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Fc:Fc INTERACTIONS REVEALED BY SPIN-LABELED IgG HETEROSACCHARIDES IN MODEL IMMUNE COMPLEXES*

Akihiro Kusumi† and Jeffrey L. Winkelhake‡

†Microphotonic Center, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, and Department of Biophysics, Faculty of Science, Kyoto University, Kyoto 606, Japan

‡CETUS Corporation, 1400 Fifty-third Street, Emeryville, CA 94608

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SUMMARY: Dynamic properties of spin-labelled heterosaccharides in the Fc-region of murine monoclonal antihapten immunoglobulin G were studied in model immune complexes (IC) as a function of the IC size. Model IC dimers, trimers and oligomers were formed using bivalent photoaffinity antigens. The ESR spectrum exhibits two components. The rotational correlation time of the less-immobilized species is shorter than 10^{-10} sec, and that of the more-immobilized component is in the order to $10^{-9} \sim 10^{-8}$ sec depending on the IC size. Fraction of the more-immobilized spin labels increases, and the mobility of this component decreases with increase in IC size (i.e., mobility: monomers $\approx$ dimers > trimers >> immune-complex precipitates). These data strongly suggest the existence of Fc:Fc interactions in IC, and provide the basis for a model in which such interactions underlie the initial mechanism by which the information of antigen binding to Fab region is transferred into organized Fc:Fc association structure for IgG effector activities.

During our previous studies on the dynamics of spin-labeled heterosaccarides on rabbit IgG, we observed that formation of immune complexes (IC) by antigen induces substantial restriction on spin label mobility (1). Only slight decrease of probe mobilities was observed in chemical aggregates of these same antibodies. Addition of purified Clq to IC (and not artificial chemical aggregates) results in further immobilization of spin labels, the rotational correlation time of which slows from the nsec range to 30-40 μsec (1).

The present study was performed with two fundamental questions in mind regarding the initial stages of immune reactions: (1) Is there a specific

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[1] Abbreviations used: IgG, immunoglobulin G; IC, immune complexes; Clq, a subcomponent of C1 molecule which binds to IC and initiate the classical complement pathway; DNP, dinitrophenyl; BSA, bovine serum albumin.

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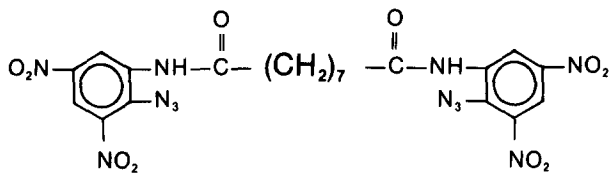

Figure 1. Chemical structure of bivalent photoaffinity hapten.

association structure of IgG (besides simple aggregation) which initiates effector activities of IC (2) What is the smallest size of IC, in which restriction of oligosaccharide mobilities are detected by Fc-specific spin probes.

To study these questions, the mobilities of spin-labeled heterosaccharides, mostly located in the Fc region (1), were evaluated as a function of size of IC (2). We and others have previously found that these IgG heterosaccharides are involved in stabilization of Fc region binding sites (anteceptors) for the first complement component C1q in the classical pathway of complement fixation (3-5) and also in structural properties of Fc regions (6).

Since polyclonal antibody:antigen complexes are heterogeneous with regard to immunochemical and physicochemical properties, they are not amenable for further physico-chemical analysis. Thus we prepared murine monoclonal anti-dinitrophenyl (DNP) IgG antibodies (IgG2a) cross-linked into model IC with a bivalent photo-affinity DNP antigen (2, the molecular structure shown in Figure 1). Such complexes possess defined stable structures (dimers, trimers, and larger oligomers) and provide ideal models for further probing the spin label immobilizing effects seen in IC.

MATERIALS AND METHODS

Murine monoclonal IgG2a anti-dinitrophenyl (DNP) antibodies with dissociation constants (K_d) of 2-5 x 10^{-9} M were prepared and purified as described in (2). Model IC dimers, trimers and multimers were prepared using a photoaffinity bivalent hapten, the arylazide analog of bis-dinitrofluorobenzene pimelate amide (2, see Figure 1) as described previously (7). Aggregates were resolved immediately on Sephacryl S300 and S500 molecular sieve columns (2).

Vicinal hydroxyls of sialic acids on monoclonal antibodies and model IC were spin-labeled as described previously (1). Neuraminidase treatment and sialic acid quantification were carried out as described in (8) and (9).

ESR spectroscopy was performed on deoxygenated samples as described previously at 25 ± 0.5°C (1).

RESULTS

Spin Labeling and Probe Localization

The number of Tempamine molecules bound to each IgG2a antibody molecules was estimated by integrating the ESR spectrum twice using 0.1 mM Tempamine as a standard. ESR analyses showed that about 90% of the spin probes are Fc-associated (Table 1) and that about 80% of the spin label was hydrolyzed by the neuraminidase (Table I) at concentrations sufficient to remove 90% of total available sialic acids from non-labeled IgG. Protein denaturation by probe attachment was not detectable: dissociation constants, quantitative precipitin analysis and the binding of rabbit C1q to immune complexes were unaffected by attachment of spin labels.

Mobility of Spin-Labeled IgG Oligosaccharides in Model Immune Complexes.

Figure 2A shows a representative ESR spectrum of an IgG2a antibody with spin labels attached to terminal saccharide residues in the Fc regions. The spectrum shows the presence of at least two subcomponents; a more-immobilized (see the spectrum recorded at a higher amplifier gain) and a less-immobilized (three sharp lines) component. The rotational correlation time of the less-immobilized component is estimated to be $\sim 1 \times 10^{-10}$ sec (calculated according to (13)). Hyperfine structure due to the coupling with proton nuclei in the piperidine ring is also observed since molecular oxygen is removed from the sample.

Table 1. Extent and selectivity of spin-labeling reaction with murine monoclonal anti-DNP IgG2a*

Protein	Total NeuNAc	Spin Label	Neuraminidase Cleavable Spin Label
IgG2a	4.7	3.2	3.9
Fab	0.2	N.D.	N.D.
Fc	4.2	3.5	3.8

*Each number is given as a mole ratio to the protein.

N.D.: No detectable ESR signal.

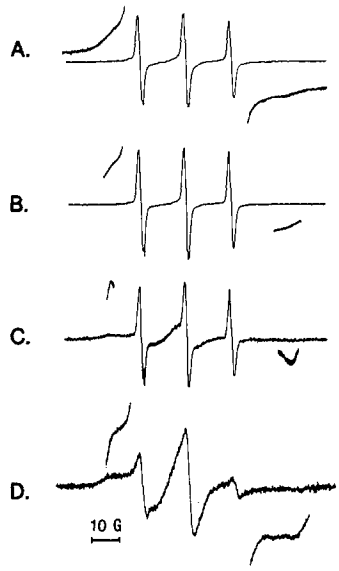

Figure 2. ESR spectra of spin-labeled oligosaccharides on IgG2a anti-DNP antibodies in solution. (A) Monomers. (B) Model IC dimers. (C) Model IC trimers. (D) Large IC pellet made of DNP_{12}-BSA and IgG2a anti-DNP antibodies.

Figure 2B shows the ESR spectrum of the same antibodies in model IC dimers. The difference between monomers and dimers is small (compare Figures 2A and 2B). The ESR spectrum of spin-labeled IC trimers is shown in Figure 2C. The more-immobilized component becomes more prominent in this figure. All complexes are soluble in aqueous buffer. These results indicate that an increase in the proportion of the more-immobilized spin probes takes place with formation of trimer IC and is not related to formation of precipitates.

Larger IC were formed by mixing DNP_{12}-BSA and murine monoclonal IgG2a anti-DNP antibodies at equivalence. The ESR spectrum of this mixture (Figure 2D) shows a further increase in the more-immobilized component.[2] Non-specific

[2]While the results presented here are consistent with our previous work (1), in which large immobilization of spin-labels on heterosaccharides was observed in large IC, these results are at variance with the data of William et al., (10) and Nezlin et al., (11,12). These investigators did not detect significant spectral change on IC formation. The reasons for differences are not clear. The periodate concentration used in our studies is 10-fold lower than those used by these workers (10,11) who also reported the detachment of spin labels from IgG. We do not observe detachment except with aged (> 2 week) samples. The molecular oxygen, which seriously affects ESR spectrum, was comprehensively removed from the ESR sample in this work. As a consequence, the ESR line shapes are more sensitive to motional changes of the spin label.

aggregation (and pelleting) does not induce this type of ESR spectral change as shown in Figure 3 where IgG2a antibody molecules have been treated with 50% saturated ammonium sulfate (3A), 50% methanol (3B), or 60% ethanol (3C) and then pelleted by centrifugation. The spin labels on monoclonal IgG2a oligosaccharides show little signs of increased immobilization by these treatments (compare with Figure 2A).

DISCUSSION

There have been several immunological observations implying the existence of Fc:Fc interactions in immune complexes. Møller (14) and Møller and Steensgaard (15) proposed Fc-mediated precipitation on the basis of their observation of a marked difference between the $F(ab')_2$ fragments and intact IgG with respect to the ability to precipitate macromolecular antigens. Rodwell et al. (16) and Schifferli et al. (17) suggested that protein:protein interactions localized in the Fc-region of the antibody molecule play a major role in precipitate formation in intact immunoglobulin molecules (16). More recently, the findings of liquid phase cooperative interactions of monoclonal antibodies (18) may be partially explained by directed interactions between specific regions (Fc) of these antibodies.

In the present study, we were able to extend these observations to the molecular level by taking advantage of the rigorous control one has over antibody heterogeneity and IC stability using model IC. Spin probes attached to terminal carbohydrate residues of monoclonal IgG2a antibody molecules show little immobilization in dimeric IC, stronger immobilization becomes apparent with formation of trimer-sized IC, and further immobilization occurs when larger IC is formed. These results cannot be explained by a decrease in the rotational diffusion of IgG molecules. Motional freedom of the spin label of both more- and less-immobilized components in soluble IC is large, with rotational correlation times in the order of 10^{-8} and 10^{-10} sec, respectively. These values are considerably shorter than the expected rotational correlation time of IgG monomer as a whole (10^{-7} sec). As we and others have found, the binding of DNP haptens to monomeric IgG has little effect on spin-labeled oligosaccharide ESR

spectra (1,10-12). Such results suggest that potential conformational changes induced by antigen binding cannot explain our observations with model IC. Further, nonspecific aggregation of IgG by ammonium sulfate, methanol, or ethanol does not induce immobilization of the spin probes seen with IC (Fig. 3). Thus, it is concluded that Fc:Fc interactions are responsible for the observed, IC-size-dependent, IC-specific ESR spectral changes.

The molecular mechanism(s) by which the information of antigen binding in the Fab region is transmitted to initiate Fc region effector functions has been a long-time puzzle. The currently favored model is that cross-linking of the antibody by multi-determinant antigens is the critical and perhaps sufficient event (19,20). Our results allow the hypothesis that the molecular basis for IC initiation of effector functions is the organization of intermolecular Fc's. Such interactions can then provide necessary structures for recognition by IC-binding molecules such as C1. The major obstacle to such a proposal involves the question of the driving mechanism for Fc:Fc associations. Since there is no evidence for large conformational changes of IgG molecules, it is possible that Fc regions have built-in associative properties (16), i.e., the free energy gained by Fc associations may be smaller than the thermal energy of random diffusion of monomeric IgG (thus monomers stay as monomers) but Fc associations

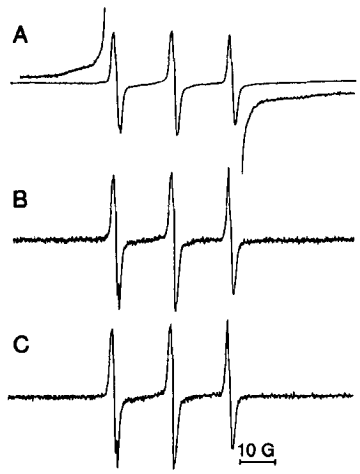

Figure 3. ESR spectra of spin-labeled oligosaccharides on IgG2a anti-DNP antibodies in nonspecific precipitates. (A) In 50% saturated ammonium sulfate. (b) In 50% methanol. (C) In 60% ethanol.

may manifest themselves when free diffusion is suppressed, e.g., when trimeric IC or larger IC are formed. Such built-in associative properties of Fc regions are consistent with the ready crystalizability of Fc fragments, the binding of C1q to (apparently) monomeric IgG, and the relative insolubility of IgG above 20 mg/ml.

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