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# Segmental flexibility and complement fixation of genetically engineered chimeric human, rabbit and mouse antibodies

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**We generated a family of chimeric immunoglobulin G (IgG) molecules having identical antigen-combining sites for the dansyl (DNS) hapten, in conjunction with nine heavy chain constant ($C_H$) regions. This family of antibody molecules allows comparison of $C_H$ dependent properties independent of possible variable region contributions to IgG function. The segmental flexibility and complement fixation activity were measured of six genetically engineered molecules (the four human IgG isotypes, mouse $IgG_3$ and rabbit IgG) and the remaining three mouse IgG isotypes, ($IgG_1$, $IgG_{2a}$ and $IgG_{2b}$), isolated previously by somatic cell genetic techniques. These properties of antibody molecules each correlate with the length of the immunoglobulin hinge region which separate the first and second $C_H$ ($C_H1$ and $C_H2$) domains. These results attribute a structural basis for two critical properties of antibody molecules.**

*Key words:* chimeric immunoglobulins/haptens/flexibility/complement fixation

## Introduction

Segmental flexibility of IgG molecules has been shown by electron microscopy (Feinstein and Rowe, 1965; Valentine and Green, 1965), hydrodynamic (Noelken *et al.*, 1965) nanosecond fluorescence polarization (Yguerabide *et al.*, 1970) and X-ray crystallographic studies (Huber *et al.*, 1976; Silverton *et al.*, 1977; Amzel and Poljack *et al.*, 1979). These studies demonstrate that the angle between the antigen-binding (Fab) arms varies over a wide range and is mediated by the hinge region which connects the Fab arms to the carboxy-terminal half of the antibody molecule (Dorrington, 1978; Metzger, 1978; Burton, 1985). Beale and Feinstein (1976) postulated that there is a correlation between hinge length and segmental flexibility based on models of the antibody molecule which they built from crystallographic data.

Segmental flexibility potentially facilitates bivalent antigen-binding and subsequent triggering of Fc-dependent effector functions. The hinge region also may act as a spacer, maintaining a required spatial relationship between the Fab arms and the sites on the Fc responsible for binding and activation of the first component of the complement cascade, C1 (Isenman *et al.*, 1975; Beale and Feinstein, 1976; Klein *et al.*, 1981). These conclusions in part were drawn from the inability of two hingeless human $IgG_1$ myeloma proteins to fix complement (Klein *et al.*, 1981). Other data supporting this hypothesis came from analyses of the complement-fixing activity of isolated human $IgG_4$ Fc fragments. Intact human $IgG_4$ does not activate the complement pathway, but the $IgG_4$ Fc fragment does. Since human $IgG_4$ has a shorter hinge than the complement fixing human $IgG_1$ isotype, Isenman *et al.* (1975) postulated that the lack of segmental flexibility about the shorter hinge leads to steric hindrance of C1 binding and activation, despite having a functional binding site. It has been shown previously that the C1 binding site is on the second heavy chain constant region ($C_H2$), proximal to the $C_H1$:$C_L$ domain of the Fab arms of the antibody molecule. This is especially clear in the structure of the hingeless $IgG_1$ Dob protein (Sarma and Laudin, 1982; Silverton *et al.*, 1977). Three proposed C1q binding sites are clustered in a region that is potentially masked by the Fab arms in 'hinge-restricted' antibody molecules (see Burton, 1985).

In order to examine systematically the molecular structures mediating biological effector functions, we have chosen to study a family of antibody molecules that share an identical antigen-combining site and differ only in their heavy chain isotype. Previously we described the generation of a family of anti-dansyl (DNS) heavy chain switch variants using somatic cell genetic techniques and fluorescence-activated cell sorting (Dangl *et al.*, 1982). We found that the segmental flexibility and complement fixing activity of the mouse $IgG_1$, $IgG_{2a}$, $IgG_{2b}$ and IgE antibodies containing the same anti-DNS combining site are correlated (Oi *et al.*, 1984). Is this correlation a general feature of immunoglobulins? We report here a study of six more members of this antibody family generated by genetic engineering. These additional immunoglobulins, human $IgG_1$, $IgG_2$, $IgG_3$ and $IgG_4$, rabbit IgG and mouse $IgG_3$ anti-DNS antibodies, retain the same immunoglobulin kappa light chain and heavy chain V region of the original mouse anti-DNS antibody. In this study of all nine IgG anti-DNS antibodies, we find that there is a strong correlation between segmental flexibility and the number of amino acids contained in the 'upper' hinge defined as the sequence between the end of $C_H1$ to the first cysteine amino acid forming an inter-heavy chain disulfide bridge. Moreover, the hinge length and segmental flexibility correlate with the complement fixation activity of these IgG molecules.

## Results

The overall strategy for generating this family of recombinant anti-DNS antibodies was to clone the mouse immunoglobulin

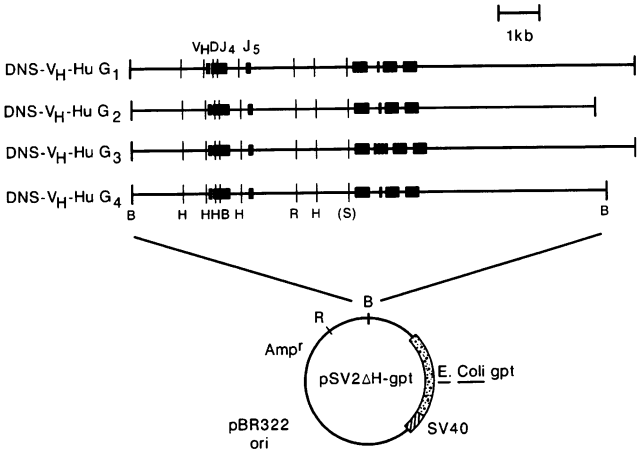


**Fig. 1.** Chimeric human IgG anti-DNS expression vectors. Heavy chain vectors are based on the pSV2ΔH-gpt expression system (Morrison and Oi, 1984; Oi and Morrison, 1986) and were constructed by standard techniques. Exons are represented as dark areas, introns by thin lines. The DNS-$V_H$ gene is carried on the same 4.2-kb fragment in each vector. Each heavy chain is cloned into the vector shown at the bottom. Complete restriction maps are shown for H = *Hin*dIII, B = *Bam*HI, E = *Eco*Ri, S = *Sal*I. See Dangl (1986) for further details. Sequences for the heavy chain constant region genes are: Honjo *et al.* (1979); Sikorav *et al.* (1980); Yamawaki-Kataoka *et al.* (1980); Wels *et al.* (1984); Ellison and Hood (1982); Ellison *et al.* (1982); Krawinkel and Rabbitts (1982); Martens *et al.* (1984).

heavy chain variable region ($V_H$) gene expressed in the DNS1 (27−44) hybridoma cell line (Dangl *et al.*, 1982) and to join this gene to the already cloned human $IgG_1$, $IgG_2$, $IgG_3$ and $IgG_4$, rabbit IgG and mouse $IgG_3$ heavy chain constant region genes. These recombinant immunoglobulin heavy chains were then expressed in a heavy chain loss variant of the DNS1 hybridoma cell line, 27−44.A5C13 (provided by Dr David Parks, Department of Genetics, Stanford University) or co-expressed with the DNS1 mouse-immunoglobulin kappa light chain ($C_\kappa$) in the immunoglobulin non-producing cell line SP2/0 (Morrison and Oi, 1984; Oi and Morrison, 1986). The chimeric mouse−human immunoglobulin heavy chain vectors are depicted in Figure 1. The rabbit IgG and mouse $IgG_3$ heavy chain vectors were constructed similarly (not shown). Stable transfectoma cell lines were generated which produce genetically engineered immunoglobulins with heavy chains of appropriate size and charge as determined by two-dimensional SDS−PAGE (data not shown). The recombinant heavy chains are glycosylated, a post-translation modification known to affect antibody function (Hickman and Kornfeld, 1978; Nose and Wigzell, 1983; Leatherbarrow *et al.*, 1985), as revealed by the shift in their electrophoretic mobility in SDS−PAGE after biosynthetic labelling in the presence and absence of tunicamycin, an antibiotic known to inhibit asparagine-linked glycosylation (not shown).

The nature of the DNS combining site of our engineered antibodies was monitored by measuring the fluorescence emission spectra of bound DNS-lysine. This hapten is a sensitive indicator of the polarity of its micro-environment. We previously showed that independently derived mouse $IgG_1$ anti-DNS antibodies with different combining sites generate different emission spectra (Reidler *et al.*, 1982). The absorption and emission spectra of DNS−lysine bound to each genetically engineered antibody were identical to the DNS1 parental hybridoma antibody (Dangl, 1986). This

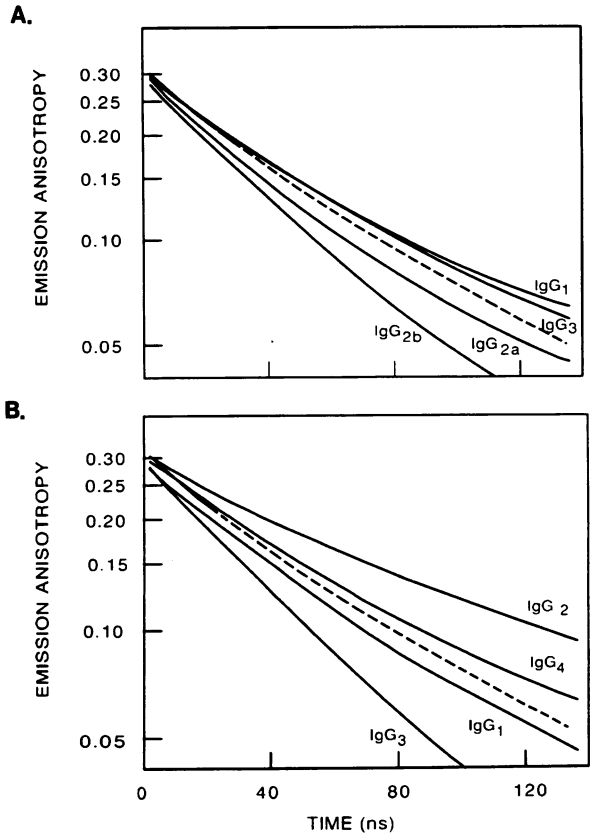


**Fig. 2.** Segmental flexibility of chimeric IgG isotypes. Nanosecond emission anistropy kinetics of ε-dansyl-L-lysine bound to chimeric IgG isotypes of mouse (**A**) and human (**B**). Data for chimeric rabbit IgG anti-DNS are shown as a dotted line in each panel to facilitate comparison.

indicates that each chimeric antibody has a properly folded $V_H$ domain, despite the heterologous junction between the mouse $V_H$ and human or rabbit $C_H1$ domains.

### *Segmental flexibility*

As a measure of segmental flexibility, the fluorescence emission anisotropy kinetics of the genetically engineered anti-DNS antibodies were determined using a single-photon-counting instrument described previously by Reidler *et al.* (1982). Excitation with picosecond pulses of polarized light preferentially excites a subpopulation of identically aligned molecules. The fluorescence emission from this population is highly polarized but depolarizes over time due to molecular motion and tumbling. The rate of fluorescence depolarization depends on the size and shape of the molecule, and also on modes of internal molecular flexibility.

The intensities of the vertically polarized $F_V(t)$, and horizontally polarized $F_H(t)$ components of the emitted light were measured, and the anisotropy decays calculated according to:

$$A(t) = \frac{F_V(t) - F_H(t)}{F_V(t) + 2F_H(t)}$$

For graphic purposes, these data were fitted to a sum of two exponential decays defined by $A(t) = a_1^{\exp.(\phi_1/t)} + a_2^{\exp.(\phi_2/t)}$. These fitted curves are plotted as log $A(t)$ versus time (in Figure 2). For convenience we express $A(t)$ in terms

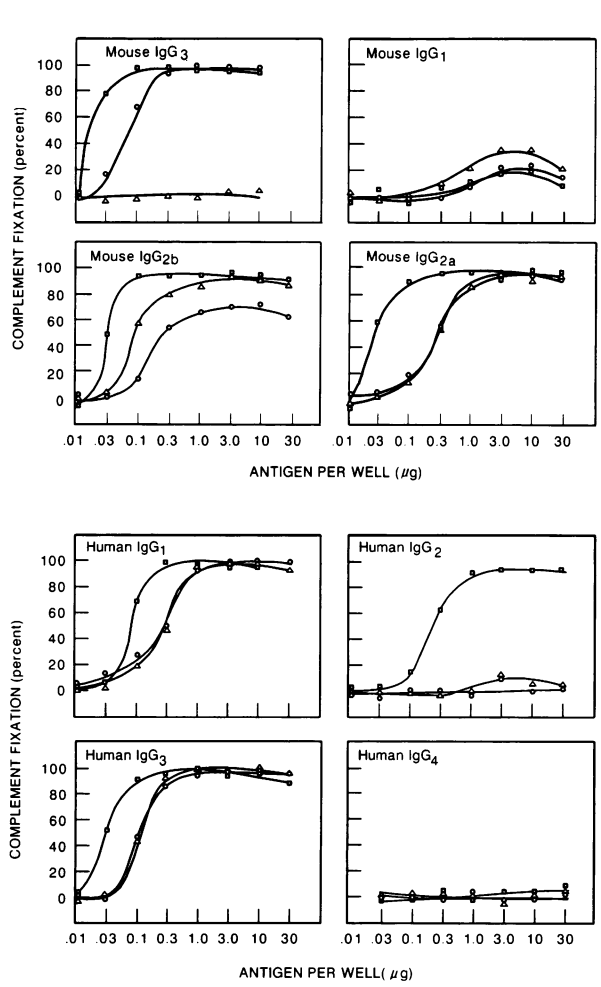


**Fig. 3.** Fixation of human (○), guinea pig (△), and rabbit (□) complement by chimeric anti-DNS IgG antibodies. Mouse IgG isotypes (**A**) and human isotypes (**B**) were tested in complement consumption assays (see Materials and methods). Each data point represents the mean of four to eight measurements.

of a single parameter, the mean rotational correlation time:

$$<\phi> = \frac{(a_1\phi_1 + a_2\phi_2)}{(a_1 + a_2)}$$

Larger values of $<\phi>$ correspond to more rigid molecules, and smaller values to more flexible ones, provided the overall size of the immunoglobulin isotypes are nearly the same.

Figure 2a shows the anisotropy decay curves for mouse IgG isotypes. The results for $IgG_1$, $IgG_{2b}$ and $IgG_{2a}$ are virtually identical to those obtained previously (Oi *et al.*, 1984). $IgG_3$ was found in this study to be slightly more flexible than $IgG_1$, so that the overall order of flexibility is:

$$IgG_{2b}(<\phi> = 55 \text{ ns}) > IgG_{2a}(<\phi> = 63 \text{ ns}) > IgG_3(<\phi> = 78 \text{ ns}) > IgG_1(<\phi> = 81 \text{ ns}).$$

The human IgG isotypes, like the mouse IgG isotypes, exhibit a broad range of segmental flexibilities (Figure 2b) with:

$$IgG_3(<\phi> = 50 \text{ ns}) > IgG_1(<\phi> = 69 \text{ ns}) > IgG_4(<\phi> = 84 \text{ ns}) > IgG_2(<\phi> = 120 \text{ ns}).$$

The range of internal motions ($<\phi>$) is remarkably similar in both species, whereas the rabbit has only one IgG isotype with intermediate segmental flexibility $IgG(<\phi> = 72 \text{ ns})$. The anisotropy decay curve of rabbit IgG is shown in Figure 2A and B to facilitate comparisons with mouse and human IgGs. Our monoclonal rabbit IgG data also provides a reference to compare this study with other reports describing the flexibility of polyclonal rabbit IgGs (Yguerabide *et al.*, 1970; Cathou, 1978; Hansen *et al.*, 1981).

### *Complement fixation*

Complement fixing activities of these recombinant anti-DNS antibodies were measured using a complement consumption assay (Oi *et al.*, 1984). Human, guinea pig and rabbit sera were used as complement sources. The complement fixation activities of mouse $IgG_1$, $IgG_{2a}$ and $IgG_{2b}$ anti-DNS heavy chain switch variants were identical to our previous data. Figure 3A illustrates these data and those obtained with the

**Table I.** Summary of mean rotational correlation times (in ns) and complement fixation results in relation to 'upper hinge' length.

| Isotype | Hinge sequences | | | | Upper hinge length | $<\phi>$[a] | Complement Fixation[c] | | |
|---|---|---|---|---|---|---|---|---|---|
| | Upper hinge | Core hinge | | | | | Human | Rabbit | Guinea pig |
| | 216 | | | 238 | | | | | |
| Mouse $IgG_3$ | EPRIPKPSTPPGSS | C | P | PGNILGGP | 14(9)[b] | 78 ± 3ns | 70 | 15 | – |
| Human $IgG_3$ | ELKTPLGDTTHT | CPRCP(EPKSCDTPPPCPRCP)$_3$ | | APELLGGP | 12 | 50 ± 2ns | 100 | 30 | 100 |
| Mouse $IgG_{2b}$ | EPSGPISTINP | CPPCKECHK | CP | APNLEGGP | 11 | 55 ± 2ns | 80 | 30 | 200 |
| Human $IgG_1$ | EPKSCDKTHT | CPP | CP | APELLGGP | 10 | 69 ± 3ns | 300 | 80 | 300 |
| Mouse $IgG_{2a}$ | EPRGPTIKP | CPPCK | CP | APNLLGGP | 9 | 63 ± 2ns | 250 | 25 | 250 |
| Rabbit IgG | APSTCSKPT | C | P | PPELLGGP | 9 | 72 ± 3ns | 300 | 400 | 450 |
| Human $IgG_4$ | ESKYGPP | CPS | CP | APEFLGGP | 7(5)[b] | 84 ± 3ns | – | – | – |
| Mouse $IgG_1$ | VPRDCG | CKPCI | CT | VPSEVS | 6 | 81 ± 3ns | +/– | +/– | +/– |
| Human $IgG_2$ | ERK | CCVECPP | CP | APPVAG P | 3 | 120 ± 5ns | – | 250 | – |

The amino acid sequence of the hinge region and the N terminus of the $C_H2$ domain are aligned from amino acid 216 to 238 (human $IgG_1$ EU numbering) adapted from Burton (1985). The 'upper hinge' as defined by Beale and Feinstein (1976) are shown as well as the 'core hinge' as defined by Endo and Arata (1985). Cysteine residues which are not part of the core hinge are involved in disulfide linkage to the light chain. The complement fixation results are taken from Figure 3 and are the amount of antigen required for 50% complement consumption.
Single letter amino acid code: A = alanine; C = cysteine; D = aspartic acid; E = glutamic acid; F = phenylalanine; G = glycine; H = histidine; I = isoleucine; K = lysine; L = leucine; M = methionine; P = proline; Q = glutamine; R = arginine; S = serine; T = threonine; V = valine; W = tryptophan; Y = tyrosine.
[a] $<\phi>$ is the mean rotational correlation time (in ns).
[b] Amino acid hinge length when polyproline helical structures are considered restricting elements defining 'upper hinge' length.
[c] Amount of $DNS_{26}$–BSA (ng) required to activate one $CH_{50}$ with 10 μg of antibody.

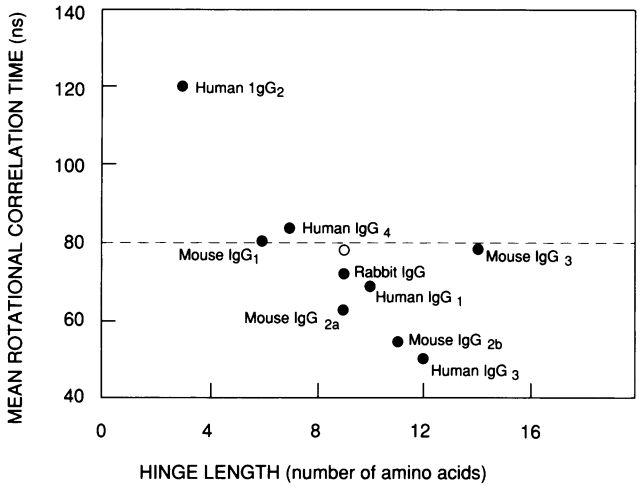


**Fig. 4.** The mean rotational correlation time ($<\phi>$) as a measure of segmental flexibility is correlated with the length of the 'upper hinge'. $<\phi>$ is plotted against the number of amino acids in the 'upper hinge' as defined in the text. The open circle represents mouse $IgG_3$ when the polyproline helical structure (see Table I) is considered to be a restricting element limiting Fab motion. The immunoglobulins listed below the dotted line have complement fixation activity; while those above the dotted line do not have complement fixation activity.

chimeric mouse $IgG_3$ antibody in conjunction with complement from all three species. The $IgG_3$ antibody activates both human and rabbit complement, but does not activate guinea pig complement. Rabbit IgG activates complement from all three species (data not shown, but see Table I).

Complement fixation by the chimeric human $IgG_1$, $IgG_2$, $IgG_3$ and $IgG_4$ antibodies is shown in Figure 3B. The $IgG_1$ and $IgG_3$ isotypes activate complement from all three species, while $IgG_4$ does not fix any complement. An interesting anomaly is that human $IgG_2$ efficiently activates rabbit complement, while not activating human or guinea pig complement.

## Discussion

The advantage of studying this family of monoclonal anti-DNS antibodies is that they share identical antigen-combining sites. Therefore, any difference in biological effector functions can be attributed solely to structures defined by the heavy chain constant region of the antibody. Beale and Feinstein (1976) first predicted that segmental flexibility and hinge length are correlated. Figure 4 and Table I show our segmental flexibility data presented as $<\phi>$ values correlated with the amino acid length of the hinge region of each heavy chain isotype. The hinge amino acid sequences in Table I are aligned to illustrate the 'upper hinge' defined by Beale and Feinstein (1976) and as depicted by Burton (1985) as the number of amino acids between the end of the first heavy chain constant region domain ($C_H1$) and the first cysteine forming an inter-heavy chain disulfide bridge. The exception to this correlation is the restricted flexibility exhibited by mouse $IgG_3$. The length of the mouse $IgG_3$ 'upper hinge' is 14 amino acids and predicts a flexible molecule; however, it is fairly rigid (see Figure 2A and Table I).

An explanation for this exceptional behavior may be that the 'upper hinge' of mouse $IgG_3$ contains the sequence Pro-Pro-Gly proximal to the single inter-heavy chain disulfide bridge. This polypeptide sequence could form a turn of a polyproline helix capable of restricting Fab motion. Nuclear magnetic resonance studies of Ito and Arata (1985), and Endo and Arata (1985) provide evidence for a 'core hinge' structure which is solvent accessible and unaffected by the presence or absence of Fab or Fc portions of the human $IgG_1$ molecule. In fact, this core sequence, Cys-Pro-Pro-Cys (residues 226–229), folds into polyproline helix in the human $IgG_1$ Kol crystal (Marquart *et al.*, 1980). All IgG hinge sequences have similar polyproline cores (see Table I). Generally, this core is bordered by inter-heavy chain disulfide bridges, except in the cases of mouse $IgG_3$ and rabbit IgG. We suggest that in addition to the inter-heavy chain disulfide bridges, polyproline helical structures may act as restricting elements defining the length of the 'upper hinge' and consequently Fab segmental motion. By this definition, the mouse $IgG_3$ 'upper hinge' has nine amino acids, which correlates with its segmental flexibility. The only other isotype which contains a potential polyproline helix sequence, Gly-Pro-Pro, proximal to the first inter-heavy chain disulfide bridge, is human $IgG_4$. This changes the length of the human $IgG_4$ 'upper hinge' from seven to five amino acids. This does not affect significantly the observed correlation between segmental flexibility and hinge length depicted in Figure 4.

There is evidence suggesting that antibody molecules have several modes of segmental flexibility (Yguerabide *et al.* 1970; Hansen *et al.*, 1981). These internal motions include: flexibility of the 'switch peptides' between the $V_H-V_x$ and the $C_H1-C_x$ domains or 'elbow motion'; Fab motion pivoting about the 'upper hinge', or 'arm waving'; and $(Fab')_2$ motion about the Fc. Yguerabide *et al.* (1970) reported that the fluorescence anisotropy curves of Fab fragments of rabbit IgG anti-DNS antibodies exhibit a single decay component with an appropriate mean rotational correlation time for a globular protein of 50 000 $M_r$. We also observed only single decay components with Fab fragments from monoclonal mouse $IgG_1$ anti-DNS antibodies (Reidler *et al.*, 1982). If there were 'elbow motion' about the 'switch peptides', then the fluorescence emission anisotropy kinetics of the Fab fragments should have more than a single decay component, therefore we believe that the nanosecond motions measured by bound DNS fluorescence anisotropy reflects either Fab or $(Fab')_2$ motion. Analyses of polyclonal rabbit IgG anti-DNS by Hansen *et al.* (1981) suggest that the major component of nanosecond segmental flexibility is Fab motion. We cannot distinguish between these modes of internal motion.

When we compared the complement fixing activities of all the chimeric IgG antibodies we found, as expected, that human $IgG_1$ and $IgG_3$, mouse $IgG_{2a}$, $IgG_{2b}$ and $IgG_3$ and rabbit IgG activate complement (Table I). These data extend our previously observed correlation between complement-fixing activity and segmental flexibility (Oi *et al.*, 1984). More flexible antibodies are able to fix complement more effectively. However, there are two anomalies (see Figures 3 and 4 and Table I). Human $IgG_2$ is the most rigid IgG molecule in the anti-DNS family yet it is able to activate rabbit complement. Mouse $IgG_3$ is somewhat flexible,

activates human, rabbit and mouse complement (Neuberger and Rajewsky, 1981) but fails to activate guinea pig complement. Human $IgG_2$ may activate rabbit complement in a novel way, potentially because of steric constraints which permit interaction only with rabbit complement. The structures of mouse $IgG_3$ and guinea pig C1 may have diverged such that they no longer function in combination. We cannot distinguish between these and other possible explanations with the present data.

The correlation between complement fixation activity and segmental flexibility may be explained by the hypothesis that a flexible IgG hinge allows a necessary spatial relationhip between the Fab arms and the C1 binding site located on the $C_H2$ domain proximal to the $C_H1$:$C_x$ domain of the Fab arms (Isenman *et al.*, 1975; Beale and Feinstein, 1976; Klein *et al.*, 1981). Restricted hinges may position the Fab arms so as to interfere with C1q binding (Burton, 1985). If this indeed were the case, the length of the entire hinge should correlate with the complement activation. Indeed, human $IgG_2$ and $IgG_4$ and mouse $IgG_1$ have shorter overall hinge lengths than the other human and mouse IgG isotypes, suggesting that overall hinge length can be important in preventing steric hindrance of C1q binding and complement activation. The rabbit IgG breaks this correlation because, in fact, it has the shortest overall hinge length. Therefore, overall hinge length is not sufficient for complement activation. The core hinge is a structural feature whose essential function may be to provide a point of attachment for the two $C_H2$ domains. Note that the lower hinge length is nearly constant. We believe that with normal IgG antibody molecules, the upper hinge is the dominant factor which determines the efficacy with which the antibody molecule and C1q interact.

Our data are consistent with two roles for the hinge being (i) the structural basis for Fab flexibility and C1q binding; and (ii) the maintenance of an appropriate quaternary relationship between antigen-binding and biological effector domains. This does not exclude the possibility that other structural constraints affect these two immunoglobulin functions. Longitudinal interactions between $C_H$ domains, particularly the juxtaposition between the $C_H1$:$C_x$ domain, and $C_H2$ domains, may affect antibody function (Schneider *et al.*, 1988). The role of the hinge as a clasp, fastening the two $C_H2$ domains at their N-termini and imposing an appropriate Fc structure for biological effector functions is an additional structural role associated with the hinge. This notion is consistent with a hingeless molecule being unable to bind and activate C1q. There is data which shows that Dob, a hingeless human $IgG_1$ molecule, does not bind and activate C1q (Klein *et al.*, 1981). In fact, we have confirmed these results using a recombinant hingeless mouse $IgG_{2a}$ molecule (W.P.Schneider and V.T.Oi, unpublished).

We have defined the structural basis for some aspects of antibody function by analyzing segmental flexibility and complement fixation activity in a family of genetically engineered anti-DNS antibodies differing only in their heavy chain constant region. Site-specific manipulation of the hinge should determine the exact structure which provides the basis for the correlation between hinge restriction and segmental motion, as well as the exceptional character of the mouse $IgG_3$ hinge. Likewise, studies of C1 binding, in conjunction with genetic manipulation of regions involved in flexibility, should clarify the significance of the observed correlation between these two important properties of antibody molecules.

## Materials and methods

### *Production of chimeric anti-DNS immunoglobulins*

The expressed $V_H$ and $V_x$ genes from the anti-DNS hybridoma, DNS1, were cloned from phage lambda libraries (Dangl, 1986). Heavy chain vectors utilizing the DNS-$V_H$ gene were constructed and transfected into either a DNS1 heavy chain loss variant cell line or co-transfected with the DNS1 kappa light chain vector into the SP2/0 hybridoma cell line by protoplast fusion (Oi and Morrison, 1986). Supernates of three transfectant cultures from each experiment were assayed for secretion of immunoglobulin by biosynthetic labelling, immunoprecipitation, and one or two-dimensional SDS−PAGE. Proteins were metabolically labelled with [$^{35}$S]methionine and labelled immunoglobulin was immunoprecipitated from culture supernates or whole cell extracts as described by Jones (1980). N-linked glycosylation was analyzed by biosynthetic labelling in the presence or absence of tunicamycin and subsequent immunoprecipitation and gel analysis. The same biosynthetic labelling procedure was used as described by Jones (1980) except that 10 μl of a 1 mg/ml solution of tunicamycin in DMSO was added and the cells incubated at 37°C for 1 h prior to the addition of [$^{35}$S]methionine.

One transfectoma culture which secreted appropriate anti-DNS antibody was chosen for further study. Each culture was subcloned using single-cell deposition by the FACS (Parks *et al.*, 1979) and supernates from these clones screened for anti-DNS antibody secretion using a fluorescence immunoassay. Anti-DNS antibody levels in culture supernates varied between 5 and 10 μg/ml.

For antibody affinity purification, we used the dansyl isomer, 2-dimethylaminoapthyl-5-sulfonamide-ε-lysine which binds to the DNS1 combining site with an affinity $\sim 10^3$ lower than that of dansyl (our unpublished observations). The isomer was coupled to AH-Sepharose 4B (Pharmacia, Piscataway, NJ) and used as the ligand and eluant for affinity purification of the anti-DNS antibodies. Bound hapten was removed from the combining site by extensive dialysis. Hapten removal was verified by fluorescence emission spectroscopy. Purified immunoglobulins were analyzed by size-exclusion chromatography using the FPLC system (Pharmacia) and a 30-cm Superose 6 column, and were >95% pure and free of detectable aggregates.

### *Segmental flexibility*

Corrected DNS fluorescence emission spectra were measured using an SLM model 8000 fluorescence spectrophotometer, with 340 nm excitation (Reidler *et al.*, 1982). All six chimeric isotypes have identical emission spectra (Dangl, 1986). Fluorescence Anisotropy Kinetics were measured as described by Reidler *et al.* (1982).

### *Complement fixation assays*

Complement fixation was measured using a complement consumption assay similar to that described in Oi *et al.* (1984). A 2-ml suspension of 10% sheep red blood cells was washed twice with 20 ml of cold gel−HBS (10 mM Hepes, 150 mM NaCl, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% gelatin pH 7.4) and resuspended in 2 ml cold gel−HBS. Two hundred microcuries of $Na_2[^{51}Cr]O_4$ (Amersham, sp. act. 20 mCi/μ mol) was added and the suspension was incubated for 1 h at 37°C. The cells were again washed twice with 10 ml cold gel−HBS and resuspended in 4 ml of the same buffer. An appropriate titred amount of hemolysin (Colorado Serum Co.) in cold gel−HBS was added and the suspension incubated at 37°C for 30 min and then at 0°C for 30 min.

After washing twice with cold gel−HBS, the cells were resuspended as a 2% suspension. Duplicate 25-μl aliquots of antibody (0.4 mg/ml) were mixed with 25 μl of $DNS_{26}$−BSA at indicated concentrations and 25 μl of complement (previously titred as 2 $CH_{50}$ units) in a 96-well round-bottom microtiter plate. The plate was incubated for 45 min at 37°C. Then, 25 μl of a 2% suspension of $^{51}$Cr-loaded, hemolysin-coated SRBC was added and the plate incubated for an additional 45 min at 37°C. The amount of complement activity in each well was determined by scintillation counting of supernatants after centrifugation of intact cells. Each assay was performed two or three times, with duplicate samples in each assay.

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