible	0

The amino acid sequence (single-letter code) of the hinge region and the N terminus of the C_H2 domain are aligned from amino acids 216–238 (human IgG, EU numbering). The upper hinge (underlined) as defined by Beale and Feinstein (9) as well as the core hinge as defined by Endo and Arata (20) are shown.

*Values are presented as the average ± SE.

sist of a mixture of H_2L_2 tetramers—i.e., tetramers joined by disulfide bridges, as well as HL half-molecules, which form tetramers without covalent bridges. An intact hinge region is required for complement binding and activation. Two hinge-deleted human IgG1 myeloma proteins (8), a hinge-deleted mouse IgG2a anti-DNS antibody (14), the hinge-deleted human IgG3 anti-DNS antibody (described in this report), and reduced and aklylated antibody do not activate complement. From these studies it is not clear whether it is the hinge region or the formation of covalent tetramers that is required. It is conceivable that the disulfide bridged IgG3 molecules with the IgG4 hinge region are as effective at activating complement as wild-type IgG3 molecules, and the noncovalent tetramers do not activate complement at all, resulting in an averaged, reduced effectiveness of the antibody population to activate complement. This interpretation of the data would suggest that the short IgG4 hinge length with its restricted flexibility does not interfere with complement binding or activation by the IgG3 C_H2 domain or any other IgG3 structure involved in complement activation.

It is difficult to explain why IgG3 in which the hinge has been further lengthened to 107 amino acids has reduced complement fixing activity. Neither the segmental flexibility of the molecule nor the lower hinge is changed and the C1q binding ability is unaltered. With the lengthened hinge only the core hinge sequence is altered to include six instead of three reiterated hinge exons. This change may influence the relative position of C_H1 and the Fc; it is possible that the binding of subsequent complement components is influenced or that the molecule cannot assume an appropriate altered conformation upon C1q binding.

Despite our ability to genetically engineer antibody molecules, the structural basis for antibody-mediated complement activation remains enigmatic. From our analyses of these genetically engineered antibodies, we conclude (*i*) segmental motion is correlated with upper hinge length; (*ii*) hinge length and segmental flexibility are not enough to effect complement binding and activation; and (*iii*) segmental flexibility does not correlate with proficiency to activate the complement cascade. In the "aggregation" model of complement activation, C1 binding to antigen–antibody aggregates is enough to activate the complement cascade; in the allostery model, antigen binding is thought to induce a conformational change that enhances C1q activation. In the "distortive" model, antigen binding is proposed to distort the Fc by displacing the Fab arms and exposing a cryptic C1q binding site. Of these three models, the aggregation model of complement activation has had the greatest support, in part because it is difficult to prove that allostery or distortion is involved in triggering antibody-mediated complement activation. It is difficult to specify which model of complement activation reflects our data best; however, it would appear that aggregation and distortion alone are not enough to describe the molecular mechanism of complement activation.

We would like to thank Dr. Lubert Stryer (Department of Cell Biology, Stanford University) for use of the instrumentation used in the fluorescence anisotropy kinetics measurements and Sherree Friend for development of the C1q binding assay. Work in the laboratories was supported by Grants AI 00408, AI 19042, CA 16858, CA 22736, and CA 13696 from the National Institutes of Health; Grant IMS-360 from the American Cancer Society; and a grant from Becton Dickinson Monoclonal Center. R.J.S. was supported by the Leukemia Society of America.

1. Brüggemann, M., Williams, G. T., Bindon, C. I., Clark, M. R., Walker, M. R., Jeffries, R., Waldmann, H. & Neuberger, M. S. (1987) *J. Exp. Med.* **166,** 1351–1361.
2. Dangl, J. L., Wensel, T. G., Morrison, S. L., Stryer, L., Herzenberg, L. A. & Oi, V. T. (1988) *EMBO J.* **7,** 1989–1994.
3. Yasmeen, D., Ellerson, J. R., Dorrington, K. J. & Painter, R. H. (1976) *J. Immunol.* **116,** 518–522.
4. Kehoe, J. M., Bourgois, A., Capra, D. J. & Fougereau, M. (1974) *Biochemistry* **13,** 2499–2504.
5. Golomb, M. & Porter, R. R. (1975) *Biochem. J.* **145,** 177–183.
6. Duncan, A. R. & Winter, G. (1988) *Nature (London)* **332,** 738–740.
7. Isenman, D. E., Dorrington, K. J. & Painter, R. H. (1975) *J. Immunol.* **114,** 1726–1729.
8. Klein, M., Haeffner-Cavaillon, N., Isenman, D. E., Rivat, C., Navia, M. A., Davies, D. R. & Dorrington, K. J. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 524–528.
9. Beale, D. & Feinstein, A. (1976) *Q. Rev. Biophys.* **9,** 135–180.
10. Woof, J. M., Nikjaafar, M., Jefferis, R. & Burton, D. R. (1984) *Mol. Immunol.* **21,** 523–527.
11. Huck, S., Fort, P., Crawford, D. H., Lefranc, M.-P. & Lefranc, G. (1986) *Nucleic Acids Res.* **14,** 1779–1785.
12. Burton, D. R. (1985) *Mol. Immunol.* **22,** 161–206.
13. Feinstein, A., Richardson, N. & Taussig, M. J. (1986) *Immunol. Today* **7,** 169–173.
14. Schneider, W. P., Wensel, T. G., Stryer, L. & Oi, V. T. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 2509–2513.
15. Oi, V. T. & Morrison, S. L. (1986) *Biotechniques* **4,** 214–221.
16. Lathe, R., Skory, S. & Kleny, M.-P. (1984) *Focus* **6** (4), 1–6.
17. Schneider, W. P., Oi, V. T. & Yanofsky, C. (1987) *Proteins* **2,** 81–89.
18. Hardy, R. R. (1986) in *Handbook of Experimental Immunology*, ed. Weir, D. M. (Alden, Oxford, England), Vol. 1, pp. 40.1–40.12.
19. Reidler, J., Oi, V. T., Carlsen, W., Minh-Vuong, T., Pecht, I., Herzenberg, L. A. & Stryer, L. (1982) *J. Mol. Biol.* **158,** 739–746.
20. Endo, S. & Arata, Y. (1985) *Biochemistry* **24,** 1561–1568.
21. Oi, V. T., Vuong, T. M., Hardy, R., Reidler, J., Dangl, J., Herzenberg, L. A. & Stryer, L. (1984) *Nature (London)* **307,** 136–140.