Activation of antibody Fc function by antigen-induced conformational changes
(complement/IgM)

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ABSTRACT IgM antibody directed against the phenyl-β-lactoside hapten was examined for its capacity to fix complement in the presence of the hapten, monohapten-substituted antigen, and multihapten-substituted antigen. Hapten was found to have no effect; monovalent antigen induced an excellent response which could be inhibited by hapten; and multivalent antigen also induced an excellent response which was related to the number of determinants added and not to the formation of antigen-antibody aggregates. The difference between the activities of hapten and monovalent antigen was reflected in their affinities for the IgM antibody. The monovalent antigen had a lower $K_d$, indicating that energy from binding was used to activate the Fc complement binding sites. These data show that the expression of IgM Fc function depends on a change in Fc conformation produced by the binding of antigen at the distant Fab combining sites.

The antibody molecule consists of two functionally distinct regions: Fab arms, which are responsible for specific antigen binding, and an Fc portion, which is responsible for the biological functions shared by all antibodies of the same class or subclass. Although these regions are spatially separated within the antibody molecule, the binding of antigen to the Fab sites is known to initiate a number of Fc reactions. The reactions include such physiologically significant processes as the binding of complement, the triggering of mast cells to release histamine, and the triggering of B lymphocytes to differentiate and synthesize antibody.

The mechanism of antigen signaling has not been satisfactorily resolved. Studies of monomeric immunoglobulins have shown that the formation of antigen–antibody aggregates is critical. For example, IgC exhibits significant complement fixation only after monomers have been aggregated to tetramers and higher polymers (1), and triggering of B lymphocytes and mast cells has been found to depend on crosslinking of their monomeric receptors with multivalent ligands (2-4). On the other hand, aggregation alone does not explain data obtained with polymeric immunoglobulins. Pentamer IgM and its nonspecific aggregates can fix complement components in the absence of antigen (5, 6), but the reaction is greatly enhanced in the presence of specific antigen. Moreover, dose response curves indicate that a single IgM molecule bound to particulate antigen is capable of fixing complement (7-9). These data suggest that conformational changes as well as aggregation may be required to activate Fc reaction sites.

To determine the contribution of conformational changes, studies were undertaken of IgM antibodies with hapten specificity. IgM antibodies had the advantage of being built-in aggregates so that conformational changes could be assayed from differences between the activities of the free molecules and those complexed with antigen under non-aggregating conditions. Hapten specificity conferred the additional advantage that antigen reagents could be tailored with respect to the number of hapten groups conjugated and the carrier properties. The present paper reports the results obtained when hapten, monosubstituted antigens, and multisubstituted antigens were compared for their capacity to bind to IgM antibody and induce complement fixation.

MATERIALS AND METHODS

Preparation of Haptenes and Hapten-Carrier Conjugates. Lac dye, $p$-(p-dimethylaminobenzeneazo)phenyl-β-lactoside, was synthesized according to the method of Kurash (10). Lac-BGG was prepared by coupling the diazonium salt of $p$-aminophenyl-β-lactoside (Lac) to carboxymethylated bovine gamma globulin at a mole ratio of 120:1 in 0.2 M carbonate buffer, pH 9.0 (11). Multi-Lac-RNase was obtained by reacting 24 μmol of Lac diazonium salt per one μmol of ribonuclease A (10 mg/ml) in 0.1 M NaHCO$_3$, pH 8.0 for 18 hr at 4°C. After coupling, the protein was reduced in 20 mM dithioerythritol, 10 M urea, 0.2 M Tris-HCl, pH 8.0, 20 mM glycine, and 2 mM EDTA for 2 hr at 25° and alkylated at 0° by adding a 2.2 M excess of $[^3]$Hiodoacetic acid (specific activity = 8.49 × 10$^5$ cpm/μmol). Mono-Lac-RNase was prepared by adding 1 μmol of Lac diazonium salt per 2 μmol of RNase under the conditions described above. The solution was dialyzed against 0.15 M NaCl, concentrated by ultrafiltration, and adjusted with glacial acetic acid to pH 4.5 for 2 hr at 4°C to decrease the amount of any hapten coupled to lysyl side chains via the diazoamino reaction (12). After this treatment an average of 0.02-0.03 mol of hapten remained bound per mol of RNase. The diazotized molecules were isolated on an immunoadsorbent column prepared by coupling 2 mg of specifically purified rabbit anti-Lac IgG antibody against per ml of CNBr-activated Sepharose 4B (13). The nonbound material was removed by overnight washing with TBS (0.02 M Tris-HCl, 0.15 M NaCl, 0.02% NaN$_3$, pH 7.5), and the bound fraction was eluted by application of a bed volume of 0.4 M lactose in TBS, followed by TBS alone. Either before or after the affinity chromatography step the RNase was completely reduced and $[^3]$Hcarboxymethylated. When the reaction was carried out after affinity chromatography, the product contained significantly less than 1 Lac group/mol, apparently because some of the diazo bonds were broken during reductive cleavage. The extent of hapten substitution in the various diazotized conjugates was determined from spectrophotometric analysis (14) and measurements of protein content by amino acid and $[^3]$Hcarboxymethylcysteine analyses (15).

Preparation of Anti-Lac IgM Antibody. New Zealand White rabbits were immunized with alum-precipitated Lac-BGG as described (11). To isolate the hapten specific anti-
bodies, the pooled antisera were passed through a column of Sepharose 4B derivatized with Lac hapten (4 μmol/ml of packed resin) and equilibrated with 0.01 M NaCl, pH 8.0. After thorough washing to remove nonspecifically adherent protein, the anti-Lac antibodies were eluted from the column by passage of a bed volume of 0.5 M lactose, pH 8.0. The eluate was concentrated by ultrafiltration to 10–15 mg/ml, and the IgM and IgG fractions were separated by gel filtration through a column of Sephadex G-200 equilibrated with 0.02 M Tris-HCl, 0.5 M NaCl, 0.02% NaN3, 1 mM EDTA, pH 8.0. The yields of IgM antibody averaged 0.05 mg/ml of pooled antisera and represented 15–20% of the specifically bound antibody against hapten. The final preparations were at least 95% pure by the criteria of sodium dodecyl sulfate-agarose-polyacrylamide gel electrophoresis, immunoelectrophoresis against goat antibody against rabbit Ig, and J chain stoichiometry.

**Microcomplement Fixation Assay.** Sheep red blood cell (Colorado Serum Co.) preparation, hemolysin (Microbiological Assoc., Inc.) titration, and whole guinea pig complement (Grant Island Biological Co.) titration were performed according to Wasserman and Levine (16) with two modifications: smaller quantities were used for titration and the diluent buffer consisted of 0.02 M triethanolamine-HCl, 0.128 M NaCl, 5 × 10−4 M Mg++, 1.5 × 10−4 M Ca++, 0.1% bovine serum albumin, μ = 0.15, pH 7.4 (TEBS).

For measurement of complement fixing activity a series of antigen or hapten dilutions was made in TEBS to a constant volume of 2.75 ml, and 20 μl of anti-Lac IgM antibody (1.55 × 10−5 μmol) were added to each tube as its contents were stirred. After equilibration for 20 hr at 4°, a series of 2-fold dilutions were rapidly made at 0° and an amount of complement in 0.5 ml that would give 0% lysis was added to each dilution. The tubes were incubated for 60 min in a 37° water bath, 0.2 ml of a 0.25% suspension of sensitized red blood cells was added at 0°, and the 37° incubation step was repeated. The solutions were then centrifuged and the absorbance of the supernatants was measured at 413 nm. Appropriate controls were included to determine the 90% endpoint and the extent of fixation by IgM alone. None of the Lac haptons or hapten-conjugates exhibited detectable anticomplementary activity.

Hapten inhibition of complement fixation was assayed by use of a similar procedure; to a series of 2.78-ml solutions containing increasing quantities of p-aminophenyl-β-lactoside were added 1.55 × 10−8 μmol of anti-Lac IgM antibody RNase antigen that gave 50% of maximum fixation. After 22 hr of equilibration at 0°, the complement fixing capacity of the solutions was determined as described above.

**Binding Measurements.** The affinity of IgM antibody against Lac for Lac dye was assayed by equilibrium dialysis in double sector cells, using an IgM concentration of 0.7 mg/ml, hapten concentration ranging from 2 to 7 × 10−8 M, and a diluent of 0.02 M Tris-HCl, 0.5 M NaCl, pH 7.5. After equilibration for 60 hr at 4°, the concentrations of free and bound Lac dye were determined from spectrophotometric measurements at 455 nm and molar extinction coefficients of 2.48 and 2.8 × 104 for the free and bound forms, respectively (10). The binding affinity of mono-Lac RNase was measured by ultracentrifugation of antigen–IgM mixtures in which the IgM concentrations ranged from 0.5 to 1.0 mg/ml and the antibody concentrations ranged from 0.5 to 5 × 10−5 M. After sedimentation for 30 min at 100,000 × g, the free antigen concentrations were determined from radioactivity measurements of the supernatants and the specificity activity of the RNase.

**Ultracentrifuge Analysis.** Velocity sedimentation was carried out on a Beckman Spinco model E analytical ultracentrifuge equipped with Schlieren optics. Double sector cells with quartz windows were used in an An-D rotor.

**RESULTS**

The results from a typical complement fixation assay are illustrated in Fig. 1. A curve was generated for each IgM–antigen mixture by plotting the percentage of complement fixed per dilution as a function of the IgM content of the dilution. The amount of IgM giving 50% fixation could then be derived from the curve and used as a measure of the extent of reaction. In duplicate experiments the 50% endpoints were found to be reproducible within 10% (see Figs. 2 and 4).

As the control titration shows, the anti-Lac IgM antibody exhibited a low level of fixation in the absence of antigen. The reaction was specific since neither the buffer solution nor the corresponding anti-Lac IgG antibody gave similar results. Moreover, the amount fixed was consistent from one IgM preparation to another, the 50% endpoint averaging 1.29 ± 0.09 μg. The cause of this reaction was not clear. Previous studies have shown that IgM can bind the first enzyme complex in the complement cascade (5, 17), but only aggregated IgM activates the subsequent enzymatic reactions that lead to cell lysis (6). When the preparations of anti-Lac IgM antibody were examined in the ultracentrifuge, a small aggregate fraction was detected (Fig. 3b). However, the...
amount varied from 2 to 15% in the different preparations and did not correlate with the fixation measurements.

In the presence of the homologous antigen, BGG derivatized with 25 hapten groups per mol, the IgM antibody fixed complement more efficiently (Table 1 and Fig. 1). The amount required for 50% fixation decreased as a function of antigen concentration to a minimum value of 0.09 μg. This relationship is illustrated more clearly in Fig. 2, where the 50% end-points determined for each IgM-antigen mixture are plotted against the logarithm of the determinant to IgM molar ratio in the mixtures. It can be seen that maximum reaction was achieved with the addition of 20 mol of Lac-BGG determinants per mol of IgM and was maintained even in extreme antigen excess, i.e., 2500 Lac groups per IgM molecule. Significantly, an identical curve was obtained when complement fixation by anti-Lac IgM antibody was measured in the presence of a small, heterologous antigen, reduced, and alkylated RNase substituted with 2.9 hapten groups per mol (Table 1 and Fig. 2).

These data indicated that antigen was not activating complement fixation by aggregating IgM molecules. The Lac-BGG and multi-Lac RNase antigens differed in their capacity to complex IgM because of differences in carrier size and hapten content; yet they were found to be equivalent on a determinant basis in inducing complement fixation. Moreover, both antigens effected maximum fixation at very high determinant to IgM ratios, where crosslinking between IgM molecules would be expected to be minimal. To obtain definitive evidence for the absence of aggregates at these ratios, ultracentrifuge analysis was carried out on anti-Lac IgM antibody in the presence of excess multi-Lac RNase (450 Lac groups per mol of IgM). The Schlieren pattern (Fig. 3a) showed only a single IgM-antigen complex which sedimented similarly to an IgM control (Fig. 3b).

The finding that fixation was independent of antigen–antibody aggregates suggested that antigen binding caused internal changes in the IgM molecule. Two different mechanisms could be envisioned. Multivalent antigen could cross-link combining sites within the pentamer and thereby alter the alignment or structure of Fc complement binding sites. Such “staple” formation has been observed for flagella reacted with anti-flagella IgM antibody (18) and for dinitrophenyl-substituted phage combined with anti-dinitrophenyl IgM antibody (19). Alternatively, antigen could simply interact at individual combining sites and induce an allosteric change at the distant Fc complement binding sites. Because it was not possible to resolve these alternative mechanisms by the use of multivalent ligands, a series of monosubstituted RNase reagents were synthesized. The preparations varied in their degree of substitution from 0.27 to 0.85 Lac groups per mol (Table 1), and all failed to precipitate anti-Lac IgM antibody over 1000-fold range of antigen to antibody ratios.

Their monovalency was substantiated by velocity sedimentation analyses in the presence of IgM antibody. Although aggregate formation was optimized at the concentrations used, 3 mol of antigen per mol of IgM, the Schlieren patterns showed only a few percent increase in the aggregate region (Fig. 3c) and the major RNase–IgM complex co-sedimented with an IgM control lacking antigen (Fig. 3b). These data also eliminated the possibility that the mono-Lac RNase molecules might associate in solution and function as a multivalent ligand.

When the monovalent antigens were tested in the micro-complement fixation assay, they were found to induce an excellent response (Fig. 4). Again the amount of fixation was related to the determinants added and not to the degree of hapten substitution. At determinant to antibody ratios below 40:1, the monovalent preparations were somewhat less effective than the homologous BGG antigen (compare Figs. 2 and 4). For example, the addition of 2 mol of mono-Lac RNase per mol of IgM was required to match the 50% endpoint obtained in the presence of one determinant mol of multi-Lac antigen. This difference, although small, was reproducible and probably reflected the relative binding affinities of the mono- and multivalent ligands. However, at determinant to IgM ratios above 40:1 the mono-Lac substituted preparations were as efficient as their multivalent counterparts in elic-
iting maximum fixation. These results showed that site filling by antigen was sufficient to generate changes in the IgM Fc structure and that crosslinking by multifunctional antigen was not required.

Supporting evidence for conformational changes was obtained by comparing the interaction of hapten and antigen with anti-Lac IgM antibody. When the small monovalent haptens, p-aminophenyl-\(\beta\)-lactoside and Lac dye, were reacted with the IgM antibody, they failed to cause any increase in complement fixation above that observed for IgM alone (Fig. 4). However, hapten was found capable of blocking the response induced by mono-Lac RNase. As the data in Fig. 5 show, relatively high concentrations of p-aminophenyl-\(\beta\)-lactoside were required; inhibition was not detected until the hapten-antigen ratio exceeded 2500:1, and complete inhibition was achieved at a twofold higher ratio.

The large amounts of hapten were necessitated by the multivalence of the IgM antibody. Since complement fixation was initiated by the binding of mono-Lac RNase to a single IgM site (Fig. 4), each of the ten sites had to be blocked by hapten to prevent reaction. In fact, it could be calculated from the binding constant of \(7 \times 10^7\) reported for p-nitrophenyl-\(\beta\)-lactoside (10) that 100% inhibition of fixation occurred when essentially all the IgM sites were theoretically occupied with hapten.

The difference in the capacity of hapten and antigen to elicit complement fixation was reflected in their binding to the IgM antibody. In equilibrium dialysis against Lac dye, the anti-Lac IgM antibody exhibited a homogeneous binding curve (Fig. 6) that extrapolated to the expected valence of 10. The \(K_a\) for the hapten was calculated to be \(1.6 \times 10^3\), in good agreement with the values reported by Karush (10).

A similar plot was obtained from ultracentrifuge measurements of the binding of mono-Lac RNase to anti-Lac IgM antibody except that the slope was flatter and gave a \(K_a\) of \(4.7 \times 10^4\). Since the affinity constant at equilibrium is a mixed function representing the net energy from binding and structural changes (20), the lower \(K_a\) obtained for mono-Lac RNase suggested that some of the energy of interaction was expended on the Fc conformational changes responsible for complement fixation. Moreover, the low \(K_a\) value excluded the possibility that the mono-Lac RNase molecules were associating after binding to the IgM and acting as internal crosslinkers. Such a model would require an affinity constant three to four orders of magnitude greater than that observed.

**DISCUSSION**

Previous attempts to demonstrate Fc conformational changes due to hapten or antigen binding have given equivocal results [see Metzger for a review of the literature (21)]. The binding of hapten has been shown to alter the volume, sedimentation rate, and circular dichroism spectrum of IgG antibodies (22-24), but analyses of the constituent Fab fragments indicate that most of these changes are confined to the combining site and its vicinity (24). Studies of multifunctional antigen binding have yielded more positive results. For example, the interaction of antigen with IgG antibody has been found to affect the environment of Fc tryptophan residues (25), and cooperativity has been observed in the binding of the first complement enzyme to antigen-antibody complexes (26). In many of these studies, however, complexities in the systems utilized made alternative interpretations of the data possible.

In the present study, interpretation of the data was simplified by using a built-in aggregate, IgM antibody, to test the effects of hapten and antigen binding. In addition, the assay for conformational changes depended on a physiologically significant reaction known to be mediated at Fc sites. Using this system, definitive evidence was obtained that antigen, but not hapten, binding alters the conformation of the IgM Fc region. The most convincing result was the finding that a monofunctional antigen induced complement fixation and the reaction could be blocked by hapten. The monovalency of the ligand eliminated the possibility that antigen was activating the Fc sites by aggregating or internally crosslinking IgM molecules, while the inhibition data showed that site-filling by antigen was responsible for the distal changes reflected in complement fixation. Moreover, the monovalent antigen was found to have a lower affinity for IgM than a nonproductive hapten ligand, suggesting that energy from antigen binding was utilized to activate the Fc complement binding sites.

Although these studies show that a carrier moiety is essential for the induction of conformational changes, the function of the carrier is not completely understood. It does not appear to contribute to the specificity of binding since equivalent fixation was obtained whether the determinant groups were conjugated to native or reduced and alkylated protein. Moreover, the binding measurements indicated that
the carrier does not greatly enhance affinity through non-specific protein-protein interaction. It is possible that a carrier group provides the small increment in affinity necessary to exceed the threshold value for conformational changes. On the other hand, the carrier may act as a bulky tail group that induces additional conformational changes over those of smaller hapten and thus drives the conformational changes from the vicinity of the combining site to the Fc region.

Because IgM antibody is a covalently linked pentamer, these studies do not define the role of aggregation in activating the Fc functions of monomeric immunoglobulins. In experiments with anti-Lac IgG antibody evidence was obtained that aggregation is required for complement fixation; mono-Lac RNase was found to be completely ineffective at determinant concentrations that gave maximum fixation with multi-Lac RNase. From similar results reported for IgG antibody by other laboratories (1, 6, 7) and the data on IgM antibody presented in this paper, it seems likely that both processes, aggregation and conformational changes, are essential for the expression of Fc function.

The information available on immunoglobulin structure leads to a similar conclusion. The IgG complement binding site has been located in the Cα2 Fc domain (27, 28), approximately 50–100 Å from the antigen combining site (29), and the IgM complement binding site may be even more removed (17). To alter the conformation of the Fc sites, energy from antigen binding must be transferred through several independent domains and the flexible hinge regions connecting the domains. These considerations suggest that effective signal transmission depends on stabilization of immunoglobulin structure. Such stabilization could be accomplished by antigen aggregation of monomer immunoglobulin or by the disulfide bonds linking the subunits of polymeric immunoglobulins. In the case of monomeric immunoglobulins, multivalent antigen would serve the dual functions of stabilizing aggregate structures and inducing Fc conformational changes.

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