Reprinted from Nature, Vol. 307, No. 5946, pp. 136-140, 12 February, 1983

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Correlation between segmental flexibility and effector function of antibodies

V. T. Oi, T. M. Vuong, R. Hardy, J. Reidler, J. Dangl, L. A. Herzenberg & L. Stryer

Department of Structural Biology, Sherman Fairchild Center and the Department of Genetics, Stanford University School of Medicine, Stanford, California 94305, USA

Mouse monoclonal anti-dansyl antibodies with the same antigen-binding sites but different heavy chain constant regions were generated. The extent of segmental flexibility in times of nanoseconds and the capacity to fix complement were greatest for IgG2b, intermediate for IgG2a, and least for IgG1 and IgE. Hence, the effector functions of immunoglobulin isotypes may be controlled in part by the freedom of movement of their Fab arms.

IMMUNOGLOBULIN G is a flexible Y-shaped molecule. The two antigen-binding Fab units of IgG are joined to an Fc unit at a hinge that allows the angle between the Fab parts to vary over a broad angular range, as shown by hydrodynamic[1], electron microscopic[2,3], and X-ray crystallographic studies[4–6]. Nanosecond fluorescence polarization measurements have demonstrated that immunoglobulin molecules exhibit segmental flexibility in the nanosecond time range[7–9]. Segmental flexibility is likely to be important in enabling immunoglobulins to bind optimally to multivalent antigens and to carry out certain effector functions. Studies of polyclonal antibody populations have suggested that immunoglobulin classes differ in their degree of segmental flexibility[10–11]. We have explored the relationship between hinge motion and effector function in specially constructed families of homogeneous immunoglobulins. Mouse monoclonal anti-dansyl antibodies with the same antigen-combining sites but different heavy chain constant regions (Fig. 1) were generated by selecting somatic variants in hybridoma cell lines. The extent of segmental flexibility in times of nanoseconds (measured by fluorescence spectroscopy) and the capacity to fix complement were greatest for IgG2b, intermediate for IgG2a, and least for IgGl and IgE. Hence, the effector functions of immunoglobulin isotypes may be controlled in part by the freedom of movement of their Fab arms.

In most antibody-producing hybridoma cell lines, variant cells arise which produce antibody containing a different heavy chain[12]. For example, cells producing IgG2b sometimes arise from a cell line producing IgGl. The frequency of these heavy-chain switch variants is ordinarily low (10^{-5}–10^{-6} per generation). These rare cells can be separated by fluorescence-activated cell sorting on the basis of the appearance of a different heavy chain on their cell surface[13,14]. In these switch variants, the light chain remains the same, whereas V_H becomes joined to a different C_H. Consequently, the antigen-combining site is the same as in the parent, despite the change in the heavy-chain constant region. Many of these switch variants are stable and can be cloned.

We chose to generate a family of homogeneous mouse anti-dansyl (DNS) antibodies for two reasons. First, the fluorescence emission spectrum of the bound dansyl chromophore is very responsive to the polarity of its environment[15], making it a convenient and sensitive indicator of whether the antigen-combining site of the switch variant antibody is in fact the same as that of the parent line. Second, the bound dansyl chromophore has an excited state lifetime suitable for determining the segmental flexibility of immunoglobulin isotypes by nanosecond fluorescence polarization spectroscopy[16]. Variant hybridoma cell lines producing different immunoglobulin isotypes were selected by using affinity-purified goat and rabbit anti-mouse heavy-chain sera and a panel of hybridoma anti-mouse immunoglobulin allotype antibodies[14]. IgG2b, IgG2a and IgE anti-DNS antibody-producing cell lines were isolated starting with the mouse IgG1 anti-DNS hybridoma cell line DNS1 (Igha allotype, also designated as 27–44). Our previous nanosecond fluorescence study[16] showed that IgGl from this mouse cell line is more rigid than is polyclonal rabbit anti-dansyl antibody. The cell lineage of the DNS1 family is: IgGl (27–44) → IgG2b (27–35) → IgGa (27–13) → IgE (27–74). A cell line containing a different IgGl anti-dansyl combining site, DNS3 (Igh^b allotype, also designated as 44–10), served as the source of another anti-dansyl IgG2b (44–32).

Two-dimensional gel electrophoresis of antibodies[17,18] produced by the DNS1 family shows that the heavy chain synthesized by each member has the electrophoretic mobility expected for γ1, γ2b, γ2a and ϵ chains (Fig. 2). Also, these gels show that the light chains produced by this family are identical. Corresponding results were obtained for the DNS3 family. Heavy chains synthesized by these variant cells in the presence of tunicamycin, a known inhibitor of asparagine-linked glycosylation[19–21], have a greater electrophoretic mobility than do those

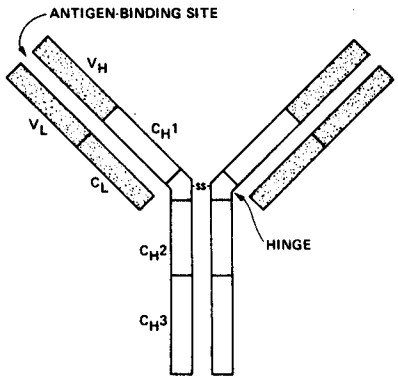

Fig. 1 Schematic diagram of immunoglobulin G. In each family of isotypes, the V_L, C_L and V_H regions are the same. The hinge is located between the C_H1 and C_H2 domains.

Table 1 Mean rotational correlation times of immunoglobulin isotypes and their Fab fragments

	⟨φ⟩ (ns) No DTT	⟨φ⟩ (ns) With DTT
DNS1 family		
Intact immunoglobulin		
IgE	124	88
IgG1	81	58
IgG2a	60	51
IgG2b	47	42
Fab fragment		
IgG1	28	28
IgG2a	28	28
IgG2b	29	29
DNS3 family		
Intact immunoglobulin		
IgG1	93	55
IgG2b	46	45

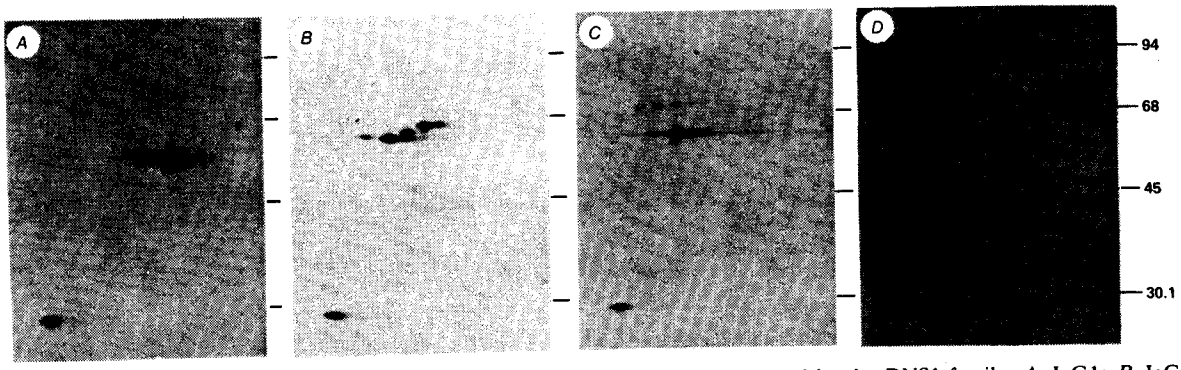

Fig. 2 Two-dimensional gel electrophoresis autoradiogram patterns of antibodies produced by the DNS1 family. *A*, IgG1; *B*, IgG2b; *C*, IgG2a; and *D*, IgE. The horizontal direction corresponds to a non-equilibrium *p*H gradient (low *p*I at the left) and the vertical direction corresponds to sodium dodecyl sulphate polyacrylamide gel electrophoresis[17,18]. Antibodies were labelled biosynthetically with ^{35}S-methionine. Secreted anti-dansyl antibodies in the culture supernatants were precipitated with monoclonal antibodies specific for γ1 (*A*), γ2b (*B*), γ2a (*C*) and a rabbit anti-mouse IgE (*D*). The spot in the lower left-hand corner appears in the same position in each autoradiogram, providing strong confirmation that the κ light chain is the same in each. The multiple spots in the upper half of each gel are typical for myeloma and hybridoma heavy chains. The heterogeneity probably arises from differences in deamidation and glycosylation.

synthesized in its absence. This finding suggests that each variant isotype is normally glycosylated in the absence of tunicamycin. The fluorescence emission spectra of ε-dansyl-L-lysine bound to switch variants of the DNS1 cell line are identical to that of the parent cell antibody (Fig. 3), strongly suggesting that the DNS1 family has the same antigen-combining site. Likewise, both DNS3 isotypes have identical dansyl fluorescence emission spectra. Also, the dissociation constant of the antibody-hapten complex is the same within the DNS family ($K_d = 17 \pm 1$ nM) and the DNS3 family ($K_d = 3 \pm 1$ nM).

Segmental flexibility

The segmental flexibility of these anti-dansyl immunoglobulins was determined by measuring their fluorescence polarization kinetics in the nanosecond time range[7,16,22]. A solution of immunoglobulin containing bound ε-dansyl-L-lysine is excited by a picosecond pulse of vertically-polarized light. Photoselection produces an ensemble of excited choromophores preferentially aligned in the vertical direction. The orientations of these excited molecules then become randomized by rotational brownian motion. The rate of randomization depends on the size and shape of the immunoglobulin, and also on its modes of flexibility. These motions can be monitored by measuring the intensities of the vertically-polarized [$y(t)$] and horizontally-polarized [$x(t)$] components of the fluorescence emission as a function of time following the excitation pulse. The most informative parameter is the emission anisotropy $A(t)$, which is defined as

$$A(t) = \frac{y(t) - x(t)}{y(t) + 2x(t)}$$

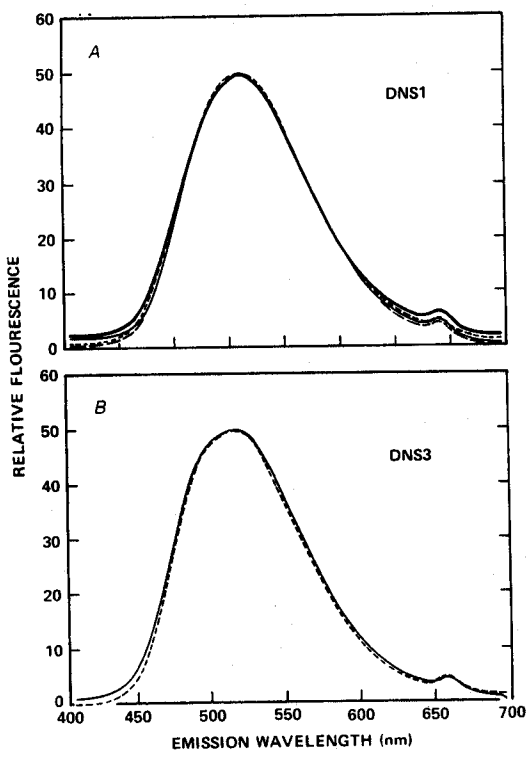

Fig. 3 Fluorescence emission spectra of ε-dansyl-L-lysine bound to isotypes of the DNS1 (*A*) and DNS3 (*B*) families. The spectra of isotypes within a family are virtually superposable. The excitation wavelength was 340 nm.

The emission anisotropy kinetics of a chromophore rotating in common with a rigid sphere is given by $A(t) = A_0 \exp(-t/\phi)$, where A_0 is the initial value of the emission anisotropy and ϕ is the rotational correlation time. For a nonspherical particle, $A(t)$ is more complex, consisting of up to five exponential terms. For a rigid ellipsoid, $A(t)$ is a sum of three exponential decays that depend on the volume and axial ratio of the ellipsoid and on the directions of the transition moment of the emitting chromophore. A rigorous theory for the emission anisotropy kinetics of rigid and segmentally flexible Y-shaped molecules has not yet been developed. Emission anisotropy data for immunoglobulins have instead been interpreted phenomenologically by comparing observed $A(t)$ curves with those calculated for rigid spheres and ellipsoids. In this regard, the mean rotational correlation time $\langle\phi\rangle$ can be informative. $A(t)$ is fitted to a sum of two exponential decays, $a_1 \exp(-t/\phi_1) + a_2 \exp(-t/\phi_2)$, to give $\langle\phi\rangle$, which is equal to $(a_1\phi_1 + a_2\phi_2)/(a_1 + a_2)$. Rigid nonspherical particles have a $\langle\phi\rangle$ greater than ϕ for a rigid sphere of the same volume. Consequently, a particle, irrespective of shape, must be flexible if its $\langle\phi\rangle$ is less than ϕ_{sphere}.

Emission anisotropy kinetics were measured using a mode-locked argon-ion laser and synchronously pumped rhodamine 6G dye laser as the excitation source and a single-photon counting detection system, as described previously[16]. Picosecond pulses at 325 nm were produced by doubling the frequency of the 650 nm output of the dye laser. All fluorescence studies were carried out at 20 °C. $A(t)$ curves for the DNS1 family are shown in Fig. 4*A*. The striking finding is that the IgG1, IgG2a and IgG2b isotypes of this family display markedly different $A(t)$ curves. Their $\langle\phi\rangle$ values are 81, 60 and 47 ns, respectively. These immunoglobulins have virtually identical molecular weights and sedimentation coefficients (6.8, 6.67, and 6.70, respectively; V. Schumaker and M. Phillips, personal communication), which means that their molecular volumes and time-averaged shapes

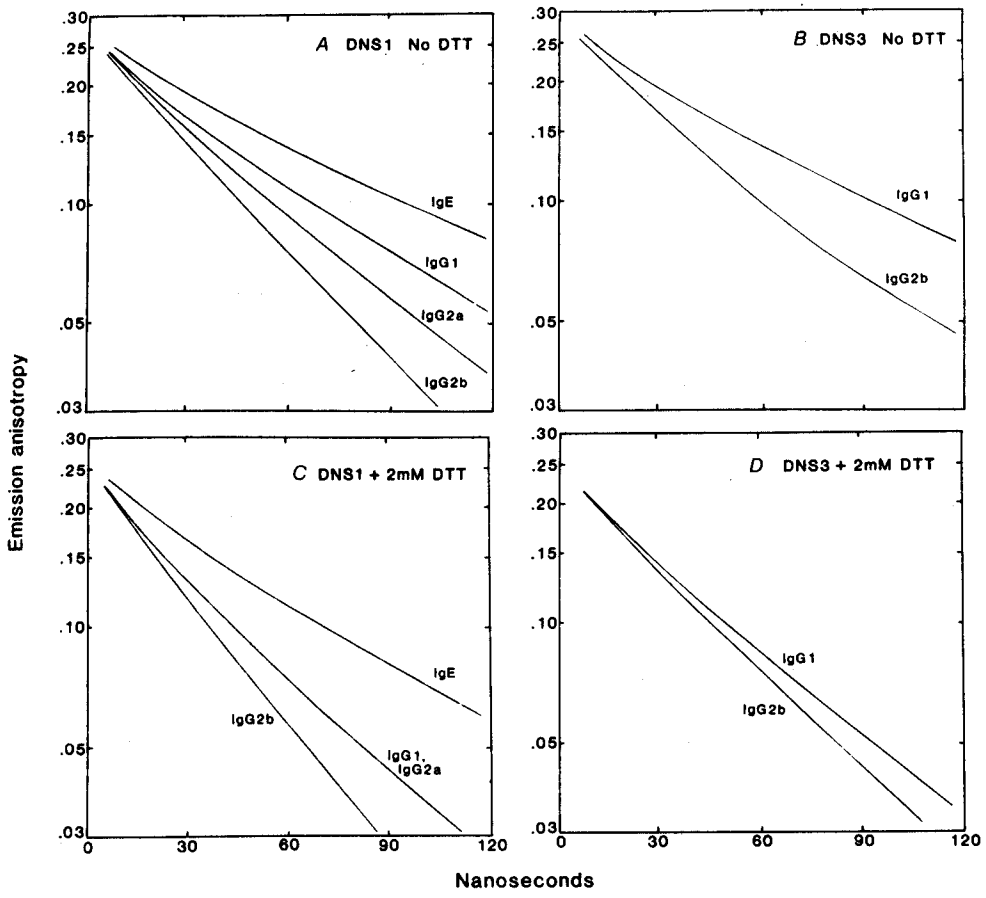

Fig. 4 Nanosecond emission anisotropy kinetics of ε-dansyl-L-lysine bound to immunoglobulins of the DNS1 family in the absence (*A*) and presence (*B*) of 2 mM DTT; and the DNS3 family in the absence (*C*) and presence (*D*) of 2 mM DTT. The temperature was 20 °C.

are nearly the same. Furthermore, the Fab fragments obtained from these isotypes have nearly the same $\langle\phi\rangle$ values (Table 1). The orientation of the bound dansyl chromophore with respect to the long axis of the Fab unit is identical or nearly so in these isotypes because they possess identical antigen-combining sites. Thus, the large difference in the $A(t)$ curves (Fig. 4*A*) are almost certainly due to different extents of segmental flexibility in the intact immunoglobulins. IgGl is the most rigid, IgG2a is intermediate, and IgG2b is the most flexible of the three isotypes.

IgE is like IgGl in being more rigid than IgG2a and IgG2b (Fig. 4*A*). The $\langle\phi\rangle$ value for IgE can be compared with those of the three IgG isotypes by scaling its $\langle\phi\rangle$ to take into account its 190,000 molecular weight[23] compared with 150,000 for IgG. The scaled $\langle\phi\rangle$ value for IgE is $124\times(150/190)=98$ ns, which is even larger than the 81 ns value for IgGl. The sedimentation coefficient of 8.35 S for this IgE (V. Schumaker and M. Phillips, personal communication) corresponds to 6.59 S when scaled for the molecular weight difference between IgE and IgG. This is consistent with the time-averaged shape (as expressed by the translational diffusion coefficient) of IgE being very similar to that of the three IgG isotypes.

Reduction of the disulphide bonds between the heavy chains in the vicinity of the hinge between the Fab and Fc units[16,24] provided additional information concerning the degree of segmental flexibility of the isotypes. The addition of 2 mM dithiothreitol (DTT) leads to a large decrease in $\langle\phi\rangle$ for IgE and IgG1 (Fig. 4*B*, Table 1). Reduction of the interchain disulphides lowers $\langle\phi\rangle$ of IgE from 124 to 88 ns, and that of IgG1 from 81 to 58 ns. DTT has no effect on $\langle\phi\rangle$ for the Fab fragment of IgG1. Thus, DTT lowers $\langle\phi\rangle$ of intact IgG1 by opening up a restricted hinge region. In contrast, DTT has almost no effect on $\langle\phi\rangle$ of IgG2a and IgG2b because the Fab units of these antibody molecules have a high degree of rotational freedom even before reduction of the interchain disulphides.

A large difference in segmental flexibility was also observed within the DNS3 family (Fig. 4*C* and Table 1). IgG2b is again much more flexible than IgG1, as evidenced by their $A(t)$ curves and $\langle\phi\rangle$ values. Also, DTT markedly decreases $\langle\phi\rangle$ of IgG1 and has little effect on $\langle\phi\rangle$ of the more flexible IgG2b (Fig. 4*D* and Table 1).

Complement-fixation effectiveness

The ability of these antibody molecules to fix complement was measured using a micro-complement consumption assay[25]. Dansylated bovine serum albumin served as the antigen. In the DNS1 family, IgG2b is most effective in fixing complement, and IgG2a is also highly effective (Fig. 5*A*). In constrast, IgG1 fixes less complement and required ten-fold more antigen to trigger complement fixation. IgE exhibits negligible complement-fixation activity. Likewise, in the DNS3 family, IgG2b fixes more complement than does IgG1 and does so at a lower concentration of antigen (Fig. 5*B*). The isotypes that fix complement most effectively, IgG2a and IgG2b, also exhibit the highest degree of segmental flexibility.

Two families of immunoglobulin isotypes with identical antigen-binding sites were constructed using somatic cell genetics techniques. Nanosecond fluorescence polarization studies of these monoclonal anti-dansyl isotypes have revealed striking differences in their extents of segmental flexibility. IgG2b is the most flexible, IgG2a is less flexible, and IgG1 and IgE are relatively rigid. The degree of molecular motion at the hinge in times of nanoseconds is correlated with the ability of these molecules to fix complement. This correlation may be more than fortuitous. Segmental flexibility may promote complement fixation by enhancing multivalent binding, facilitating the transmission of conformational changes from the Fab units to the effector domain on the Fc unit, or enabling the Fc unit to assume a conformation favourable for complement fixation. It will be very interesting to further explore the relationship between flexibility and effector function by engineering a series of immunoglobulins in which the hinge is deleted, elongated, or modified to alter the rotational mobility of the Fab arms. For

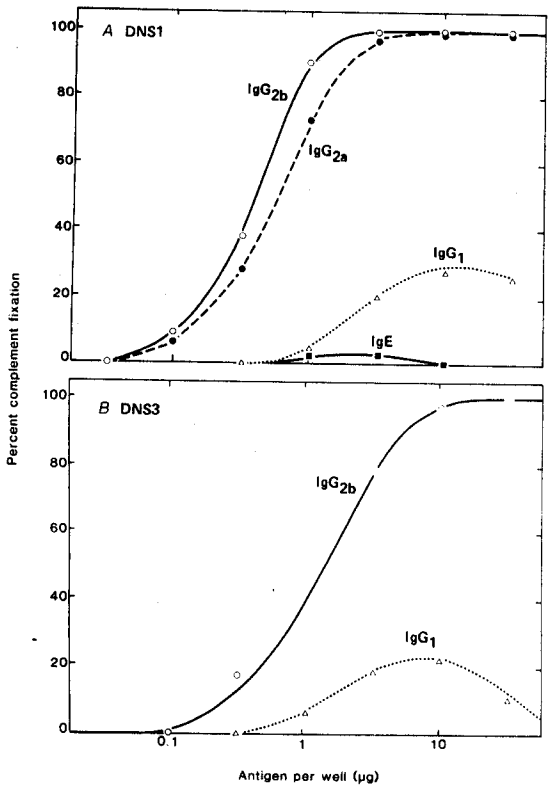

Fig. 5 Complement-fixation by the DNS1 (*A*) and DNS3 (*B*) family. A microcomplement consumption assay using ^{51}Cr release from haemolysin-treated sheep erythrocytes similar to that described previously[25] was used to measure complement fixation by the DNS1 and DNS3 family. Immunoglobulin (10 μg per 25 μl), DNS-bovine serum albumin dilutions (in 25 μl) and complement (2 CH-50 units in 25 μl) were combined in wells of a 96 well microtitre plate and incubated for 45 min at 37 °C. Next, 50 μl of a 2% solution of activated, ^{51}Cr-loaded erythrocytes was added to each well and after a further 45 min incubation at 37 °C the non-lysed cells were pelleted by centrifugation of the plates. 50-μl aliquots of supernatant were collected from each well and counted on a gamma counter set for ^{51}Cr.

example, it would be instructive to study a chimaeric immunoglobulin containing an IgG1 hinge and IgG2b C_H2 and C_H3 domains. We predict that such an immunoglobulin would fix complement less effectively than a normal IgG2b molecule. Segmental flexibility is likely to be one factor correlated with complement fixation. The construction of immunoglobulin genes with novel combinations of domains or with site-specific mutations and their subsequent insertion into lymphoid cell lines should lead to deeper insights into how immunoglobulins mediate their distinctive functions. Methods for expressing designed immunoglobulins have recently been developed[26,27].

Monoclonal antibodies directed against tumour-specific antigens are now being used to treat certain malignancies[28]. It will be important to learn whether the therapeutic effectiveness of a tumour-specific antibody depends on its isotype as well as on its antigen-binding properties. The capacity of such antibodies to interact optimally with tumour-specific antigens on cell surfaces may be influenced by their segmental flexibility.

We thank Tammy Guion for technical assistance and Drs Verne Schumaker and Juan Yguerabide for discussions. This research was supported by research grants from NIH (GM-24032 to L.S. and CA-04681, AI08917, and GM-17367 to L.A.H.). Fellowship support was provided by NIH (GM-07276 to T.M.V. and AI-06144 to V.T.O.), the Cystic Fibrosis Foundation (to J.R.) and the California Division of the American Cancer Society (J-22-82 to R.R.H.).

Received 22 August; accepted 5 October 1983.

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