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Complement C1q Binding Affects Spin-labeled Heterosaccharides of Rabbit Antibodies in Immune but Not Artificial Immunoglobulin G Aggregates*

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Jeffrey L. Winkelhake, Akihiro Kusumi‡, Larry McKean, and William J. Mandy

From the Department of Microbiology and National Biomedical ESR Center, Department of Radiology, Medical College of Wisconsin, Milwaukee, Wisconsin 53226 and the Department of Microbiology, University of Texas, Austin, Texas 78712

IgG anti-hapten antibodies were purified from the sera of rabbits homozygous for allotypic determinants *d*11 and *d*12 in the constant region of the heavy chain. Correlative with this determinant is the absence (*d*11) or presence (*d*12) of an oligosaccharide chain just below the hinge region of the IgG molecule. Both *d*11 and *d*12 molecules contain a complex heterosaccharide chain located near the carboxyl terminus of the second constant region domain. The two populations of IgG antibodies were thus selectively labeled with the spin probe Tempamine in their second constant region domains by reductive amination primarily of terminal *N*-acetylneuraminic acid residues. Chemical and enzymatic cleavages showed about 80% of the attached spin labels were *N*-acetylneuraminic acid-associated. Analysis of probe adducts by ESR spectrometry showed the presence of slower and faster moving subcomponents. Formation of immune complexes by antigen induces slight but significant restrictions of spin label mobility for both *d*11 and *d*12 IgG molecules. This restriction is qualitatively different from that seen in glutaraldehyde-, carbodiimide-, or ethanol-induced aggregates of the same IgG antibodies. The addition of purified complement C1 subcomponent C1q to immune aggregates resulted in marked immobilization of spin labels, the rotational correlation time of which was 30–40 μs for both *d*11 and *d*12 molecules (evaluated by saturation transfer spectroscopy). A similar spin probe immobilizing effect is not seen when C1q binds to chemically aggregated IgG antibodies (which also do not activate C1). A novel model is proposed in which C1q is hypothesized to juxtapose Fc moieties in a discrete fashion required for subsequent C1 activation processes mediated by immune complexes.

Despite the remarkable conservation of primary sequence location and concomitant functional implications for immunoglobulin-linked oligosaccharides (1), several recent studies indicate that carbohydrate moieties are not *directly* involved in the familiar antibody functions of antigen binding and C[1] activation by the classical, C1q-mediated pathway (2–4). Heterosaccharide moieties of immunoglobulins studied, however, may be involved in clearance of IgG antibodies from the circulatory system (5) or in interactions with cell surfaces and most likely contribute to physicochemical properties of immunoglobulins such as hydrophobicity, solubility (6), or isoelectric point (especially for the more highly glycosylated forms such as IgM and IgE). Oligosaccharides probably contribute as well to the initial folding of polypeptide chains during protein biosynthesis (7) and to stabilizing protein tertiary structure. As a consequence, immunoglobulin heterosaccharides, especially inner region sugars near the polypeptide backbone of the protein, may participate indirectly in the formation and stabilization of Fc region structures which are bound by the first C component, C1 (3).

The C1 macromolecule is composed of three subcomponents; C1q, C1r, and C1s which complex in a 1:2:2 stoichiometry after C1q binds to IgG in immune complexes (8). While C1q is not hydrolytically altered, the subsequent Ca^{2+}-dependent binding of C1r and C1s is accompanied by hydrolysis of these latter subcomponents to give two disulfide-bonded heavy and a light chains (9, 10). This hydrolysis and appearance of protease catalytic sites converts C1 to its active form, $C\bar{1}$. The process is temperature dependent (37 °C) (11) and can be separated from C1 binding which occurs at 0 °C. It is possible for C1q binding to occur without C1 activation. For example, this will happen if the IgG molecule is chemically altered or cross-linked by glutaraldehyde (12, 13). Such phenomena suggest that physicochemical integrity of the C1q anteceptor (Fc region binding site for the C1q active site) is essential for C1 activation processes. However, details of the mechanisms and requirements for intramolecular processing which occurs during conversion of C1 to $C\bar{1}$ are not known nor are the effector roles of IgG (or C1q) in this multifaceted first step in the classical C pathway.

In the present study, we decided to look more closely at the initial C1q encounter with its anteceptor by taking advantage of the Cγ2 localization of rabbit IgG oligosaccharides to selectively attach spin (mobility) probes. All rabbit IgG molecules have a complex, *N*-glycosidically linked heterosaccharide located near the carboxyl terminus of the Cγ2 domain at residue 297 (Eu human myeloma IgG numbering system). In

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[1] The abbreviations and trivial name used are: C1, the first component of the classical complement (C) pathway; $C\bar{1}$, the activated form of C1; Cγ2, the second about 110-amino acid domain in the constant region of the γ heavy chain of IgG; DTT, dithiothreitol; DNP, dinitrophenyl; NTCB, 2-nitro-5-thiocyanobenzoic acid; Tempamine, 4-amino-2,2,6,6,-tetramethylpiperidin-1-oxyl; BSA, bovine serum albumin; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.

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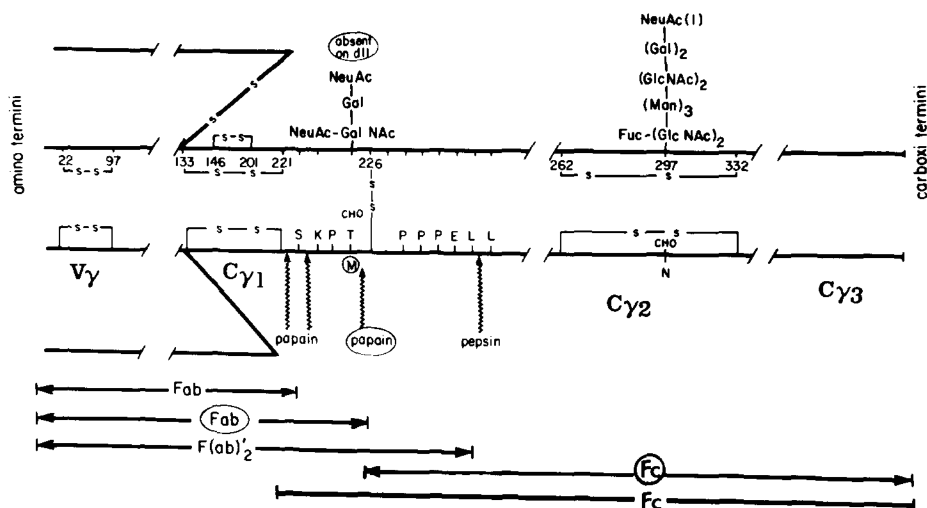

FIG. 1. **Schematic profile of rabbit IgG primary structure(s).** Allotypic structures at position 225 are indicated for *d*12/*d*12 molecules while differences between *d*12 and *d*11 molecules are indicated by including *d*11 structures and fragments inside *circles*.

addition, 40% of pooled rabbit IgG has an *O*-glycosidically linked heterosaccharide including NeuNAc[2] in the hinge region carboxyl-terminal to the locus of papain cleavage, and 15% have an oligosaccharide with no associated sialose located asymmetrically on one of the γ chains above (NH_2-terminal to) the hinge (14, 15). The distribution of this *O*-linked carbohydrate is probably related to the allotypic antigens *d*11 and *d*12 which are defined at a molecular level by the existence of the hinge region methionine (*d*11) or a threonine (*d*12) on the NH_2-terminal side of the inter-γ chain disulfide bond (16) at position 226 (see Fig. 1). In the case of the *d*12 molecule, the threonine participates in a mucin-type (*O*-glycosidic) linkage found on pooled rabbit IgG.

After selective spin labeling of these heterosaccharides (primarily terminal NeuNAc) on IgG antibodies from rabbits homozygous for *d*11 and *d*12 allotypes, the present study was performed to determine whether such structures might be involved in some way in the C1q anteceptor and to evaluate potential differences in the mobilities of Cγ2 domains when comparing the binding of C1q to native immune complexes and to artificial (chemical) IgG aggregates.

EXPERIMENTAL PROCEDURES

Materials

Organic reagents were obtained from Aldrich Chemical Co., Milwaukee, WI. All reagents for peptide sequencing were Sequanol grade (Pierce Chemical Co., Rockford, IL). Enzymes were purchased from Worthington, Bedford, MA (2× crystallized papain), and from Boehringer Mannheim, Indianapolis, IN (*Vibrio cholerae* neuraminidase). Electrophoretic materials and supplies were from Bio-Rad, Richmond, CA.

Methods

Antibody Preparation and Characterization—New Zealand White rabbits were serotyped for *d*11 and *d*12 allotypic specificities by inhibition of agglutination as described by Mandy and Todd (17, 18). Four *d*11 and three *d*12 homozygous animals were selected for immunization with dinitrophenylated porcine globulin (22 mol of hapten/carrier molecule) in complete Freund's adjuvant. Protocols for immunogen preparation, vaccination, and affinity purification of hyperimmune anti-DNP antibodies were as previously described (19, 20). Hapten elution of antibodies from affinity columns was accomplished using 100 mM 2,6-dinitroaniline (K_{rel} to ϵ-2,4-DNP-L-Lys = $0.0025x$) followed by exchange dialysis against 50 mM DNP-OH (pH 8.0) and Dowex 1-X8 ion-exchange chromatography. An AcA-34 molecular sieve column was used to separate IgG from IgM. This procedure removes up to 98% of the hapten from moderate/high affinity anti-DNP antibodies. The average intrinsic association constants for *d*11 and *d*12 molecules were determined by equilibrium dialysis using [^{3}H]ϵ-DNP-L-Lys (20). Protein purity was determined by analysis of polyacrylamide slab gel electrophoretograms run in sodium dodecyl sulfate and 8.0 M urea at neutral pH. Immunoglobulin concentrations were determined spectrophotometrically using 1% extinction coefficients of 15 (IgG), 15.5 (Fab), and 12.5 (Fc) at 278 nm.

Enzymatic and Chemical Hydrolyses—Limited papain fragmentation of IgG antibodies was performed by modifying the general methods (22, 23) using papain immobilized on 1,1′-carbonyldiimidazole-activated, cross-linked agarose (REACTI-GEL, Pierce). This modification provides a highly stable, enzymatically active reagent which can be removed rapidly from the reaction mixture. A second modification was then to limit the time of proteolysis to provide about 50% cleavage (as evaluated by molecular sieve chromatography). Enzymic activity was determined using benzoyl-Arg ethyl ester (24). After cleavage, products were separated from intact IgG (Sephadex G-100), and Fab fragments were removed from Fc fragments using DNP_{12}BSA-cellulose affinity adsorbent prepared as described by Robbins *et al.* (25).

Neuraminidase treatments were performed using affinity-purified, commercial enzyme (26) after denaturing IgG preparations by reduction (0.15 M DTT in 0.2 M Tris-Cl, pH 8.0, with 8.0 M urea), *S*-carboxymethylation (iodoacetamide to 0.3 M) and separation of polypeptides from reagents (Bio-Gel P-10, 1.6 × 10 cm in 0.05 M phosphate, pH 8.0). Hydrolyzed NeuNAc was quantitated by high performance liquid chromatography of aminopyridine derivatives of *N*-acetylmannosamine released by NeuNAc aldolase.[3] Total protein-bound sialic acid was determined colorimetrically (27). Glycosidase-susceptible spin label was determined by ESR spectrometry of the supernatant fraction from a 50% ethanol precipitation of the hydrolysis reaction.

The inter-γ chain disulfide bond of rabbit IgG Fc fragments was reduced with DTT and resulting cysteine sulfhydryl groups were selectively cyanylated and cleaved with NTCB prepared as described by Degani *et al.* (28). Optimal conditions for modification and cleavage were determined from studies on IgG, IgM (29), and IgE[4] as follows: Fc molecules (30 μmol) in 1.0 M Tris-buffered saline, pH 8.0, were treated for 15 min with 100 μmol DTT followed by the addition of 1.0 mmol NTCB (room temperature). The modified protein was then separated from other reagents on a short (20 ml) Sephadex G-25 column in 0.1 M Tris, pH 8.0, and then incubated at 37 °C overnight. A second G-25 filtration step was used to separate cleavage products which were detected by absorptivity (205 and 350 nm) and quantitated as described by Lowry *et al.* (30).

Peptide Analysis—The small NTCB cleavage product (1.0 mg) was subjected to end group analyses by dansylation (31) and hydrazinolysis (32). Peptide and hydrolysates (about 2 nmol) were dried *in*

[2] A nomenclature replacing sialose for sialic acid and SiaNAc for *N*-acetylneuraminic acid has been proposed by Scott *et al.* (Scott, R., Yamashina, K. & Jeanloz, V. (1982) *Biochem. J.* **207,** 367–368), but is not being used in this paper.

[3] D. Willett, L. McKean, and J. L. Winkelhake, manuscript submitted for publication.

[4] L. McKean and J. L. Winkelhake, unpublished observations.

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vacuo, dissolved in 0.2 M sodium bicarbonate, redried *in vacuo* over CaO pellets for 5 days, and then subjected to anhydrous hydrazine or resuspended in water, adjusted to pH 9.0, and dansylated with 20 μl of dansyl chloride (5 mg/ml in acetone) for 2 h at room temperature. Total peptide composition was determined after acid hydrolysis by thin layer chromatography on Cheng Chin polyamide plates in two dimensions using as solvent I a mixture of water and formic acid (50:1.5) and as solvent II benzene and acetic acid (9:1). Dansylated amino acids were identified by comparison with commercial standards and dansyl-OH using a long ultraviolet wavelength lamp.

IgG Aggregates, C Components, and C1 Assays—Chemical aggregates of *d*11 and *d*12 IgG molecules were prepared using glutaraldehyde at a final concentration of 0.1% (33) and a water-soluble carbodiimide (34). Larger, soluble aggregates were isolated by molecular sieve chromatography using Sephacryl S-500 just prior to use.

Immune complexes were formed using monomeric DNP_6BSA at equivalence ratios of antibodies and antigen (determined as described previously; Ref. 3). For ESR studies, immune aggregates and complexes were isolated after centrifugation (10,000 × *g*, 5 min), while for Cl binding and activation assays, antibodies were added to provide slight antibody excess.

Rabbit C1q was purified also as previously described (3) or by neutral euglobulin precipitation (35). The precipitate was dissolved in 0.5 volume of 500 mM NaCl, 20 mM EDTA, dialyzed against 50 mM phosphate, pH 7.5, containing 2 mM EDTA and 82 mM KCl (mmho = 13) prior to Bio-Rex 70/Bio-Gel A-5m chromatography (36). Purified C1q was radiolabeled using Iodogen (Pierce)-coated 10 × 75 mm glass tubes to a level of about 0.5 mol of I/mol of protein (determined spectrophotometrically at 278 nm using a 1% extinction coefficient of 6.8). The binding of C1q to immune or chemical aggregates was determined as previously described (3) in 2.5% polyethylene glycol. For ESR studies, C1q was added to aggregates for 30 min at 4 °C prior to spectroscopy. During this time, disruption of aggregates was not detectable in control studies.

The nonactive form of C1 was partially purified from neutral euglobulin (35) without inhibitors. All steps were performed at 0–4 °C and the C1 complexes were stored in 50 mM acetate, pH 5.0, with 2 mM CaCl, 150 mM NaCl at 0.5 mg/ml. Total Cl protein was determined using a 0.1% extinction coefficient of 0.94 assumed from a C1q (0.68):C1r (1.15):C1s (0.98) ratio of 1:2:2 (35, 37). Just prior to use, C1 was prepared by adjusting pH to 6.0 with Tris-Cl containing 10 mM $CaCl_2$ and centrifugation (10,000 × *g*, 10 min).

C1 esterolytic activity was determined using the method of Sim and Reboul (38) by combining 150 μg of *N*-benzyloxycarbonyl-L-lysine-*p*-nitrophenyl ester (Sigma), a substrate which is semispecific for C1s, with immune or chemical aggregates at 0 °C in 100 mM phosphate-buffered saline, pH 6.0 (no EDTA), in plastic Microfuge capsules. The addition of 1.0 mg of C1 was followed by a 30-min incubation at 0 °C and then transfer to 37 °C to initiate activation. Duplicate reactions were terminated by the addition of diisopropylphosphorofluoridate (Sigma) in propan-2-ol to 10 mM, centrifuged (10,000 × *g*, 1 min) and supernatants were monitored for *p*-nitrophenol release (340 nm). Data were corrected for spontaneous *N*-benzyloxycarbonyl-L-lysine-*p*-nitrophenyl ester hydrolysis, and only C1 preparations giving less than 15% spontaneous activation (compared with immune complexes) over a 30-min period were used.

Spin Labeling and ESR Spectrometry—IgG was spin labeled prior to aggregate formation by modifications of the procedures outlined by others (39–41). Briefly, 32.5 mg of IgG (3.0 mg/ml in 150 mM acetate-saline, pH 5.5) was mixed with 10 mg $NaIO_4$ at 0 °C for 15 min followed by rapid dialysis/concentration.[5] After the addition of 32.5 mg of 4-amino-2,2,6,6,-tetramethylpiperidin-1-oxyl (Tempamine), 5 mg of $NaCNBH_3$ were added and the solution was stirred for 16–20 h at 4 °C. Reagents were removed by dialysis/concentration against an XM-100 membrane in an Amicon N_2-pressurized cell, and the spin-labeled IgG was passed over a Sephacryl S-300 column to remove any potential aggregates.

Samples (10 μl) were drawn into a capillary tube (0.9 mm inner diameter) made of a methylpentene polymer called TPX (42). This plastic is permeable to nitrogen, oxygen, and carbon dioxide and is substantially impermeable to water . Samples were deoxygenated by equilibration with nitrogen gas obtained from the liquid nitrogen source during temperature control. ESR spectra were obtained with a Varian E109 X-band spectrometer with Varian temperature control accessories and E-231 multipurpose cavity (rectangular TE_{102} mode). All measurements were performed at 25 °C (unless otherwise stated) after allowing 15 min for complete oxygen removal. Integration of ESR spectra was accomplished using a Tracer Northern NS-570A digital signal analyzer.

Saturation transfer ESR measurements were made using a loop-gap resonator (43) instead of the E-231 cavity. Dispersion mode and phase-sensitive detection 90° out of phase with respect to field modulation (44) were employed. A 24-kHz field modulation was used with 5 G amplitude. Incident microwave power was either 2 or 10 milliwatts which gave microwave fields on the sample of 0.4 and 0.9 G, respectively. Effective rotational correlation times were estimated by comparisons with reference curves obtained using spin-labeled hemoglobin in aqueous glycerol solutions (43).

[5] Periodate concentrations were 10-fold lower than those used by other investigators (39, 40) who reported the detachment of spin labels from IgG and did not detect the spectral change on immune complex formation by analyzing h_s/h_b. They also did not compare spectra with nonspecific (chemical) aggregates of IgG.

RESULTS

Spin Labeling and Probe Localization—Homozygous rabbit *d*11 and *d*12 IgG antibodies had average intrinsic association constants ranging from 8.5 to 12.1 × 10^7 M^{-1}. The range of heterogeneity indices calculated from Sips (45) plots was 0.49 to 0.56. These values were unaltered by spin-labeling reactions as were the abilities of the antibodies to precipitate DNP_6BSA.

The number of spin labels bound to each IgG was estimated by integrating ESR spectra twice using 0.1 mM Tempamine as the standard. This analysis indicated an average of 2.2 mol of Tempamine/mol of *d*11 IgG and 4.2 mol of spin probe/mol of *d*12 IgG with standard errors of 10%. These values correspond well with total NeuNAc present on the antibody populations determined colorimetrically before imine formation and are about 25–30% lower than the values reported by Fanger and Smyth (14, 15).

The binding of Tempamine to NeuNAc was confirmed by probe susceptibility to glycosidase. Neuraminidase treatments followed by protein precipitation and ESR peak height comparisons of hydrolysate supernatant with 0.1 mM Tempamine solutions in 50% ethanol showed that about 80% of the spin label was hydrolyzed by this glycosidase when the enzyme was used at concentrations sufficient to remove >90% of the total NeuNAc present on nonlabeled controls. The value of 80% cleavage may be a lower limit estimate since covalent attachment of Tempamine to NeuNAc is likely to adversely affect cleavage by the enzyme which recognizes exocyclic hydroxyls (susceptible to periodate) for optimal activity (46). While it is possible that saccharides other than NeuNAc (with unsubstituted C-3 hydroxyls) were also nominally labeled, comparisons of spectral peak heights between Tempamine-sialose complexes and Tempamine does tend to underestimate the concentration of the former because of its larger molecular size. In fact, the ESR signal of protein precipitates from the sialosidase hydrolysates was very weak and accounted for less than 10% of the attached spin labels. In addition, control studies showed the Gal and Man are about 100 times less susceptible to periodate oxidation than is NeuNAc in that the hexose susceptibility range begins at $NaIO_4$ concentrations about 10 times higher than used here.

Preparation of Fab and Fc fragments followed by analysis of spin label content showed about 90% of the spin probes are Fc-associated. Once again, comparisons between intact IgG and Fc by integration may be subject to errors since the Fab pieces accounted for <10% of the total spin label. Subsequent treatment of DTT-reduced Fc fragments with NTCB resulted in the appearance of two peptide subfragments from *d*12 Fc populations and only one peak from *d*11 Fc populations when reaction mixtures were resolved on molelcular sieve columns (Fig. 2). All NTCB products had spin label associated, and

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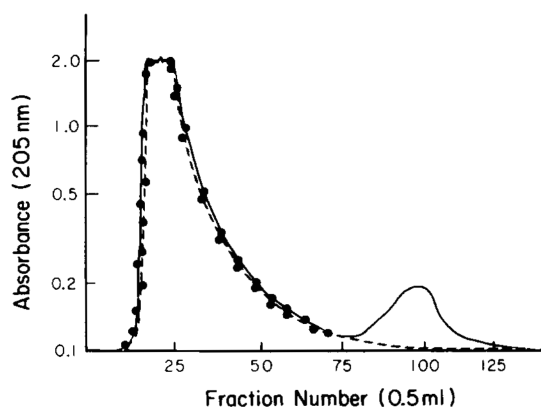

FIG. 2. **Molecular sieve chromatographic profiles of reduced Fc polypeptides from *d*11 and *d*12 homozygous rabbit IgG antibodies after treatment wqith NTCB.** Profiles correspond to (- - -) *d*11 and (——) *d*12 peptides.

the addition of $K_3Fe(CN)_6$ to 1.0 mM did not enhance spectra, thereby indicating that probes were not significantly reduced by the mild DTT treatments used here.

When evaluated in the ESR spectrometer, the mobility of the small fragment-associated spin label from *d*12 Fc populations was markedly increased compared with those of the large cleavage product, of the intact Fc or of intact IgG (data not shown). Analyses of peak height ratios showed approximately 0.7 mol of Tempamine/small peptide and between 1.3 and 1.5 mol of spin probe/large peptide (for both *d*11 and *d*12 molecules).

Amino acid analyses of the small peptide NTCB cleavage product showed the *d*12 piece contained about equimolar amounts of proline, lysine, serine, and a threonine-like spot when compared with commercial standards.

C1 Binding and Activation—Binding of radioiodinated C1q to spin-labeled *d*11 and *d*12 antibodies in both immune and chemical aggregates is shown by Fig. 3*A*. When aggregates are incubated with incremental amounts of C1q and equivalent IgG aggregates (based on weight of IgG present), C1q is bound decreasingly from an optimal value depending upon the type of aggregate. For subsequent ESR studies, the amount of C1q added/mg of total IgG present was chosen such that an identical percentage of C1q (75%) molecules present were bound in the spectrometer tube/mg of IgG present. Spin labeling of IgGs did not affect C1q bindability when compared with non-probe-labeled controls.

When the same aggregate solutions were mixed with partially purified C1, the amount of $C\bar{1}$ produced is shown by Fig. 3*B*. In this case, while native immune complexes activate C1 virtually completely within 30 min, chemical aggregates activate at less than 10% of that rate. Table I shows the inhibitory effects of incubating C1q with immune complexes or chemical aggregates prior to assessing C1-activating capacities. After 30 min, all IgG aggregates with C1q bound were able to inhibit C1 activation about equally (50–75% inhibition), suggesting that potential differences in the affinity of C1q for the different aggregates are not responsible for differential abilities of these aggregates to activate. This result confirms the more detailed binding studies of Folkerd *et al.* (13) which showed that the K_d of C1q binding to its anteceptors in different types of IgG aggregates is not related to C1 activation.

The Mobilities of Spin-labeled IgG Heterosaccharides—ESR spectra of all IgG aggregates (immune as well as chemical) exhibit essentially two major features; namely, more and less

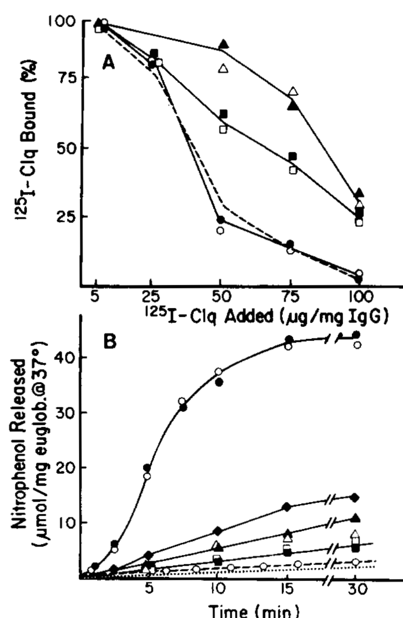

FIG. 3. **Binding and activation of complement.** *A*, binding curves for C1q:rabbit IgG aggregates after 30-min incubation at 4 °C. *Open symbols*, *d*11; *closed symbols*, *d*12. ○, ●: data for native immune complexes; △, ▲: data for gluteraldehyde-aggregated IgG; □, ■: data for carbodiimide cross-linked antibodies. - - -, data for native immune complexes without spin-labeled oligosaccharides. *B*, activation kinetic curves of complement C1 by rabbit IgG aggregates. Conditions are as in *A* except that - - - represents data for C1 alone and ○- - -○ shows data obtained with *d*11 IgG antibodies in immune complexes incubated with C1 which had been previously heated at 56 °C for 30 min. ◆: activation by gluteraldehyde-aggregated antigen (no antibodies present). The amount of C1q added ranged between 30 and 65 μg/mg IgG present based on the type of aggregate used.

TABLE I

Inhibition of C1 activity by Clq:IgG aggregates

Test proteins	Cl activity
	%
Controls	
*d*11 or *d*12 IgG alone	<5
Purified Clq alone	<2
Glutaraldehyde-aggregated DNP_6BSA	3
Glutaraldehyde-aggregated *d*11 or *d*12 IgG	7
Carbodiimide-aggregated *d*11 or *d*12 IgG	9
*d*11 or *d*12 IgG in immune complexes	100
Aggregates + Clq	
Glutaraldehyde-aggregated *d*11 or *d*12 IgG	3
Carbodiimide-aggregated *d*11 or *d*12 IgG	<2
*d*11 IgG in immune complexes	43
*d*12 IgG in immune complexes	37
*d*11 IgG in immune complexes + glutaraldehyde-aggregated *d*11[a]	23
*d*12 IgG in immune complexes + carbodiimide-aggregated *d*12	16

[a] Incubation of immune complexes was accomplished with equimolar (IgG) amounts of chemical aggregates.

immobilized components (shown for immune complexes by Figs. 4 and 5).

These spectra are a superposition of the individual spectra of 4.2 Tempamine/*d*12 and 2.2/*d*11 IgG. Although this multiplicity overrides the possibility of exact spectral analysis, qualitative and semiquantitative interpretations were possible and led to meaningful conclusions as follows. The mobility of more immobilized spin labels (which display the broader lines) is characterized by maximum splitting values and rotational correlation times (evaluated as described in Ref. 47) in Table

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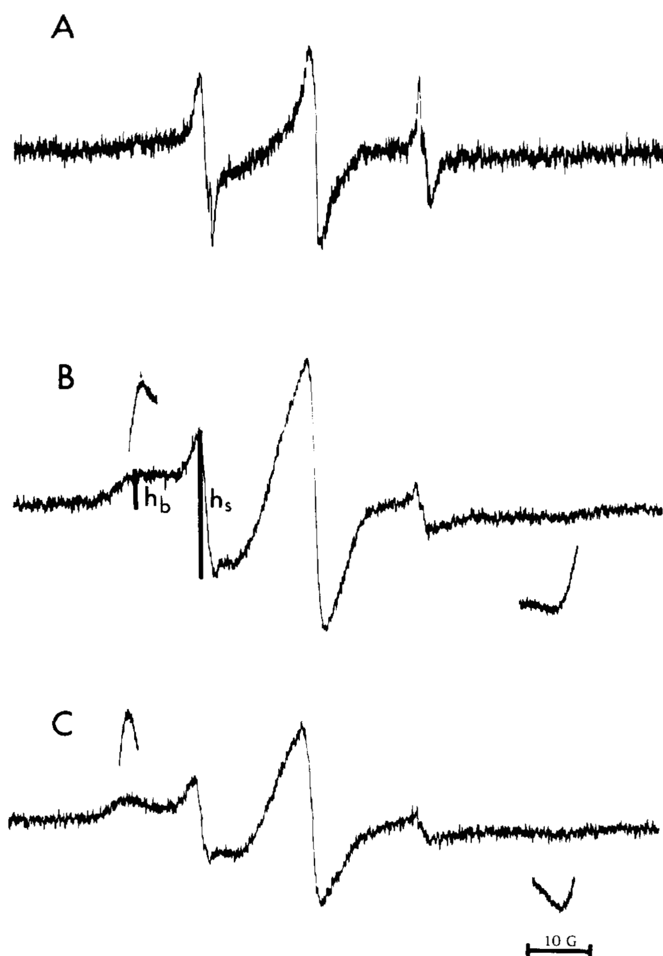

FIG. 4. **Spin-labeled oligosaccharide ESR spectra of IgG antibodies from rabbits homozygous for the *d*11 allotypic determinant.** *A*, spectrum obtained for monomeric IgG (10 mg/ml) in solution. *B*, spectrum obtained for *A* in immune complexes with DNP_6BSA. *C*, spectrum obtained for *B* with C1q added. Microwave power of 0.5 milliwatts and field modulation widths of 0.5 G were employed. Enhanced peaks shown by *insets* in *B* and *C* were obtained with increased power (5 milliwatts) and field modulation widths (4 G). All buffers (*A–C*) contained 2.5% polyethylene glycol. *Spectra B* and *C* were obtained using immune precipitates, which were loosely pelleted by brief centrifugation (12,000 G, 30 s).

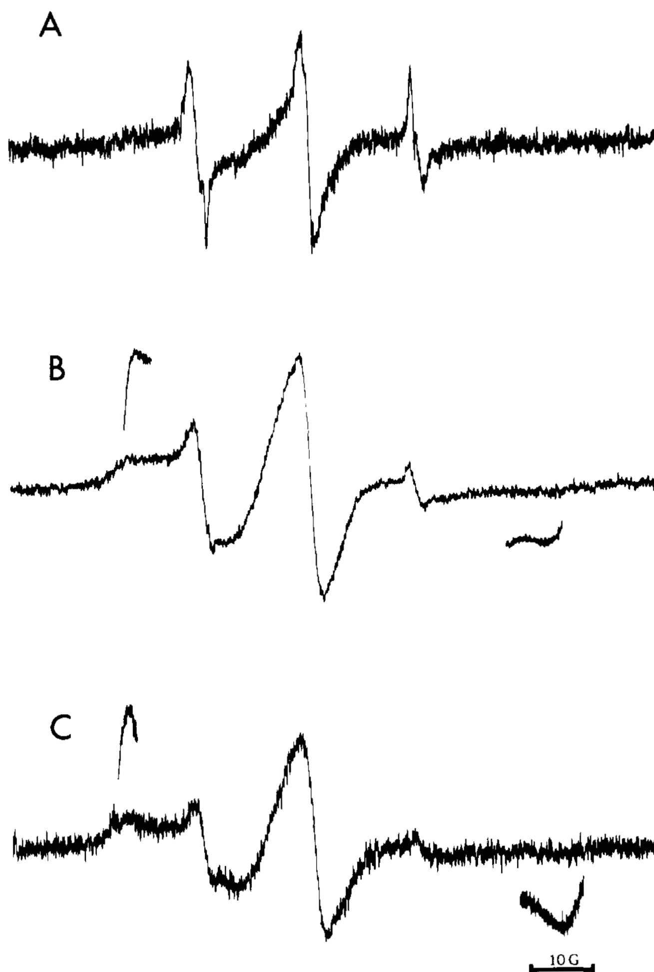

FIG. 5. **Spin-labeled oligosaccharide ESR spectra for IgG antibodies from rabbits homozygous for the *d*12 allotypic determinant.** *Insets A–C* are as described for Fig. 4.

II. The mobility of the less immobilized spin labels (which display sharper lines in the ESR spectra) was very large (almost isotropic) with rotational correlation times in the range of 10^{-10}. Hyperfine splitting by protons is seen in Figs. 4*A* and 5*A* and was confirmed by using a small field modulation (0.04 G) and by expanding the abscissa display (10 G scan) using O_2-free samples.

One convenient way to qualitatively compare ESR spectra is to evaluate the ratios of less immobile (h_s: sharp line) peak heights and more immobile (h_b: broad line) peak heights of low field lines ($M_1 = +1$) (see *e.g.* Ref. 48). This ratio (h_s/h_b) decreased greatly in immune precipitates, less so in chemical aggregates, and even less in nonspecific (ethanol) precipitates (Table II), indicating that spin label mobility is more restricted in immune complexes. These results suggest, at least empirically, that some directed protein:protein (Fc:Fc) interactions may be occurring in immune complexes which do not occur in nonspecific protein aggregates.

Antigen-induced IgG aggregates also exhibited a loss of hyperfine splitting by protons in less immobilized lines, and while the changes in peak height ratios were presumably caused by a broadening of the sharp lines (reflecting a small decrease in probe mobility), it is possible that some subpopulation of less immobilized spin probes experiences a large decrease in mobility upon the formation of immune complexes, alternatively explaining the observed increase in the broader peak.

A convenient way to compare the mobility of more immobilized components between experimental samples is to measure the maximal splitting value for each sample compared with its potential upper limit of immobilization (39, 40). Such splitting reflects motional averaging of the anisotropy of the nitrogen hyperfine tensor, and the upper limit of splitting is influenced by probe environment, *e.g.* polarity of solvent, formation of hydrogen bonds, etc. (49, 50). The upper limit was determined by freezing each sample at −120 °C (no differences were observed between −76 and −158 °C). By comparing the ratio of the sample's splitting value at 25 °C ($2T'_{zz}$) with that obtained for the sample at −120 °C ($2T_{zz}$), it is possible to conclude whether an experimental manipulation of the sample decreases spin probe mobility toward the right limit (*i.e.* $2T'_{zz}/2T_{zz}$ approaches 1.0). As shown in Table II, the formation of immune complexes has a slight but significant increasing effect on this ratio. However, unlike h_s/h_b, this analysis does not discriminate types of IgG aggregates.

Another useful way to determine the mobility of slowly moving spin labels is to adopt the approach of McCalley *et al.* (47) with the assumption that the rotational diffusion is

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TABLE II

Effects of aggregate formation and Clq binding on spin-labeled heterosaccharides of rabbit IgG antibodies

	h_s/h_b	$2T'_{zz}$[a]	$2T_{zz}$[a]	Ratio[b]	$\tau 2$
		G			*ns*
*d*11 antibodies					
IgG alone	13.6	60–63	73.9	0.85	<7
IgG + antigen	3.7	63.7	72.3	0.88	8
IgG + antigen + Clq	3.0	66.4	72.2	0.92	13[c]
IgG + antigen + non-immune IgM	3.3	64.4	72.8	0.88	8
Glutaraldehyde-aggregated IgG alone	4.9	63.5	72.5	0.87	8
Glutaraldehyde-aggregated IgG + Clq	5.0	62.9	72.6	0.88	7
Ethanol-precipitated IgG	5.4	63.2	71.7	0.88	8
*d*12 antibodies					
IgG alone	14.9	60–63	73.7	0.85	<7
IgG + antigen	4.6	63.3	72.5	0.87	8
IgG + antigen + Clq	4.2	65.2	72.3	0.91	10[c]
IgG + antigen + non-immune IgM	4.8	63.5	72.5	0.88	8
Glutaraldehyde-aggregated IgG alone	5.2	63.9	72.5	0.88	8
Glutaraldehyde-aggregated IgG + Clq	5.4	63.3	72.4	0.87	8
Ethanol-precipitated IgG	5.7	62.7	72.4	0.85	7

[a] Maximal splitting value of the more immobilized component.

[b] $2T'_{zz}/2T_{zz}$ (S.D. < 0.02 except IgG alone).

[c] Rotational correlation times of more immobile component were calculated as in Ref. 47. The rotational correlation time estimated by saturation transfer ESR (see Fig. 6) was in the range of 30–40 μs for both *d*11 and *d*12 antibodies in immune complexes and incubated with Clq. Discrepancies in the two techniques may be due to overestimation of $2T_{zz}$ due to H bonding (49, 50), low sensitivity of conventional ESR, or overestimation of spin trapping-ESR data due to smaller maximal splitting than seen for the rigid limit.

isotropic without hindrance by the environment (*i.e.* no potential wall). As shown in Table II, the formation of immune complexes has a slight but significant effect by such analyses. ESR spectra and the effect of immune complex formation on ESR signals are very similar between *d*11 and *d*12 antibodies.

Effects of C1q Binding on Spin-labeled Heterosaccharides of IgG Aggregates—While C1q binding to chemical aggregates of IgG has little effect on splitting values or peak height ratios, addition of C1q to immune complexes increased maximum splitting of immobilized components by 2 to 3 G (Figs. 4*C* and 5*C*), increasing thereby $2T'_{zz}/2T_{zz}$ ratios toward 1.0 and further decreasing mobile/immobile peak height ratios (Table II). To obtain a better appreciation for the effective rotational correlation time of spin probes in the C1q:immune complex mixtures, saturation transfer spectroscopy was performed in the dispersion mode (reviewed in Ref. 51). An excellent signal/noise ratio was attained by using the loop-gap resonator with only 0.6 μl of the sample. By comparing the resulting spectrum (Fig. 6) to solutions of hemoglobin in glycerol, an empirical value for the correlation time was determined to be 3 to 4 × 10^{-5} s (see Footnote *c* in Table II), indicating a very strong immobilizing effect on the spin labels. This effect was not seen when C1q is bound to spin-labeled IgG antibodies in chemical aggregates (Table II). In control experiments, the addition of a large protein, normal rabbit IgM, to immune complexes had only a slight immobilizing effect on spin label spectra. While, it is striking that C1q binding affects spin labels on *d*11 and *d*12 antibodies very similarly (perhaps because the C1q molelcule (M_r = 600,000) has binding sites in structures of about M_r = 50,000 interacting with a very small subdomain region of Cγ2, thereby potentially capable of affecting all Cγ2 domain structures), cumulatively, these

FIG. 6. **Representative saturation transfer dispersion spectrum of rabbit (*d*11) IgG antibodies in immune complexes and incubated with C1q.** Very similar profiles were obtained for *d*12 antibodies (data not shown). Incident microwave power was 10 milliwatts.

results indicate that C1q-binding effects occur as a result of close, specific protein:protein interactions between C1q heads and Cγ2 domains and that the type (immune *versus* chemical) of IgG aggregates is reflected in the type of spin probe spectra.

DISCUSSION

There are basically three models which attempt to explain how antibodies in immune complexes might be recognized by Fc-binding macromolecules after antigen binding: (*a*) $V_H - V_L$ interactions may alter *V/C* switch angles (52, 53); (*b*) antigen binding may strain hinge region structures, thereby altering spatial relationships of Cγ2 domains (54, 55) perhaps expelling carbohydrates (56); or (*c*) cross-linkage of Fabs may alter Fab:Fc interactions, releasing steric hindrance between swiveling Fab arms and the Fc (3, 57). This latter proposal is a modified version of the "click open" hypothesis (58).

While none of these models is supported or rejected by firm data, the first is unlikely as a means for IgG activation of C via the classical pathway, although such a mechanism could be involved in alternative pathway activation. Current opinion favors a dual involvement of subtle conformational or motional alterations along with the aggregation process. Furthermore, since chemically aggregated IgG does not activate C1 but is a good substrate for C1q (13, 59), it appears that optimal conversion to $C\bar{1}$ requires IgG molecules with functional, flexible hinge regions. This would mean that either Fc:Fc interactions must occur or that the C1q molecule must be able to re-orient Fcs to affect C1r/C1s (which bind 10-fold better to C1q:immune complexes than to C1q:chemical aggregates of IgG; Ref. 60).

One way to begin to resolve these possibilities would be to attach physicochemical probes specifically to glycoprotein structures within the Cγ2 domain and to then evaluate conformational and (possibly) sequential changes in Cγ2 upon antigen binding and then upon C1q binding. In the present study, the spin label Tempamine was attached to vicinal hydroxyls on periodate-oxidized carbohydrate moieties (predominantly NeuNAc) of rabbit IgG anti-hapten antibodies. Advantage was taken of the knowledge that rabbit IgG allotypic determinants *d*11 and *d*12 include the absence (*d*11/*d*11) of an oligosaccharide containing NeuNAc in the hinge region while *d*12 molecules contain such heterosaccharides. Since each allotypic variant contains a complex oligosaccharide (with NeuNAc) further down the γ chain near the Cγ2-Cγ3 junction (see Fig. 1), spin probes can be attached at the COOH-terminal region (*d*11) or the NH_2 and COOH-terminal regions (*d*12) of the Cγ2 domain.

Under the chemical modification conditions utilized here, probe adducts occurred on 75–80% of the total detectable

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NeuNAc moieties (*i.e.* about 80% of the spin labels could be removed from denatured IgG with neuraminidase). Studies with limited papain-digested Fab and Fc fragments and with NTCB cleavage products of those Fc pieces, showed that more than 90% of the spin labels were attached to the Fc with about twice as many probes detectable on *d*12 heavy chains as on *d*11 chains.

The ESR spectra of rabbit IgG oligosaccharides in solution reflect heterogeneity and confirm previous results (40, 41), showing that terminal sugars of IgG heterosaccharides are moving with respect to the protein (Figs. 4*A* and 5*A*). This pattern is no different when the antibodies are mixed with monovalent haptens and is not greatly different when the IgG is either aggregated chemically or precipitated with ethanol (Table II). Such results indicate that protein concentration as well as nonspecific aggregation has little effect upon spectral profiles.

All ESR spectra show the presence of significant portions of faster and slower moving components (*e.g.* Figs. 4*B* and 5*B* and Table II). The molecular basis for these subcomponents is not clear. However, differences in peak height ratios between immune and nonspecific chemical aggregates of IgG (Table II) suggest that the mode of aggregation plays an important role in the nature of the ESR spectra observed. Because of this, we propose that peak height spectral changes seen with immune complexes may be explained by protein:protein interactions which are more than nonspecific. We suggest calling these "directed" Fc:Fc interactions.

Addition of C1q to either *d*11 or *d*12 immune complexes, but not to chemical aggregates of these same antibodies, shifts maximum splitting of the immobilized component from 64 to 66–67 G with a concomitant decrease in peak height ratio (Figs. 4*C* and 5*C* and Table II). While both chemical and immune aggregates bind C1q (Fig. 3*A*), the fact that chemical aggregates do not activate C1 (Fig. 3*B* and Table I) suggests that C1q binding to immune complexes alters Fc:Fc interactions in some fashion.

Even upon C1q binding, all spectra show both faster and slower moving components. There may be several reasons for this. First, while unlikely, it is possible that the chemical-modifying conditions we have used have modified sugar moieties other than terminal NeuNAc. In such a case, labeling of more rigidly fixed, core region sugars could account for much of the more immobile component seen with IgGs in solution and amplified in all aggregates. We do not think that periodate provided amino acid modifications which would allow for spin labeling of peptide moieties because, as pointed out by Nezlin and Sykulev (39) and our own data (Table II), $2T'_{zz}/2T_{zz}$ ratios for sugar probes are in the range of 0.8–0.9 (close to the rigid limit of the protein) while peptide probes (*e.g.* maleimide or anhydride spin label amino acids) give ratios of about 0.74.

A second explanation for the appearance of two, particularly the small amount of residual fast moving, components in C1q:immune complex samples is that it is likely that not every probe-labeled Fc is bound by a C1q active site. Thus, heterogeneity in ESR spectra can be attributed to possible labeling of core region sugars in addition to (more mobile) NeuNAc, to carbohydrate microheterogeneity in general and, in the case of C1q-bound aggregates, to nonperturbed Fcs.

Based on these analyses, we propose that the change in ESR spectra seen on the binding of C1q to immune complexes reflects a C1q-induced rearrangement of the initial Fc:Fc interactions which occur during aggregation. This proposal suggests that the process of C1 activation has at least two discrete initiating steps: namely (i) an antigen-induced cessation of Fab:Fc steric hindrance (exposing the C1q anteceptor) which allows C1q to bind and (ii) an organization or aligning of Fcs by the large, globular binding units of the C1q molelcule.

Such a proposal emphasizes the importance of assessing antibody effector activities in immune rather than artificial aggregates (21) and supports the argument that the mechanisms of antibody effector functions involve subtle conformational changes and aggregation. We conclude that the present study shows that some very important changes occur during the C1q-binding process and that probing Fc region carbohydrates is a valid, although empirical, approach to beginning to understand the precise mechanisms of antibody effector functions.

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