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Immunology

Evidence for a long-range conformational change induced by antigen binding to IgM antibody

(J chain exposure/IgM conformational change)

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ABSTRACT **The effects of antigen binding on IgM antibody conformation were investigated by measuring the immunological reactivity of the Fc-bound J polypeptide. For such measurements anti-azophenyl-β-D-lactoside and anti-azobenzenearsonate IgM antibodies were examined in a J chain radioimmunoassay before and after complexing with various hapten-conjugates. The assays showed that (*i*) the accessibility of J chain determinants is very limited in uncomplexed IgM and (*ii*) their accessibility is significantly enhanced in the presence of an excess of specific antigen. In both antibody systems, enhanced J chain exposure was achieved with the homologous multi-hapten-substituted antigen (1.9-fold), with a small multivalent antigen in which three to four hapten groups were coupled to a heterologous carrier (1.55-fold), and with monohapten-substituted antigen (1.4-fold). Because the J chain is located in the terminal C_H4 Fc domains, these data provide direct evidence that a change in Fc conformation is induced by the binding of antigen to the distant Fab combining sites. Moreover, the data indicate that the changes in J chain exposure do not depend on crosslinking by antigen, but can be induced by the interaction of antigen at individual IgM combining sites.**

Evidence that the binding of ligand at antibody Fab sites induces conformational changes in the distant Fc portion has been difficult to obtain. Studies of hapten–antibody interactions have consistently given negative results: the binding of small monovalent ligands was found to cause local changes in the Fab domains (1, 2), but no significant effects on Fc structure or activity could be demonstrated (3). Studies with large polyvalent antigens, on the other hand, have generally given inconclusive data. Although the binding of such antigens to Fab sites is known to initiate Fc reactions, it has not been possible to distinguish whether these effects are mediated solely by antigen aggregating antibody molecules (4–8) or whether antigen-induced conformational changes may be required as well.

Recently, however, several new approaches to the problem have provided more positive evidence for conformational modification of Fc structure. By use of circular polarization of fluorescence, spectral changes have been detected in monomer IgG antibody following reaction with either mono- or multivalent antigen (9–11). These changes appeared to be located in the Fc domains because quite different spectroscopic patterns were observed when the constituent Fab fragments were reacted with antigen. Moreover, a new flexibility has been demonstrated in the hinge structure of IgG antibody that could allow an antigen signal to be transmitted to the Fc portion by a cascade of domain interactions (12–15).

Evidence for Fc conformational changes has been obtained in our laboratory by using a built-in antibody aggregate, hapten-specific pentamer IgM, and complement fixation as the index of Fc activation (16). Multi-hapten substituted antigens were found to induce a maximum response at very high determinant-to-IgM ratios at which crosslinking between IgM molecules was minimal. More importantly, a mono-hapten-substituted antigen was essentially as efficient as the multi-substituted preparations in effecting complement fixation and the reaction could be blocked by hapten. These results suggested that with built-in aggregates such as pentamer IgM the interaction of antigen at single sites was sufficient to activate complement binding in the Fc domains.

The present studies were undertaken to substantiate the complement fixation data by a different and more direct method of measurement. The choice of method was based on the observation that the J chain in mammalian IgM is folded within the Fc structure so that only a few antigenic determinants are available for reaction (17, 18). This observation suggested that the accessibility of the J chain determinants is a function of Fc conformation and thus changes in J chain accessibility could be used to assay the allosteric effects of antigen binding. For such measurements, the IgM fractions from rabbit anti-phenyl-β-D-lactoside and anti-benzenearsonate antibodies were incubated in the presence of excess hapten-carrier conjugate. The soluble complexes formed were then compared with uncomplexed IgM for their capacity to inhibit the binding of ^{125}I-labeled rabbit J chain to its specific antiserum.

METHODS AND MATERIALS

Preparation of Hapten-Carrier Conjugates. The immunizing antigens, Lac-BGG and Ars-BGG, were synthesized by coupling the diazonium salt of *p*-aminophenyl-β-D-lactoside (Lac) to carboxymethylated bovine γ globulin (BGG) and the diazonium salt of *p*-aminobenzenearsonate (Ars) to unmodified BGG at a mole ratio of 120:1, in pH 9.0 carbonate buffer (19). Crossreacting multi-substituted antigens were prepared by reacting Lac or Ars diazonium salt with reduced and alkylated bovine ribonuclease A (RNase) at mole ratios, 20–30:1, that yielded an average of three to five hapten groups per mol of RNase. Monofunctional antigens were obtained by reacting 1 μM diazonium salt to 2 μM RNase pretreated with succinic anhydride to block lysine ε-amino groups. The preparations were then reduced and alkylated and the diazotized molecules were isolated by affinity chromatography as previously described (16).

Preparation of Anti-Lac and Anti-Ars IgM Antibodies. Lac- and Ars-BGG were coprecipitated with alum and the mixture was injected into New Zealand White rabbits in increasing doses over a 4-week period (19). The animals were bled out 4 days after the last injection and the hapten-specific antibodies were isolated from the serum by passage through successive columns

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Abbreviations: Lac, *p*-azophenyl-β-D-lactoside; Ars, *p*-azobenzenearsonate; BGG, bovine γ globulin; RNase, ribonuclease A.

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of Sepharose 4B derivatized with Lac and Ars haptens (packed gel at 4 μmol/ml). The antibodies were eluted by application of a column volume of 0.5 M lactose or 0.3 M benzenearsonate and the IgM fractions were separated by gel filtration through a column of Sephadex G-200 or Sepharose 6B equilibrated with 0.02 M Tris·HCl/0.5 M NaCl/0.05% NaN_3/1 mM EDTA, pH 8.0 (16). The yields of Lac-specific IgM antibody ranged from 0.02 to 0.05 mg/ml of pooled antisera, and those of Ars-specific IgM antibody from 0.005 to 0.02 mg/ml. The purity of the preparation was assayed by sodium dodecyl sulfate/agarose/polyacrylamide gel electrophoresis, Ouchterlony analyses with anti-rabbit IgM and IgG antibodies, and J chain stoichiometry. By these criteria, the anti-Lac IgM antibody was contaminated with less than 5% IgG, whereas the anti-Ars preparations consistently exhibited a larger IgG contamination ranging from 10 to 15%.

J Chain Radioimmunoassay. The indicator protein, rabbit J chain, was purified by preparative gel electrophoresis of completely reduced and carboxymethylated rabbit secretory IgA (20), and 100-μg samples were radioiodinated by the lactoperoxidase method (21) under conditions that provided ^{125}I specific activities of 4 to 6 × 10^3 cpm/ng of protein. Antibodies to the completely reduced and alkylated rabbit J chain were raised in goats by injecting 10- to 100-μg doses of the protein emulsified in complete Freund's adjuvant. The IgG fraction was isolated from the goat antiserum by successive precipitation with 35%, 40%, and 40% neutral $(NH_4)_2SO_4$ and subsequent chromatography on a column of DEAE-Sephadex A-50 equilibrated with 0.0175 M PO_4, pH 6.9. The eluted material was then absorbed by passage through a column of Sepharose 4B conjugated with rabbit light chain, dialyzed against 0.01 M Tris·HCl/0.05 M NaCl/0.02% NaN_3, pH 7.5 and concentrated by ultrafiltration to approximately 10 mg/ml.

The amount of anti-J chain antibody that would give 50% binding was determined by titrating 1–2 ng of the ^{125}I-labeled J chain against doubling dilutions of the antibody. After incubation for 1 hr at 37° and overnight at 4°, precipitation of the immune complexes was facilitated by the addition to each tube of 12 μg of normal goat IgG and 60 μl of rabbit anti-goat IgG (Miles–Pentex). The reaction mixtures were allowed to stand for 1 hr at 37° and 30 min on ice and the precipitates were then separated by centrifugation and washed once with cold phosphate-buffered saline (75 mM PO_4/73 mM NaCl, pH 7.2).

For the inhibition assays the IgM antibodies (approximately 1 mg/ml) were preincubated for 1 hr at room temperature either alone or with an excess of hapten-carrier conjugate. In the case of multisubstituted antigen, a minimum of 500 determinants was added per mol of IgM because lower concentrations gave insoluble aggregates that interfered in the radioimmunoassay. In the case of the monosubstituted antigens, 10 to 30 determinants were added per mol of IgM. The solutions were then serially diluted and to each tube was added the amount of anti-J chain antibody selected to give 50% binding of the ^{125}I-labeled J chain. After incubation for 1 hr at 37°, 1–2 ng of radioiodinated indicator antigen was added to each tube and the incubation was continued for 1 hr at 37° and overnight at 4°. The complexes were then precipitated and washed as described above. All dilutions were made in 3% bovine serum albumin/0.1 mM phenylmethylsulfonyl fluoride/0.02% NaN_3/75 mM PO_4/73 mM NaCl, pH 7.2, and the reaction volume was kept constant at 0.5 ml. The total ^{125}I content of each tube and the amount of radioactivity precipitated were determined in a Nuclear-Chicago γ counter, model no. 1085, to an accuracy of at least 2%. The percentage of inhibition was calculated from the percentages of the radioactive material precipitated in the presence and absence of IgM or IgM–antigen complexes.

RESULTS

From previous studies it was known that reductive cleavage releases the J chain from Ig polymers without greatly altering the structure of its antigenic determinants (22). Use was made of this finding to assess the exposure of J chain determinants in rabbit IgM antibody. Intact IgM was examined for its inhibitory capacity in the J chain radioimmunoassay and the data obtained were then compared with the inhibitory capacity of the preparation after complete reduction and alkylation. As the anti-Lac IgM analyses in Fig. 1 illustrate, the intact pentamer was found to be a very poor inhibitor of J chain binding; 460 μg, containing 7.5 μg of covalently bound J chain, was required to block the binding of 1 ng of radiolabeled indicator by 50%. In contrast, the reduced and alkylated preparation was a highly effective inhibitor; 220 ng, containing 3.6 ng of free J chain, was sufficient to achieve 50% inhibition. From the magnitude of these differences it was clear that very little of the bound J polypeptide was accessible to reaction with the anti-J chain antiserum.

The poor inhibitory capacity observed for the anti-Lac antibody was characteristic of the behavior of rabbit IgM in the J chain radioimmunoassay. The 50% endpoint for five anti-Lac IgM preparations averaged 490 μg with a standard deviation from the mean of 21 μg (Table 1). The average value for five anti-Ars IgM preparations was higher, 580 ± 13 μg, but when corrections were made for the amount of IgG present, 10–15% in the anti-Ars preparations compared to less than 5% in the anti-Lac IgM, the anti-Ars and anti-Lac IgM values were indistinguishable. These results indicated that the limited exposure of the J polypeptide was a consistent feature of rabbit IgM antibody and thus measurements of accessible J chain determinants could be used to monitor the conformation of the surrounding Fc structures.

Significant changes in J chain accessibility were observed when the rabbit IgM antibodies were reacted with an excess of the homologous hapten-BGG conjugate. In the presence of 500 to 1000 determinants per mol of IgM the capacity of the IgM antibodies to inhibit J chain binding was essentially doubled (Fig. 2 and Table 1). The 50% endpoint of the anti-Lac IgM decreased from 450 to 250 μg, and that of the anti-Ars IgM decreased from 570 to 315–260 μg. These changes occurred only in the presence of the specific antigen. The addition of an equivalent excess of Lac or Ars hapten did not alter the IgM inhibitory capacity, nor did the addition of the heterologous

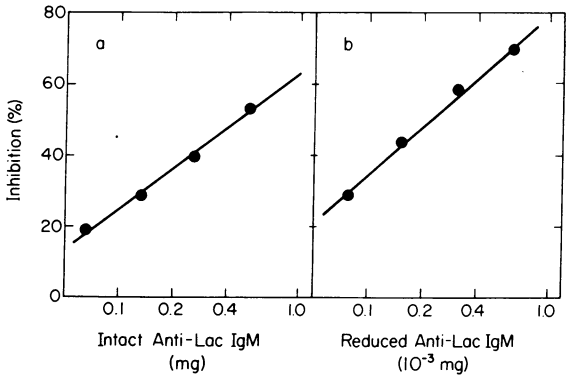

FIG. 1. Inhibition of ^{125}I-labeled rabbit J-chain binding to goat anti-rabbit J chain by (*a*) intact and (*b*) completely reduced and alkylated anti-Lac IgM antibody.

Table 1. Summary of J chain radioimmunoassays

Inhibitor	Anti-Lac IgM 50% end point, μg	Anti-Lac IgM Control/ complex	Anti-Ars IgM 50% end point, μg	Anti-Ars IgM Control/ complex
IgM control	450		575	
IgM–multihapten-BGG complex	220	1.80	290	2.00
IgM control	540		580	
IgM–multihapten-RNase complex	350	1.54	370	1.56
IgM control	540		620	
IgM–monohapten-RNase complex	380	1.42	450	1.38

hapten-BGG conjugate. As the data in Fig. 2 show, the 50% endpoint of anti-Lac IgM remained unchanged in the presence of 1000 Ars-BGG determinants per mol of IgM, and conversely, the 50% endpoint of anti-Ars IgM was not affected by incubation with 1000 Lac-BGG determinants per mol.

The mechanism of the antigen-induced change was pursued by examining the effect of hapten conjugates with different crosslinking properties. Small, multivalent antigens were constructed by coupling three to four Lac or Ars groups to a heterologous carrier, completely reduced and alkylated RNase. When these preparations were tested in the J chain radioimmunoassay, an enhancement of IgM inhibitory capacity was again observed (Fig. 3). The response was 82% of that obtained with the homologous BGG conjugate; at equivalent excess, 1000 determinants per mol of IgM, the RNase antigens increased the exposure of J chain determinants by an average factor of 1.55 compared to an average factor of 1.9 for the homologous antigen (Table 1). On the basis of these data, it appeared unlikely that antigen was producing Fc changes by aggregating IgM molecules. The RNase conjugates were almost as efficient as the homologous BGG antigens in exposing J chain determinants despite the difference in their crosslinking potential, and both types of conjugates were effective at high concentrations that minimized intermolecular bridging. Supporting evidence was provided by ultracentrifuge analysis of anti-Lac IgM antibody in the presence of excess multi-Lac RNase (450 determinants per mol of IgM). The Schlieren patterns showed only two peaks, one that corresponded to free RNase antigen and a second that

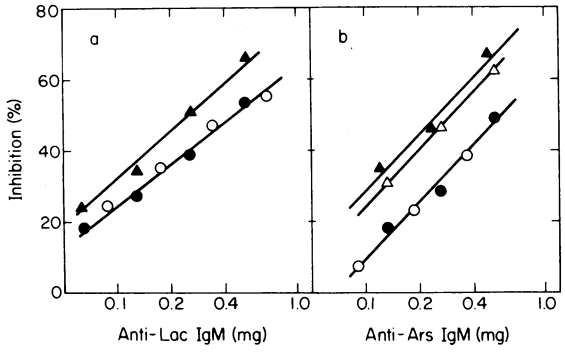

FIG. 2. Effect of homologous antigen binding on the J chain inhibitory capacity of IgM antibody. (*a*) Anti-Lac IgM in the absence (●) and in the presence of 1000 determinants per mol of IgM of multi-Lac BGG (▲) or multi-Ars BGG (○). (*b*) Anti-Ars IgM in the absence (●) and in the presence of 500 determinants per mol of IgM of multi-Ars BGG (△) or 1000 determinants per mol of IgM of multi-Ars BGG (▲) or multi-Lac BGG (○).

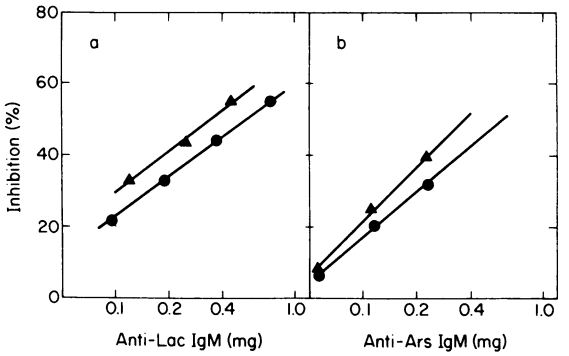

FIG. 3. Effect of small multivalent antigen binding on the J chain inhibitory capacity of IgM antibody. (*a*) Anti-Lac IgM in the absence (●) and presence (▲) of 1000 determinants per mol of IgM of multi-Lac RNase. (*b*) Anti-Ars IgM in the absence (●) and presence (▲) of 1000 determinants per mol of IgM of multi-Ars RNase.

sedimented similarly to unreacted IgM and thus represented single IgM–antigen complexes (16).

Monosubstituted conjugates were then tested to determine whether antigen was inducing changes in J chain exposure by binding at individual sites or by crosslinking combining sites within the pentamer molecules. The conjugates were prepared by diazotizing native or succinylated RNase under conditions that substituted an average of 0.02 to 0.03 hapten groups per mol. The resulting preparations were completely reduced and alkylated, and the singly substituted populations were selected by affinity chromatography. The final preparations contained an average of 0.6 to 0.75 azo groups per mol and were judged to be monovalent by precipitin and ultracentrifuge analysis (16). At low determinant/IgM ratios, these reagents were found to have no effect on the ability of the IgM antibodies to inhibit J chain binding (Fig. 4). However, when the antigen concentrations were increased to 25 to 30 determinants per mol of IgM, significant enhancement was obtained. As the radioimmunoassays in Fig. 5 show, the 50% endpoint of the anti-Lac IgM was reduced from 540 to 380 μg, and that of the anti-Ars IgM from 620 to 450 μg, an average 1.4-fold increase in inhibitory capacity (Table 1). Although the effects of higher doses were not determined because of the difficulties in preparing the amounts of antigen required, the positive responses observed in limited antigen excess indicated that antigen binding to single sites was sufficient to generate Fc conformational changes.

DISCUSSION

The titration of J chain determinants offers several advantages for measuring the allosteric effects of antigen binding to IgM

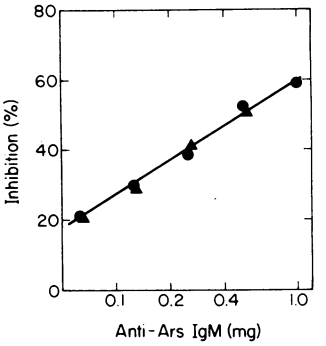

FIG. 4. Effect of monovalent antigen binding on the J chain inhibitory capacity of anti-Ars IgM antibody. No antigen (●); 10 determinants of mono-Ars RNase per mol of IgM (▲).

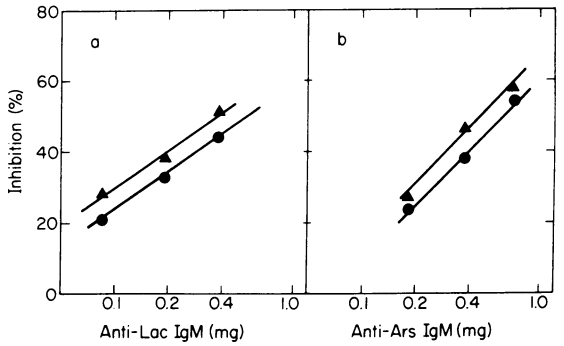

FIG. 5. Effect of monovalent antigen binding on the J chain inhibitory capacity of IgM antibody. (*a*) Anti-Lac IgM in the absence (●) and presence (▲) of 30 determinants per mol of IgM of mono-Lac RNase. (*b*) Anti-Ars IgM in the absence (●) and presence (▲) of 25 determinants per mol of IgM of mono-Ars RNase.

antibody. First, the J chain is located in a portion of the Fc far removed from the antigen combining sites. It is disulfide bonded to the terminal C_H4 domains (23), which form the inner core of the pentamer molecule and are separated by 150–200 Å from the outer rim of combining sites and by 70–100 Å from the flexible hinge connecting the Fab arms to the Fc region (24, 25). Because of these structural relationships, changes in J chain reactivity cannot be effected as a direct consequence of antigen binding, but must be mediated through conformational changes induced in the C_H4 domains. Second, the arrangement of the J polypeptide within the C_H4 domains is not easily altered. The analysis of rabbit IgM antibodies showed that the exposure of J chain determinants is consistent from one preparation to another and is not affected by large quantities of heterologous antigen. Studies with dissociating agents (18) have shown that the immunological activity of the J polypeptide is not increased by exposing the IgM to 8 M urea or 1 M propionic acid. Thus, the possibility of nonspecific changes in the C_H4 tertiary structure does not pose a serious problem in evaluating the data on antigen binding. Finally, the J chain has distinctive antigenic properties which are retained during reductive cleavage and isolation of the chain. This allows the determinants on the covalently bound J polypeptide to be unequivocally identified by their capacity to compete with free J chain in a radioimmunoassay.

By use of such assays, the binding of antigen to IgM antibody was found to increase the exposure of J chain determinants. Although the changes were small relative to the total antigenic potential of the free J chain, the values obtained in the presence of antigen were significantly different from those of the uncomplexed controls. Moreover, the increases were observed in two different antibody systems, one specific for the uncharged phenyl-β-D-lactoside moiety and the other specific for the negatively charged benzenearsonate group, and they were effected by hapten conjugates constructed with carriers of different size, structure, and net charge. The sum of these measurements makes it difficult to explain the enhancement of J chain reactivity by any other mechanism than an antigen-induced conformational change.

The J chain measurements provide only a qualitative index of the magnitude of changes in the three-dimensional structure of the C_H4 domains. The C_H4 conformation could be altered in regions that did not affect J chain exposure and such alterations would not be detected by the J chain radioimmunoassay. Moreover, the assay would not detect an increased exposure of immunosilent regions of the J polypeptide and might not detect changes in determinants of low affinity for the anti-J chain antibody. In view of these limitations it is significant that antigen binding was found to enhance the inhibitory capacity of the J polypeptide by factors ranging from 1.4 to 1.9.

The J chain measurements also corroborate previous findings from our laboratory on the mechanism of the antigen-induced changes (16). When allosteric effects in anti-Lac IgM antibody were monitored by complement fixation assays, the three hapten-conjugates, multi-Lac BGG, multi-Lac RNase, and mono-Lac RNase, elicited a good response. In each case, maximum fixation was achieved by the addition of 20 to 40 determinants per mol of IgM and was maintained over 100-fold higher determinant concentrations. A similar pattern of results was obtained in the present study; increases in J chain exposure were observed in the presence of both multi- and mono-substituted ligands and under comparable conditions of antigen excess, i.e., determinant/IgM ratios ranging from 25 to 1000.

Several questions could be raised concerning the evidence that monosubstituted antigens are capable of evoking Fc responses. For example, it could be argued that the preparations contained a small amount of disubstituted RNase that constituted the effective ligand. Although this possibility cannot be rigorously excluded because of the methods used to prepare the monovalent RNase, it appears unlikely from the similarity of the Fc responses to mono- and multi-substituted RNase. The two types of conjugates were found to be essentially equivalent on a determinant basis, not only at the excess levels used in the J chain measurements, but also at the very low determinant concentrations used in the complement fixation experiments. Moreover, recent studies in our laboratory have shown that a monovalent antigen prepared by coupling hapten to the single free SH at the active site of papain induces Fc changes comparable to those observed with the monosubstituted RNase ligands (H. C. Chiang and M. E. Koshland, unpublished observations). A second argument could be made that once monosubstituted antigen was bound to the IgM antibody, the carrier proteins interacted and thus effectively created a multivalent reagent. Again this possibility appears unlikely from the data obtained with modified carrier; succinylation of the RNase did not alter the effectiveness of the conjugate, although the large net negative charge introduced by this procedure would be expected to minimize carrier–carrier association. Thus, the evidence from two independent assays, complement fixation and accessibility of J chain determinants, indicates that Fc responses in IgM antibody are determined by the ability of antigen to interact at single sites and not with its ability to aggregate or internally crosslink IgM molecules.

The precise mechanism by which the binding of monovalent antigen at IgM Fab sites induces conformational changes at a distance of 150–200 Å remains to be determined. It seems highly unlikely that the energy from antigen binding could be transferred over that distance through strain induced in a flexible system. The energy would damp out too rapidly. A more likely mechanism of domain interaction has been suggested by x-ray crystallographic analyses of an IgG myeloma protein. From the data obtained it has been postulated (15) that antigen binding alters the angle between the V and C_H1 domains, bringing them in close proximity, and the energy transferred by this process in turn causes a folding back of the Fab-Fc hinge and allows contact and energy transfer from the C_H1 to the C_H2 domains. Presumably a similar series of domain interactions could occur in pentamer IgM, although the details of the contacts might differ because of the additional domain present. However, these gross changes in shape must be accompanied by intradomain conformational changes in order

to explain the activation of complement-fixing sites and the enhancement of J chain reactivity observed after monovalent antigen binding. Such conformational changes could be initiated at each domain contact or they could be restricted to domain interactions in the Fc region.

The essential role of the carrier moiety in the induction of IgM conformational changes also remains to be explained. A mechanism in which the carrier structure is specifically recognized by the IgM antibody is excluded because equivalent Fc responses were obtained with the hapten-conjugates containing heterologous or chemically modified carrier. Moreover, nonspecific carrier effects, such as ion atmosphere changes, appear to be ruled out by the equivalent effectiveness observed for conjugates of widely different charge and structure. A conceivable explanation is that the carrier acts as a bulky tail group that alters the hydrodynamic properties of the Fab arms and thus facilitates Fab–Fc contact.

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