The Use of Gadolinium as a Probe in the Fc Region of a Homogeneous Anti-(Type-III Pneumococcal Polysaccharide) Antibody

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The binding of gadolinium [Gd(III)] to a homogeneous rabbit anti-(type-III pneumococcal polysaccharide) IgG (immunoglobulin G) and its Fab (*N*-terminal half of heavy and light chain) and Fc (*C*-terminal half of heavy-chain dimer) fragments was demonstrated by measurements of solvent-water proton relaxation rates in the appropriate Gd(III) solutions. At pH 5.5 the binding of Gd(III) to the Fc fragment is much tighter (K_D approx. $5\ \mu\text{M}$) than binding to the Fab fragment (K_D approx. $250\ \mu\text{M}$). The binding of Gd(III) to the whole IgG molecule (K_D approx. $4\ \mu\text{M}$) is very similar to that for the Fc fragment alone. This specificity of binding to the Fc region allows the use of Gd(III) as a probe of the Fc conformation. The environment of the Gd(III) in the Fc region of whole IgG is not affected by the presence of octasaccharide derived by hydrolysis of type-III pneumococcal polysaccharide, but the corresponding 28-unit saccharide does cause detectable changes. The addition of 16-unit saccharide to anti-(SIII polysaccharide) IgG in the presence of Gd(III) does not change the solvent water proton relaxation rate, although aggregation does occur. The effects of the 28-unit saccharide may be explained therefore by a change in the tumbling time of the IgG. From a study of the effect of various antigen/antibody ratios, it is concluded that the 28-unit-saccharide-induced changes in the Gd(III) environment in the Fc region are caused by the specific geometrical structure of the antigen–antibody complexes formed, and not simply by occupancy of the combining sites on the antibody.

It has been proposed that antigen-dependent activities of the Fc‡ region of immunoglobulins may be brought about by conformational changes occurring in the Fc region as a result of antigen binding. Very little direct evidence has been obtained for this hypothesis (Metzger, 1974), but two studies have supported the idea of conformational changes in the Fc region. First, Brown & Koshland (1975) showed that ribonuclease monosubstituted by coupling with the diazonium salt of *p*-aminophenyl β -lactoside will activate complement fixation with IgM antibody directed against the phenyl β -lactoside hapten. Secondly, Schlessinger *et al.* (1975) have studied the circular polarization of luminescence emitted by the tryptophan residues of various antibodies. By a comparison of the changes in the circular polarization spectra of IgG and the Fab fragments on addition of antigen, it was found that the intact antibodies showed additional spectral

changes that were attributed to the presence of the Fc region. Schlessinger *et al.* (1975) assumed that the Fc contribution comes directly from a change in the asymmetry of the environment of the chromophores in the Fc region, indicating a conformational change. An objection to this would be that the Fc fragment may be acting indirectly, possibly by imposing some structural constraints on the Fab regions of the intact IgG which are not present in the Fab fragments free in solution.

An alternative hypothesis to account for antigen-induced Fc-fragment activities is that activation depends on the formation of suitable antibody aggregates. This has received strong support from work on bivalent dinitrophenyl haptens with IgG antibody against the dinitrophenyl group (Hyslop *et al.*, 1970) and also on non-specifically aggregated immunoglobulins (Metzger, 1974).

Dower *et al.* (1975) have shown that non-immune rabbit IgG has specific lanthanide-binding sites, and that those sites on the Fc fragment have much higher affinity than those in the Fab-fragment 'arms'. This has prompted us to study the lanthanide-binding properties of a homogeneous anti-(type-III pneumococcal polysaccharide) IgG antibody. If the lanthanides have a higher affinity for Fc than for Fab sites in this system, this would provide a probe of the con-

‡ Abbreviations: Fc, *C*-terminal half of the heavy-chain dimer of IgG (see Scheme 1); IgG, immunoglobulin G; IgM, immunoglobulin M; Fab fragment, *N*-terminal half of the heavy chain and whole of the light chain; SIII polysaccharide, type-III pneumococcal polysaccharide [$\rightarrow 3$] β -D-glucuronic acid(1 $\rightarrow 4$) β -D-glucose(1 $\rightarrow$) $_n$, where for the octasaccharide, $n = 4$, and for the 28-unit saccharide, $n = 14$.

Scheme 1. Structure of IgG and its proteolytic fragments

formation of the Fc region which could be used directly with the whole IgG.

Materials and Methods

Isolation of IgG and fragments

The antibody against type-III pneumococcal polysaccharide, designated 'antibody 45-394', was raised by the method of Kimball *et al.* (1971) and purified by immunoabsorption and preparative agarose-block electrophoresis as previously described (Braun & Jaton, 1973). Fab and Fc fragments were prepared by papain digestion and isolated by ion-exchange chromatography (Porter, 1959). Rabbit non-immune IgG was prepared from pooled non-immune serum (Wilkinson, 1969).

Preparation of oligosaccharides from SIII pneumococcal polysaccharides

The preparation of oligosaccharides by acid hydrolysis of the polysaccharide, followed by gel filtration through columns of Sephadex G-25 and G-100, was described previously (Jaton *et al.*, 1976).

Proton relaxation studies

The preparation of solutions of Gd(III) has been described elsewhere (Dower *et al.*, 1975). To minimize the effect of Gd(III) binding to glassware, disposable plastic tubes and plastic pipettes were used

for all experiments. Proton spin lattice relaxation times were measured at 20 MHz and at $19 \pm 1^\circ\text{C}$ in a spin-echo machine constructed in this laboratory. Experiments were performed at pH 5.5, either in unbuffered solution or in the presence of 50 mM-Pipes [piperazine-*NN'*-bis-(2-ethanesulphonic acid)].

Two different types of experiment were performed to follow the binding of Gd(III) to the proteins, one in which the water proton spin lattice relaxation rate is followed at constant Gd(III) concentration and various protein concentrations (P-titration), and the other at constant protein concentration and various metal concentrations (M-titration). The method of analysis of these titrations has been described elsewhere (Dower *et al.*, 1975) and consists of curve-fitting to the experimental data by using binding curves calculated theoretically by assuming values for the number of gadolinium-ion-binding sites (n), the dissociation constant (K_D), ϵ_b , the enhancement of the water-proton relaxation in the binary metal-protein complex, defined as the ratio of the relaxation rate of the water protons in a solution containing metal ion bound to protein ($1/T_{1B}$) to the relaxation rate in a solution containing the same amount of metal ions free in solution ($1/T_{1F}$).

$$\epsilon_b \equiv \frac{1/T_{1B}}{1/T_{1F}}$$

The enhancement of the paramagnetic contribution to the water-proton relaxation rate by a macromolecule is defined as

$$\epsilon^* = \frac{1/T_{1P} \text{ (in the presence of macromolecule)}}{1/T_{1P} \text{ (in the absence of macromolecule)}}$$

where $1/T_{1P}$ is the paramagnetic contribution to the water proton relaxation rate.

The longitudinal relaxation rate, $1/T_1$, of the solvent-water protons is increased by interaction with paramagnetic ions. This dipole-dipole interaction is characterized by a correlation time τ_c which modulates this interaction. The explicit dependence of $1/T_1$ on τ_c is discussed fully elsewhere (Dwek, 1973), but it is sufficient to note that τ_c contains a contribution from τ_R , the rotational correlation time for the paramagnetic ion complex. The τ_c values at various frequencies are known for the Gd(III) complex with the Fc sites of rabbit non-immune IgG (Burton *et al.*, 1976), and at 20 MHz τ_c is about 500 ps. In this range of τ_c values, it was shown (Dwek, 1973) that an increase in τ_c , which could result, for example, from an increase in τ_R due to aggregate formation, increased the relaxation rate $1/T_1$ of the solvent-water protons.

Results and Discussion

Gadolinium-binding studies

Fc fragment. Water proton spin lattice relaxation rates are shown in Fig. 1 as a function of the con-

Fig. 1. Titration of Gd(III) with Fc fragment of anti-(SIII polysaccharide) IgG by following the paramagnetic contribution to the water proton spin lattice relaxation rate. The continuous curve is calculated theoretically by assuming $n = 2$, $K_D = 5.0$ (μM), $\epsilon_b = 9.5$; $[\text{Gd(III)}] = 20 \mu\text{M}$; 0.15M-NaCl, pH 5.5.

Fig. 3. Titration of Fab fragments of anti-(SIII polysaccharide) IgG with Gd(III) by following the difference between the paramagnetic contribution to the water proton spin lattice relaxation rate in the presence ($1/T_{1P}^*$) and absence ($1/T_{1P}$) of Fab fragments.

The continuous curve is calculated theoretically by assuming $n = 2$, $K_D = 400$ (μM), $\epsilon_b = 7.5$; [Fab fragment] = 48 μM ; 0.15M-NaCl, pH 5.5.

Table 1. Summary of parameters obtained from proton-relaxation studies of Gd(III) binding to IgG and its fragments

Fragment	Binding sites (n)	K_D (μM)	ϵ_b values
Fab	2	$250 \pm 150^*$	$6.5 \pm 1.0^*$
Fc	2	5.0	9.5
IgG	2	$3.75 \pm 1.25^*$	$8.5 \pm 1.0^*$

* These values represent the spread of best-fit curve parameters obtained in several different titrations, and with different protein preparations.

Fig. 2. Titration of Gd(III) with Fab fragment of anti(SIII polysaccharide) IgG by following the paramagnetic contribution to the water proton spin lattice relaxation rate (P-titration).

The continuous curve is calculated theoretically by assuming $n = 2$, $K_D = 400$ (μM), $\epsilon_b = 7.5$; $[\text{Gd(III)}] = 60 \mu\text{M}$; 0.15M-NaCl, pH 5.5.

centration of Fc fragment of anti-(SIII polysaccharide) IgG at a fixed Gd(III) concentration (P-titration). The results were analysed by fitting curves generated for various ϵ_b and K_D values to the experimental points, assuming two equivalent Gd(III)-binding sites per molecule of Fc fragment (Dower *et al.*, 1975). The values of ϵ_b and K_D giving the best-fit line, as judged by eye, are shown in Table 1.

Fab fragment. Experiments performed with Fab fragment of the anti-(SIII polysaccharide) IgG are shown in Fig. 2 (P-titration) and Fig. 3 (M-titration). The average best-fit values of ϵ_b and K_D (obtained with two different preparations) are given in Table 1. These values were obtained by assuming $n = 2$ (by analogy with Dower *et al.*, 1975), since the binding was too weak to allow a direct determination of ϵ_b from the P-titration. An unequivocal determination of n and K_D from the M-titration data requires that the value of ϵ_b is known.

IgG. The results of one P-titration of anti-(SIII polysaccharide) IgG are shown in Fig. 4, and the average best-fit parameters (obtained with two different preparations) are given in Table 1. The parameters obtained are similar to those obtained for the Fc fragment. This is to be expected, since the binding of Gd(III) to the Fc sites is much tighter than binding to the Fab sites.

Effect of oligosaccharides on the water proton relaxation rate in the presence of Gd(III) and antibody

When the total Gd(III) concentration is less than twice the IgG concentration, the Gd(III) will be bound essentially only to the Fc portion of the IgG. This therefore provides a probe of the Fc region.

Fig. 4. Titration of Gd(III) with anti-(SIII polysaccharide) IgG by following the paramagnetic contribution to the water proton spin lattice relaxation rate

The continuous curve is calculated theoretically by assuming $n = 2$, $K_D = 5.0$ (μM), $\epsilon_b = 9.33$; $[\text{Gd(III)}] = 37.5 \mu\text{M}$; 0.15M-NaCl , pH 5.5.

Varying amounts of an IgG–saccharide complex were added to a constant amount of Gd(III) (Fig. 5). The complex was prepared by mixing antibody with either octasaccharide or 28-unit saccharide, so that the saccharide was in threefold molar excess with respect to binding sites on the antibody. The relaxation data for the octasaccharide complex have been fitted assuming that $n = 2$, $K_D = 6.0$ (μM) and $\epsilon_b = 8.5$. These parameters are almost identical with those for IgG alone, and we conclude that the octasaccharide has no effect on the Gd(III) environment in the Fc region of the IgG.

By contrast, addition of the 28-unit saccharide does result in changes in the titration curve (Fig. 5). The difference from the expected value of $1/T_{1P}$ for IgG alone becomes greater as the concentrations of IgG and 28-unit saccharide are increased at a constant antigen/antibody ratio, suggesting that it is caused by the formation of high-molecular-weight complexes. This hypothesis is supported by the time-dependence of the effect, since it is known that formation of these aggregates is time-dependent (for example, precipitin assays normally require 24–72 h at 4°C). Since negligible precipitation (<5%) could be detected in any of the samples, the low values of $1/T_{1P}$ observed at high IgG–28-unit-saccharide complex concentrations cannot be caused by removal of Gd(III) from solution by insoluble complexes.

To rule out the possibility of direct binding of Gd(III) to the 28-unit saccharide or a non-specific interaction of the Fc region with the 28-unit saccharide, a control titration was carried out by adding varying amounts of a mixture of saccharide and

Fig. 5. Titration of Gd(III) with complexes of saccharide and anti-(SIII polysaccharide) IgG (the ratio [antigen]/[binding sites on antibody] = 3:1) by following the paramagnetic contribution to the water proton spin lattice relaxation rate

The upper curve is calculated theoretically by assuming $n = 2$, $K_D = 6.0$ (μM), $\epsilon_b = 8.5$; $[\text{Gd(III)}] = 33.3 \mu\text{M}$, $[\text{NaCl}] = 0.15\text{M}$, pH 5.5; 50mM-Pipes . $\circ$, 28-unit saccharide; the samples were left for 2 h at 20°C after mixing. $\triangle$, 28-unit saccharide; after further storage at 4°C for 50 h. $\square$, 28-unit saccharide; after further storage at 20°C for 10 h. $\bullet$, Octasaccharide; the points shown represent average values for the samples read at various times after mixing, as described for the 28-unit saccharide.

rabbit non-immune IgG in a molar ratio of 6:1 to a constant amount of Gd(III) (Fig. 6). No change in the titration curve was caused by the presence of octasaccharide or 28-unit saccharide from the titration curve for non-immune IgG alone (Dower *et al.*, 1975) even after 72 h standing at 4°C . The effect of the 28-unit saccharide on the relaxation rates in the anti-(SIII polysaccharide)–Gd(III) system must therefore be due to a change in the Gd(III) environment in the Fc region.

The effect of varying the antigen/antibody ratio was examined by adding varying amounts of octasaccharide or 28-unit saccharide to solutions containing a fixed concentration of Gd(III) and anti-(SIII polysaccharide) antibody (Fig. 7). The octasaccharide has little effect on $1/T_{1P}$ at any antigen/antibody ratio, although the value does decrease somewhat from that for IgG and Gd(III) alone as the amount of octasaccharide added is increased. This small effect may be due to a small amount of impurity in the saccharide, or to weak, non-specific octasaccharide–Gd(III) interaction. It may be concluded, therefore, that the octasaccharide does not affect, at any antigen–antibody ratio, the environment of Gd(III) bound to the Fc region.

Fig. 6. Titration of Gd(III) with a mixture of oligosaccharide and rabbit non-immune IgG (6:1 molar ratio) by following the paramagnetic contribution to the water proton spin lattice relaxation rate

The continuous curve is calculated theoretically by assuming $n = 2$, $K_D = 6.0$ (μM), $\epsilon_b = 10$, $[\text{Gd(III)}] = 33.3 \mu\text{M}$, $[\text{NaCl}] = 0.15 \text{ M}$, $\text{pH } 5.5$, 50 mM Pipes . Octasaccharide, 90 min standing at 20°C after mixing ($\circ$) and after a further 72 h at 4°C ($\bullet$). 28-unit saccharide, 90 min standing at 20°C after mixing (Δ) and after a further 72 h at 4°C ($\square$).

The 28-unit saccharide does produce definite effects on the relaxation rates. At low antigen/antibody ratios ($[\text{antigen}] \approx [\text{antibody}]$) a large decrease in $1/T_{1P}$ [with respect to the value in the presence of IgG and Gd(III) alone] is observed. This is consistent with our proposal above that the formation of high-molecular-weight complexes does alter the environment of Gd(III) in the Fc region. Since only 25% of the protein is present as a precipitate (as shown by centrifugation at 10000 rev./min ($r_{av} = 20 \text{ cm}$) for 20 min, where the very large effect on $1/T_{1P}$ is seen), it is unlikely that precipitation alone is responsible for the decrease in $1/T_{1P}$, although detailed interpretation of the data at antigen/antibody ratios near equivalence is difficult.

Over the range of (28-unit saccharide)/(combining site) ratios from 3:1 to 10:1, $1/T_{1P}$ is lower than that for IgG and Gd(III), either in the absence of any saccharide or in the presence of octasaccharide. To investigate this effect further, the time-dependence of $1/T_{1P}$ was followed as a function of antigen/antibody ratio, both with the octasaccharide and the 28-unit saccharide (Fig. 8). Again, the environment of the Gd(III) in the Fc region is not affected by the octasaccharide, but is affected by the 28-unit saccharide over the whole range of antigen/antibody ratios.

It is possible that the observed changes in $1/T_{1P}$ in the presence of 28-unit saccharide do not reflect a

Fig. 7. Titration of a solution containing Gd(III) and anti-(SIII polysaccharide) IgG with oligosaccharides by following the paramagnetic contribution to the water proton spin lattice relaxation rate

Measurements were taken within 90 min of sample preparation. $[\text{Gd(III)}] = 9 \mu\text{M}$; $[\text{anti-(SIII polysaccharide) IgG}] = 12.5 \mu\text{M}$; 0.15 M-NaCl ; $\text{pH } 5.5$. $\bullet$, Octasaccharide; $\circ$, 28-unit saccharide; Δ , 16-unit saccharide.

change in the Gd(III) environment in the Fc region, but are brought about by the appearance of new 'tight' sites in the Fab region in the saccharide-IgG complexes. These new metal-binding sites may then compete with those in the Fc region for Gd(III) binding, which could account for the observed decrease in $1/T_{1P}$. To test this, $1/T_{1P}$ was measured by using mixtures of anti-SIII (Fab'_2) and rabbit non-immune IgG in which Gd(III) was bound to the Fc sites. On addition of 28-unit saccharide, no effect on $1/T_{1P}$ was observed, showing that 28-unit saccharide binding to the Fab region of intact anti-(SIII polysaccharide) IgG causes a direct effect on the Gd(III)-binding site of the Fc region within the same IgG molecule.

To further characterize the dependence of $1/T_{1P}$ on the antigen/antibody ratio, sedimentation-velocity and -equilibrium experiments were carried out by using protein concentrations approximately the same as those in Fig. 8. At a (28-unit saccharide antibody)/(combining site) ratio of 10:1, the IgG is present as monomers (150000 daltons; 30%), monomers/dimers (220000–290000 daltons; 50%) and trimers (440000 daltons; 20%), whereas at a ratio of 3:1 the species present are monomers (20%) and trimers/tetramers (80%).

Fig. 8. Titration of a solution containing Gd(III) and anti-(SIII polysaccharide) IgG with oligosaccharides by following the paramagnetic contribution to the water proton spin lattice relaxation rate

[Gd(III)] = 30.8 μ M; [anti-(SIII polysaccharide) IgG] = 37.0 μ M; 0.15M-NaCl; pH 5.5, 50mM-Pipes. The samples were measured 1 h after mixing (●, octasaccharide; ○, 28-unit saccharide), after 8 h standing at 20°C (▲, octasaccharide; △, 28-unit saccharide), after a further 15 h at 4°C (▽, octasaccharide; ▼, 28-unit saccharide), and after a further 7 h at 20°C (■, octasaccharide; □, 28-unit saccharide). ϵ^* , Enhancement of the paramagnetic contribution to the water proton relaxation rate.

Over the range 3:1–10:1 antigen/(antibody-combining site) ratios with 28-unit saccharide, a decrease in the water proton relaxation rate is observed with respect to that in the absence of antigen (Fig. 8). The fact that this decrease is greater at a 10:1 antigen/antibody ratio (where the complexes are mainly monomers and dimers) than at a 3:1 ratio (mainly trimers and tetramers present) shows that the effect cannot be simply explained by the increased molecular weight of the complex to which the Gd(III) is bound. Further, as already discussed in the Materials and Methods section, an increase in molecular weight in this system would increase τ_r and hence τ_c , resulting in an increase in the relaxation rate of the solvent water protons, whereas a decrease is always observed. This suggests that the changes in relaxation rates do not reflect the changes in rotational correlation times of the different complexes.

Addition of a 16-unit saccharide to anti-(SIII polysaccharide) IgG in the presence of Gd(III) has no effect on the observed $1/T_{1P}$ of the water protons. However, sedimentation studies at an antigen/antibody ratio of 3:1 show the presence of complexes

with sedimentation coefficients of 6.5 to 11.1S (monomers/trimers). This reinforces the conclusion that the effect of the 28-unit saccharide on the solvent water proton relaxation rate cannot be explained simply on the basis of the formation of high-molecular-weight complexes.

It may be concluded that the 28-unit saccharide causes a change in the environment of the Gd(III) bound to the Fc region, which does not occur with the octasaccharide. It is noteworthy that 28-unit saccharide-IgG complexes have been shown to fix complement, whereas the octasaccharide-IgG complexes do not (Jaton *et al.*, 1976). Some conclusions may be drawn regarding the nature of these Gd(III)-environment changes. The data do not support the hypothesis that a conformational change brought about simply by occupancy of the binding site by 28-unit saccharide is being observed, since in this case a dependence of $1/T_{1P}$ on the antigen/antibody ratio would not be expected. The existence of such a dependence shows that the Gd(III) environment in the Fc region is sensitive to the nature of the antigen-antibody complexes.

Two alternative hypotheses could account for this dependency of $1/T_{1P}$ on the nature of the complexes: First, the Gd(III) probe may be detecting a conformational change in the Fc region which is brought about only when the Fab-Fab angle is strained. Alternatively, it is possible that the geometry of complexes of certain sizes allows an Fc-Fc interaction within the complex, resulting in decreased access of bulk water to Gd(III) and so decreasing $1/T_{1P}$. If this latter hypothesis is correct, the Fc-Fc interactions would have to be tight and specific, to account for the very large decreases in $1/T_{1P}$ observed.

We have used electron microscopy to look at the complexes in an attempt to distinguish between these two alternatives of Fab-Fab strain and Fc-Fc interactions, but the resolution has not been sufficiently good to define the structure of the various complexes. However, we conclude that the 28-unit saccharide brings about a change in the Gd(III) environment in the Fc region, which is due to the specific geometrical structure of the complexes.

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