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Rotational Dynamics of Monoclonal Anti-dansyl Immunoglobulins

The rotational motions of monoclonal mouse anti-dansyl immunoglobulins were studied by nanosecond fluorescence emission anisotropic spectroscopy using a mode-locked argon-ion laser as the pulsed excitation source. Three homogeneous antibodies of the immunoglobulin G1 (IgG1) subclass containing different V regions were prepared. The fluorescence emission maxima of these antibodies (designated as DNS1, DNS2 and DNS3) are at 515, 480 and 500 nm, respectively. Their mean rotational correlation times, $\langle\phi\rangle$, are 84, 109 and 96 ns, respectively. The binding of protein A or a monoclonal anti-allotype antibody to the F_c unit of DNS1 increased $\langle\phi\rangle$ to 142 and 150 ns, respectively, whereas reduction of the disulfide bond between the heavy chains decreased $\langle\phi\rangle$ to 48 ns. These nanosecond measurements show that the rotational motion of the F_{ab} arms in mouse IgG1 is restricted.

Hydrodynamic and electron microscopic studies led to the proposal that immunoglobulin G (IgG) is a Y-shaped flexible molecule and that antigen binds near the ends of the F_{ab} segments (Noelken *et al.*, 1965; Feinstein & Rowe, 1965; Valentine & Green, 1967). According to this model, the F_{ab} and F_c segments of the antibody molecule are joined by a hinge that allows the angle between the F_{ab} parts to range from nearly 0 to 180 degrees. X-ray crystallographic analyses have confirmed some key structural aspects of this model (Silverton *et al.*, 1977; Matsushima *et al.*, 1978; Amzel & Poljak, 1979). Moreover, nanosecond fluorescence emission anisotropy measurements have shown that IgG exhibits segmental flexibility in the nanosecond time range (Yguerabide *et al.*, 1970). The rotational dynamics of a wide variety of immunoglobulins have subsequently been investigated by this technique (Cathou, 1978). A recent analysis suggests that the angular range of motion of the F_{ab} arms of IgG is even larger than previously inferred (Hanson *et al.*, 1981). Most previous studies of segmental flexibility were carried out with heterogeneous antibodies. The discovery that somatic cell hybridization can be used to produce monoclonal immunoglobulins of a defined specificity (Köhler & Milstein, 1975) stimulated us to explore antibody dynamics further by nanosecond fluorescence spectroscopy. In particular, we were attracted by the prospect of examining the segmental flexibility of homogeneous immunoglobulins belonging to a single class or subclass. We report here nanosecond fluorescence studies of the rotational motions of monoclonal anti-dansyl antibodies of the IgG1 subclass.

C3H.SW and BAB/14 mice were immunized by intraperitoneal injection of 100 μg of dansyl-keyhole limpet hemocyanin (DNS-KLH) on alum with 2×10^8 heat-killed *Bordetella pertusis* organisms. Aqueous DNS-KLH (10 μg) was injected intraperitoneally seven days later to boost the antibody response. Immune spleen cells from these mice were hybridized with NS-1 myeloma cells three days after the booster dose. Somatic cell hybridization and selection were carried out as described

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previously (Köhler & Milstein, 1975; Oi & Herzenberg, 1979). Anti-dansyl-producing hybridoma cell lines were screened by solid-phase radioimmunoassay using dansyl-ovalbumin as the target antigen. Culture supernatants containing anti-dansyl antibody were detected with radioiodinated anti-mouse immunoglobulin allotype hybridoma antibodies. Hybridoma cell lines found to secrete anti-dansyl antibodies were cloned using the fluorescence-activated cell sorter (Parks *et al.*, 1979). (C3H.SW × Balb/c)F_1 or BAB/14 mice were injected with 10^6 anti-dansyl-producing hybridoma cells to grow subcutaneous tumors in order to produce large quantities of antibody. Sera containing 5 to 10 mg homogeneous anti-dansyl antibody/ml were collected and stored at −70°C.

Antibodies were purified by gel filtration on either AcA34 or AcA22 (LKB) in 50 mM-Tris·HCl, 0·15 M-NaCl (pH 8·1). Fractions containing immunoglobulin were loaded on a QAE-Sephadex G-50 column equilibrated with the same buffer. Antibody eluted by a linear 0·15 M to 0·5 M-NaCl gradient was more than 95% homogeneous. The immunoglobulin isotype of each anti-dansyl antibody was established with hybridoma anti-allotype antibodies. The immunoglobulin chain composition of each anti-dansyl antibody was determined by two-dimensional gel electrophoresis to determine whether the immunoglobulin light chain of the parental myeloma cell line, NS-1, was being synthesized in addition to the anti-dansyl antibody heavy and light chains of the immune spleen cell.

Three anti-dansyl clones were selected and grown. The anti-dansyl immunoglobulins produced by these mouse cell lines will be designated as DNS1, DNS2 and DNS3. All three hybridoma cell lines produced one heavy and one light chain of immune spleen cell origin; the NS-1 light chain was not synthesized by these cells. Thus, each antibody studied was a homogeneous anti-dansyl immunoglobulin. Analysis using anti-isotype antibodies showed that all three are of the IgG1 subclass. Their affinities for dansyl lysine were higher than $10^8\ M^{-1}$. As previously observed with heterogeneous anti-dansyl antibodies, the fluorescence quantum yield of ε-dansyl-L-lysine increased markedly on binding to these monoclonal immunoglobulins and the emission maximum of the bound hapten was shifted to shorter wavelengths. The quantum yields of the bound hapten were 0·60, 0·54 and 0·70 and the wavelengths of the emission maximum were 515, 480 and 500 nm for DNS1, DNS2 and DNS3, respectively (Fig. 1). The differing emission spectra of these antibodies reflect variations in the dynamic polarity of their combining sites. It is evident that these three antibody molecules have different V regions. The allotype analysis showed that the C region of DNS1 is different from that of DNS2 and DNS3. DNS1 is of C3H.SW origin and hence expresses the Igh-4a allotype, whereas DNS2 and DNS3 are of BAB/14 origin and therefore expresses the Igh-4b allotype.

A mode-locked argon-ion laser (Spectra–Physics, model 171/342) served as the excitation source. Green (514·5 nm) argon-ion pulses (700 mW average power) synchronously pumped a rhodamine 6G dye laser (Spectra–Physics, model 375/376B) to produce 15-picosecond pulses at 650 nm. The repetition rate was decreased from 81·9 to 0·82 MHz by a cavity dumper (Spectra–Physics, model 344) in the dye laser. Focused pulses were frequency-doubled to 325 nm by a KDP doubler (Inrad no. 531-120, type C). The 325 nm pulses were separated from the

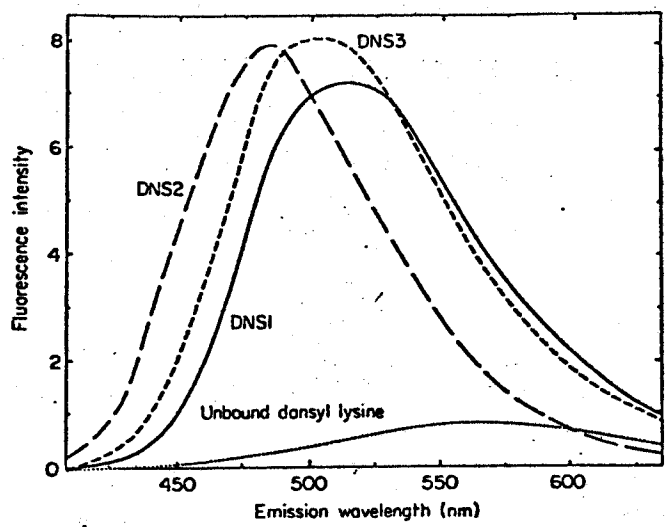

FIG. 1. Fluorescence emission spectra of unbound dansyl lysine and of dansyl lysine bound to DNS1, DNS2 and DNS3. The excitation wavelength was 340 nm.

650 nm pulses by a quartz prism and then directed into the sample compartment. A portion of the red beam triggered a photodiode (FND no. 100) to signal the arrival of a light pulse. A single-photon counting apparatus that has been described previously was used to measure the nanosecond emission kinetics (Yguerabide, 1972; Munro *et al.*, 1979). Anisotropy kinetics with a good signal-to-noise ratio to five excited-state lifetimes (~120 ns) were obtained in about 20 minutes. The decay curves contained at least 3×10^5 counts in the peak channel. The full width at half maximum of the instrument response function, determined by using a Ludox solution as a light scatter, was 600 picoseconds. The finite duration of the light pulse was taken into account by a convolution analysis. A non-linear least-squares algorithm (Marquardt, 1963; Bevington, 1969) was used to fit the data to the sum of two exponential decays (Vuong & Stryer, unpublished data).

The nanosecond fluorescence and emission anisotropy kinetics of dansyl lysine bound to DNS1 are shown in Figure 2. The excited-state lifetime of the bound hapten is 25·1 nanoseconds. The emission anisotropy curve of DNS1 can be fitted to the sum of two exponentials with: $a_1 = 0{\cdot}062$, $\phi_1 = 23$ nanoseconds, $a_2 = 0{\cdot}248$, $\phi_2 = 99$ nanoseconds. However, this fit is by no means unique. Nearly identical $A(t)$ curves, where $A(t) = a_1 \exp(-\phi_1/t) + a_2 \exp(-\phi_2/t)$, can be produced by a range of values of these parameters. In contrast, the mean rotational correlation time $\langle\phi\rangle$, given by $(a_1\phi_1 + a_2\phi_2)/(a_1 + a_2)$, is quite well-defined. For DNS1, $\langle\phi\rangle$ is 84 nanoseconds at 20°C. A 150,000 molecular weight rigid sphere in water at 20°C, would have a ϕ value of 60 nanoseconds, assuming a hydration of 0·3 cm^3/g. The rotational correlation times of a rigid Y-shaped molecule of the size of IgG have not yet been calculated in a rigorous fashion. Thus, the observed $\langle\phi\rangle$ value of 84 nanoseconds does not in itself reveal whether this immunoglobulin has modes of flexibility in times of nanoseconds.

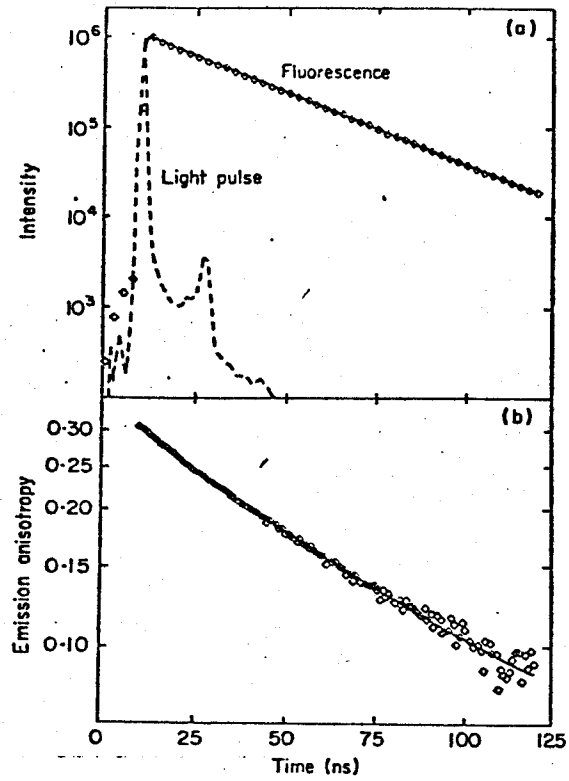

FIG. 2. (a) Nanosecond fluorescence kinetics and (b) emission anisotropy kinetics of DNS1 at 20°C. The unbroken line is the least-squares fit of the observed emission anisotropy data to the sum of 2 exponential decays.

Additional experiments were therefore carried out to gain an under-standing of the dynamics of this molecule. The binding of a large protein (an "anchor") to F_c would markedly increase the rotational correlation time of a probe bound to F_{ab} if the immunoglobulin were rigid. In contrast, there would be little effect on $\langle\phi\rangle$ if the F_{ab} units rotated independently of the whole immunoglobulin. It is assumed that the anchor does not affect the hinge itself. As shown in Figure 3, the binding of a monoclonal IgG2a anti-allotype antibody (Oi & Herzenberg, 1979) specific for a site on the F_c unit of DNS1 increased $\langle\phi\rangle$ from 84 to 150 nanoseconds. The concentration of DNS1 was $1{\cdot}9\times10^{-7}$ M and much of anti-allotype antibody was $1{\cdot}6\times10^{-6}$ M. Likewise, protein A from *Staphylococcus aureus*, which is known to bind to the F_c unit (Forsgren & Sjöquist, 1966; Lancet *et al.*, 1978; Mota *et al.*, 1981; Deisenhofer, 1981), increased $\langle\phi\rangle$ to 142 nanoseconds. The concentration of DNS1 was $1{\cdot}9\times10^{-7}$ M and that of protein A was $2{\cdot}5\times10^{-6}$ M. No turbidity was evident in these anchoring experiments. Also, higher concentrations of either anchor protein did not elicit any additional change in $\langle\phi\rangle$. These experiments showed that the rotational motion of the hapten at the tip of the F_{ab} unit was highly responsive to the anchoring of F_c.

Reduction of the disulfide bond between the heavy chains in the vicinity of the

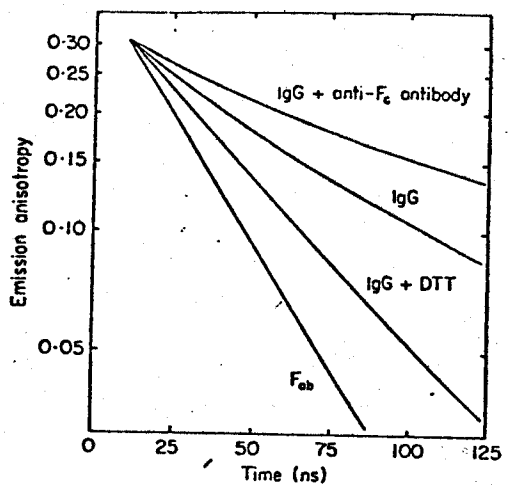

FIG. 3. Nanosecond emission kinetics of dansyl lysine bound to: (a) a complex of DNS1 with a monoclonal anti-F_c allotype IgG; (b) DNS1; (c) DNS1 reduced with 2 mM-dithiothreitol (DTT); and (d) the F_{ab} fragment of DNS1; at 20°C.

hinge region between the F_{ab} and F_c units afforded another means of assessing the significance of $\langle\phi\rangle$ of intact DNS1. As previously observed for heterogeneous rabbit anti-dansyl IgG (Chan & Cathou, 1977), the emission anisotropy of DNS1 decays much more rapidly following reduction with 2 mM-dithiothreitol (Fig. 2). The value of $\langle\phi\rangle$ for DNS1 changes from 84 to 48 nanoseconds on reduction, whereas the ϕ value of the F_{ab} fragment is unaltered. In fact, the emission anisotropy decay of reduced DNS1 resembles that of the F_{ab} fragment of this immunoglobulin (Fig. 2), which exhibits a single ϕ value of 32 nanoseconds. This large effect of reducing the disulfide bond in the vicinity of the hinge of DNS1 suggests that the F_{ab} units of this immunoglobulin do not rotate independently of the whole molecule.

Further support for this conclusion comes from a comparison of the observed $\langle\phi\rangle$ value of DNS1 with the value calculated for a highly flexible Y-shaped molecule. Expressions for the end-over-end rotational correlation time, ϕ_e, of a prolate ellipsoidal unit attached by a universal joint to two identical segments have recently been derived (Wegener *et al.*, 1980; Wegener, 1982). In such a molecule, an ellipsoid with an axial ratio of 2·5 would have a ϕ_e value 1·78 times as large as that of the completely free unit. This model predicts that the rotational correlation time of DNS1 would be $32 \times 1{\cdot}78$ or 57 nanoseconds if IgG1 were freely jointed at the junction of the F_{ab} and F_c units. The observed $\langle\phi\rangle$ value of 84 nanoseconds is substantially larger than this calculated value. The effect on $\langle\phi\rangle$ of anchoring the F_c unit of a flexibly jointed Y-shaped molecule can also be calculated. The ϕ_e value of a molecule containing an immobile F_c unit would be $32 \times 2{\cdot}5$ or 80 nanoseconds if its F_{ab} units were joined to F_c by universal joints. The observed $\langle\phi\rangle$ value of 142 nanoseconds for the protein A–DNS1 complex and of 150 nanoseconds for the complex of DNS1 with an anti-F_c antibody are much larger than 80 nanoseconds, showing that the F_{ab} units of DNS1 do not rotate independently of the rest of the immunoglobulin.

The dependence of the emission anisotropy kinetics on temperature is also

TABLE 1

Rotational correlation times of monoclonal anti-dansyl immunoglobulins

	Mouse immunoglobulins		
	DNS1	DNS2	DNS3
Subclass Allotype	IgG1 a	IgG1 b	IgG1 b
$\langle\phi\rangle$ (ns) of intact IgG			
at 8°C	123	160	—
20°C	84	109	96
37°C	55	74	—
ϕ (ns) of F_{ab}			
at 8°C	45	57	—
20°C	32	37	—
37°C	21	29	—
$\langle\phi\rangle$ (ns) at 20°C			
Intact IgG	84	109	96
Intact IgG + protein A	142	148	—
Intact IgG + anti-F_c IgG	150	—	—
Intact IgG + dithiothreitol	48	77	44

informative (Table 1). For DNS1 and its F_{ab} fragment, $\langle\phi\rangle$ is proportional to η/T (where η is the viscosity and T is the absolute temperature) in going from 8 to 20 to 37°C. The ratio of $\langle\phi\rangle$ to the value calculated for a rigid, hydrated sphere at these temperatures is 1·42, 1·40 and 1·40, respectively. Thus, the degree of flexibility of the F_{ab} units of DNS1 does not change appreciably with temperature in this range.

The emission anisotropy decay of DNS3 was similar to that of DNS1 (Table 1). Reduction of DNS3 with dithiothreitol also led to a large decrease in $\langle\phi\rangle$. DNS2 exhibited a $\langle\phi\rangle$ value of 109 nanoseconds at 20°C, compared with 84 and 96 nanoseconds for DNS1 and DNS3, respectively. Reduction of the inter-heavy chain disulfide of DNS2 had less effect on $\langle\phi\rangle$ than did reduction of DNS1 and DNS3. Another difference is that the F_{ab} fragment of DNS2 exhibited a longer ϕ value than did the F_{ab} fragment of DNS1. These differences in the emission anisotropy kinetics could arise from differences in the orientation of the dansyl transition moment with respect to the long axis of the F_{ab} unit in these immunoglobulins. Specifically, the dansyl transition moment might be more aligned along the long F_{ab} axis in DNS2 than in DNS1 and DNS3.

These nanosecond emission anisotropy measurements suggest that the F_{ab} units of mouse IgG1 do not rotate entirely independently of the F_c unit in times of nanoseconds. Rather, the segmental motions of the F_{ab} arms are restricted. Calculations of the rotational correlation times of rigid and partially flexible Y-shaped molecules, using recently developed theories (Garcia de la Torre & Bloomfield, 1981), are needed to determine the actual angular range of motion of the F_{ab} arms. These calculations will be especially interesting in view of our recent finding that the degree of segmental flexibility of anti-dansyl immunoglobulins

containing identical V regions is highly dependent on the nature of the constant region of the heavy chain (Oi *et al.*, 1981).

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REFERENCES

Amzel, L. M. & Poljak, R. J. (1979). *Annu. Rev. Biochem.* 48, 961–967.
Bevington, P. R. (1969). In *Data Reduction and Error Analysis for the Physical Sciences*, pp. 235–240, McGraw-Hill, New York.
Cathou, R. E. (1978). *Compr. Immunol.* 5, 37–83.
Chan, L. M. & Cathou, R. E. (1977). *J. Mol. Biol.* 112, 653–656.
Deisenhofer, J. (1981). *Biochemistry*, 20, 2361–2370.
Feinstein, A. & Rowe, A. J. (1965). *Nature (London)*, 205, 147–149.
Forsgren, A. & Sjöquist, J. (1966). *J. Immunol.* 97, 822–827.
Garcia de la Torre, J. & Bloomfield, V. (1981). *Quart. Rev. Biophys.* 14, 81–139.
Hanson, D. C., Yguerabide, J. & Schumaker, V. N. (1981). *Biochemistry*. 20, 6842–6852.
Köhler, G. & Milstein, C. (1975). *Nature (London)*, 256, 495–497.
Lancet, D., Isenman, D., Sjödahl, J., Sjöquist, J. & Pecht, I. (1978). *Biochem. Biophys. Res. Commun.* 85, 608–614.
Marquardt, D. W. (1963). *J. Soc. Ind. Appl. Math.* 11, 431–441.
Matsushima, M., Marquart, M., Jones, T. A., Colman, P. M., Bartels, K., Huber, R. & Palm, W. (1978). *J. Mol. Biol.* 121, 441–459.
Mota, G., Moraru, I., Sjöquist, J. & Ghetie, V. (1981). *Mol. Immunol.* 18, 373–378.
Munro, I., Pecht, I. & Stryer, L. (1979). *Proc. Nat. Acad. Sci., U.S.A.* 76, 56–60.
Noelken, M. E., Nelson, C. A., Buckley, C. E. & Tanford, C. (1965). *J. Biol. Chem.* 240, 218–224.
Oi, V. T. & Herzenberg, L. A. (1979). *Mol. Immunol.* 16, 1005–1017.
Oi, V. T., Reidler, J., Dangle, J., Vuong, M., Herzenberg, L. A. & Stryer, L. (1981). *J. Cell Biol.* 91, 75a.
Parks, D. R., Bryan, V. M., Oi, V. T. & Herzenberg, L. A. (1979). *Proc. Nat. Acad. Sci., U.S.A.* 76, 1962–1966.
Silverton, E. W., Navia, M. A. & Davies, D. M. (1977). *Proc. Nat. Acad. Sci., U.S.A.* 74, 5140–5144.

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Valentine, R. C. & Green, N. M. (1967). *J. Mol. Biol.* 27, 615–617.
Wegener, W. A. (1982). *Biopolymers*, 21, 1049–1080.
Wegener, W. A., Dowben, R. M. & Koester, V. J. (1980). *J. Chem. Phys.* 73, 4086–4097.
Yguerabide, J. (1972). *Methods Enzymol.* 26C, 498–577.
Yguerabide, J., Epstein, H. F. & Stryer, L. (1970). *J. Mol. Biol.* 51, 573–590.

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