INTRAMOLECULAR SIGNALING IN IMMUNOGLOBULINS - NEW EVIDENCE EMERGING FROM THE USE OF SUPRAMOLECULAR PROTEIN LIGANDS

INTRODUCTION

After the overall structure of immunoglobulin was recognized it became apparent that its structurally distinct fragments play different, specific role in the function of the antibody. While Fab fragments appeared to bind antigen, Fc was found to participate in complement fixation and the initiation of other effector activities. Whereas antigen binding does not seem to depend functionally on the Fc fragment, the latter reveals activity which in physiological conditions is triggered by attachment of the Fab fragment to the antigen. Some transfer of information must be postulated to explain such interdependence (1-4). The structural character of this signaling has been accepted in studies since the very
beginning, and attention has been focused particularly on the hinge region connecting fragments Fab and Fc (5-9). The suggestion of the central role of the hinge region in signal generation and transduction arose not only from its special structural position in the immunoglobulin molecule; support came particularly from the observation that subgroups of human immunoglobulin G differ in their ability to initiate effector phenomena and that this difference is associated with the length of the hinge peptide fragment (10-13). Further support has come from findings that antibodies with reduced interchain disulphides located in the hinge region failed to trigger effector activities (3,14). Although all these findings point to the hinge region as a key element in signal transduction, its exact role in this process has remained obscure. Signal generation has commonly been connected with torsional effects, based on the observed failure of small monovalent haptens to trigger effector activities, and on the known phenomenon of diminishing complement fixation ability under an excess of antigen (15).

Two basic mechanisms of signal generation have been commonly considered. The "distortive mechanism", with the assumed tension generated by antigen binding which forces structural alterations involving finally the Fc fragment and the "allosteric model", with the postulated operation of a structural "all or none" switching mechanism in antibodies that trigger effector activation.

As knowledge of immunoglobulins and their biological function has developed, however, immunologists have become aware that accumulation of antibodies in immune complexes is necessary to induce effector activities. This idea has opened a new page in understanding the role of immunoglobulins in complement activation and has led to the elaboration of the so-called "associative model" as the third possible mechanism of signaling (16,17).

The known multivalence of the C1q component clearly played a role in establishing this hypothesis. Meanwhile, the lack of convincing arguments to unequivocally support the allosteric and/or torsional models has pushed forward the associative model as apparently the only reliable mechanism explaining complement activation by immune complexes (18-19).

Hope of finding new evidence supplying a structural basis for intramolecular signal transduction returned with the improvement of methods to produce monoclonal antibodies and also crystallographic techniques allowing detailed identification of the 3-D structure of Fab fragments and their complexes with the antigen. Unfortunately, the small and diverse changes found to be generated by binding of antigens in most observed cases cannot be generalized or identified with signaling (20-27). This has disappointed those who expected to find arguments for an intramolecular signaling model as a mechanism independent of the one represented by associative model.

This failure could to some extent have been expected since the torsions derived from antigen binding performed on isolated Fab fragments, instead of on the whole antibody molecule, cannot generate as significant alterations in antibody structure as could be produced by a bivalent antibody upon
complexation with randomly distributed antigenic determinants. Such an explanation is strongly supported by observations that only complete immunoglobulin molecules that become attached to a large antigen may initiate the complement cascade (28-30).

The huge genetic progress in protein engineering techniques attained in the 1980's again directed attention to the intramolecular signaling problem and stimulated many efforts to verify the idea of the torsion-derived origin of the signal. These works confirmed the structural and functional relation between the CH$_1$ and CH$_2$ domains mediated by the hinge fragment which determines antibody flexibility and finally effector activation and thus is supposed to represent part of the signaling pathway. They also indicated that simple aggregation of immunoglobulins is itself not enough to induce the fullest possible effector activation (31-33).

Surprisingly, these elegant genetic studies also produced some apparently contradictory data challenging the theory of hinge participation in signaling, showing how really complicated the problem is (34-41). The essential role of the hinge region and particularly its upper part in complement activation seemed grounded, but some works presented observations of still active immunoglobulins deprived of the hinge region.

Other discordant data, obtained in respect to complement activation by IgG4, revealed unexpectedly that the short hinge is not the exact site responsible for the inactivity of this immunoglobulin subclass, and that after suitable alteration of a single amino acid in the CH$_2$ domain its activity becomes restored (34-41).

Our group used antibodies chemically recombined from Fab and Fc fragments separated after proteolysis and rejoined by disulphide bridging of the hinge peptide stumps, and showed that artificial chemical coupling of Fab and Fc fragments may produce some immunoglobulin derivatives still able to trigger some effector activity. However, the molecules combined from single Fab and Fc fragments having almost unlimited rotational possibilities, allowing optional contact and interaction of Fc with Fab, appeared completely inactive. Surprisingly, with a second (nonspecific) Fab arm introduced, these antibody molecules showed reasonably high activity in triggering effector phenomena, likely due to a special arrangement of the hinge peptide stumps, which may limit the rotational movement of Fc. This result indicated that although the antibody molecule seems to need increased flexibility to obtain efficient effector activity, excessive rotational freedom is unprofitable (42,43). It is also consistent with findings that with a very long hinge fragment or reduced inter-heavy chain disulfide the effector activation deteriorates (33,44).

Finally, although the majority of these studies supported the existence of intramolecular signaling, which seems sufficiently identified at least in the CH$_1$-CH$_2$ step of signal transduction, the whole phenomenon remains unclear. In this situation it has become obvious that to identify the antigen binding-generated structural changes accompanying the signaling process, they should be directly,
chemically determined and if possible arrested by complexation with a specific ligand to prove their connection with the biological function. The enhancement of antigen binding affinity should then be taken as evidence of the torsional origin of the structural changes fixed by binding of the dye-ligand.

Support for such an expectation has come from studies in which antibodies raised against primary immune complexes caused an increase of their affinity (45,46). In that case, however, the enhancement cannot be connected exclusively with stabilization of structural changes extorted in primary antibodies as the result of antigen binding, since the secondary antibodies may simply disturb dissociation of the primary immune complexes (by steric hindrance). It is also possible that antibodies modify the structure of an antigenic protein (47).

Evidence that supramolecular protein ligands recognize signal-specific conformational changes in immunoglobulins

A promising solution of the problem came with the finding of special protein ligands which appeared to attach to specific receptor sites in antibodies, distinct from the antigen binding site and accessible only in antibodies actually engaged in immune complexation (48,49). As could be expected, they were found to have an effect on both antigen binding and complement fixation (50).

These ligands were bis-azo dyes with liquid crystalline properties, represented by Congo red, Evans blue and some other related compounds known for years as amyloid markers (51). The basis for more general use of these dyes emerged from the finding that they may interact with proteins as supramolecular but compact

![Fig. 1. Model of supramolecular Congo red ligand anchored between polylysine peptides (shown as backbone chains) created by molecular dynamics simulation (59).](image)
ligands with particular new properties different from those of single monomeric molecules (52-55). Their ribbon-like micellar entities which form a mesophase may create highly stable complexes by adhesion to peptides of $\beta$-structure (Fig. 1) (56-59). A few-molecule micellar dye ligand replaces unstable polypeptide fragment, competing with it efficiently, or else penetrates between the polypeptide chains of the $\beta$-pleated sheet (58). Local instability may be induced in the protein molecule, allowing the dye to penetrate, by moderate heating or by other unfolding conditions applied. It may also occur as a result of function-derived constraints that generate structural changes in the protein. Such events do happen in antibody molecules upon antigen binding (48-50). This is what seems to make the antibodies accessible to dye complexation, which fixes the changes in structure extorted by antigen binding in the antibody, and is likely responsible for the observed enhancement of the antibody's affinity to antigen. This was

Fig. 2. The enhancement effect of bis-azo dyes on binding of anti-SRBC (sheep red blood cells) antibodies to red cells. The number of antibodies bound to erythrocytes was determined using radiiodinated antibodies (48). The initial point of the curve corresponds to the number of antibodies bound in the absence of Congo red. Inset: Correlation of the self-assembling capability of the dye with the enhancement effect on antigen-antibody interaction. Two isomeric dyes were compared: Evans blue (a, □) - high self-assembling tendency, and Trypan blue (b, Δ) - low self-assembling tendency. The enhancement of antigen-antibody interaction in the presence of the dye is shown as the increase of the maximum dilution of antibodies still yielding agglutination in the SRBC-antiSRBC system (49).
registered as increased engagement of low-affinity antibodies in immune complex formed by polyclonal antiserum (Fig. 2) (48).

The enhancing effect was absent, however, when Fab as well as (Fab)$_2$ fragments were used instead of whole IgG (50). This coincides with findings of significant structural alterations only in whole antibody molecules (28-30), again supporting the suggestion that these structural changes play the fundamental role in intramolecular signaling. Only strongly self-assembled dyes with a ribbon-like micellar organization may form complexes of sufficient stability with proteins and become specific indicators of signal-related structural changes in immunoglobulins (30,53). This particular property ensures the formation of stable complexes of antibodies with self-assembled dyes, unlike with single-molecule compounds. It arises from the plasticity of the supramolecular ligand (57-59) and is due to the large area of low-polarity interaction contact, allowing strong adhesion to chain-like periodic polymers (including peptides of $\beta$ structure). The dyes may differ in protein binding affinity even if they are closely related structurally. This is correlated with their self-assembling activity. The two dyes compared for this particular purpose were the isomeric bis-azo dyes Evans blue and Trypan blue. The first represents a high self-assembling tendency and also appeared to significantly enhance antigen binding; the second, with a low self-
assembling tendency, weakly affected antigen-antibody interaction (Fig.2, inset) (49,60). The critical role of supramolecularity for protein complexation was recently confirmed based on a large group of self-assembling dyes (53).

Immune complexation-induced dye binding occurs at ambient temperature, supporting the expectation that an antibody molecule accessible to a large ligand basically preserves native structure. This means that binding susceptibility involves rather a defined local area. The known 3-D structure of immunoglobulin molecules helped suggest the site responsible for binding the dye. Studies intended to identify this site were performed on isolated L lambda chain dimer subjected to unfolding conditions (gradually increased heating) in a search for the low-stability locus (61). The dye-receptor site found in this way appeared to be located in the V domain, representing the cavity which in native, naturally packed protein is occupied by the N-terminal chain containing residues 6-12 and 17-21 (61,62). The increased mobility of this polypeptide fragment at elevated temperature allows penetration and binding of the dye in its supramolecular

\[ \text{Fig.4. Heat-induced formation of IgG complexes with dyes of different self-assembling capabilities (54). Dye-IgG complexes formed under 20 min incubation of IgG-dye mixture (0.6µM IgG, 60 µM dye, in 0.05 M Tris-HCl pH 8.0, 0.15 M NaCl) at the given temperature. Complexes (characterized by increased electrophoretic mobility) were separated by agarose gel electrophoresis from dye-free IgG. The fixed and stained gel was analyzed quantitatively [Sigmagel software]. ANS (8-anilino-1-naphthalenesulfonic acid) binding was determined in a separate experiment (6 µM IgG, 24 µM ANS in 0.05 M Tris-HCl pH 8.0, 0.15 M NaCl, 20 min incubation at the given temperature) by measurement of the increased ANS fluorescence emission (λ_{exc} = 374 nm).} \]
ligation form (Fig. 3). For dye complexation, the polypeptides to which the dye ligands adhere need to have β-structure (58,59). A four molecule dye ligand was found to be attached to the binding locus in this case (61). Few-molecule Congo red ligation to L chain was observed also in other studies (63). The N-terminal polypeptide fragment, which cannot be repacked to its locus occupied by the dye ligand, becomes fully exposed and may easily be split off by digestion (61). That some polypeptide fragments of low packing stability are really present in the whole immunoglobulin molecule proves the observed higher accessibility for binding supramolecular dyes revealed in human IgG upon heating at temperatures below melting, in contrast to poorly or not self-assembled compounds which need fully destabilized protein for complexation (Fig. 4) (54). Unfortunately, the binding of Congo red to antibodies in the immune complex cannot be measured simply, because the different binding engagement of individual immunoglobulin molecules produces different distributions of torsion-derived structural alterations, making the results unreliable.

Even so, the dye complexation made possible by antigen binding was evidenced. This was done by electrophoresis, which revealed the higher migration rate of soluble immune complexes formed by interaction of antibodies with bivalent haptens in the presence of dye (Fig. 5) (64). Comparative proteolysis of rabbit IgG anti-TNP immune complexes performed in the presence and absence of Congo red revealed extra exposure of the polypeptide chain to proteolysis in the CH1 domain (to be published). This may support suggestions of inter-domain V-C coupling in the signaling process.

Whether packing destabilization of the N-terminal polypeptide really initiates signaling transduction in antibodies remains to be verified. The specific location of this polypeptide fragment in immunoglobulin makes such a role very likely. Its extensive mobility may affect antigen binding and the interdomain hinge fragment as well (65). Such a location makes it particularly exposed to rotational movements of the Fab domains, which naturally associate bivalent antibody binding to antigen determinants distributed and oriented randomly on the antigen surface.

The engagement of V-C interdomain hinge fragments in antigen binding and as well as effector activation is commonly noted. This may suggest their essential role in signal transduction (66-73).

Genetic studies basically connect the segmental flexibility of antibodies with the CH1 domain and in particular with the hinge fragment. Surprisingly, however, no special changes associated with antigen binding have been convincingly shown in Fc fragments (3,74). Congo red attachment to this fragment is poor even upon heating. This may mean that the Fc fragment remains beyond the direct scope of the signal-derived structural alterations. Such a conclusion sounds contradictory since the Fc fragment is indeed known to interact directly with Clq to initiate the complement cascade. A possible explanation for this apparent contradiction is that the exposure as well as the increased rotational freedom of
the whole Fc, rather than intra-molecular changes in this fragment, create the readiness of the immunoglobulin molecule to initiate the complement cascade. The need for the high rotational freedom of Fc fragments makes sense if one expects effective C1q attachment by antibodies gathered on the antigen but randomly oriented. The polyvalence of the C1q component and low association constants of individual IgG - C1q interactions clearly substantiate the need for the highest possible Fc rotational freedom to optimize the contacts (11). This may be

Fig. 5. Binding of Congo red to immune complexes as revealed by agarose gel electrophoresis (64). A - Complexes of IgG anti-TNP with bivalent TNP hapten (oxidized glutathione with amino groups substituted with TNP) bind Congo red and migrate faster (arrow) than noncomplexed IgG. Control - sample with no antigen added. 1-5 samples with increasing antigen concentration. The Congo red solution was applied directly to agarose gel along the whole width of the plate. B - Quantitative analysis of the experiment presented in A.
done by extension of the hinge region, with a subsequent increase of flexibility within the immunoglobulin molecule. The extension of the hinge region was proved to occur in some cases upon binding of antibodies to antigen. It was observed using specific antibodies risen against hinge peptides of target immunoglobulin molecules (75).

Thus, C1q binding seems to require that antibody molecules gather on the antigen and that torsion-dependent structural alterations increasing the flexibility of the molecule occur. They probably are universal restriction mechanisms in controlling highly diverse immunological phenomena. This combined signaling system forms an efficient restriction barrier protecting the immune system against generation of random signals.

The protein distortion-derived mechanism of signal discrimination seems widespread in the immune system. It was recently suggested to occur also in cell-cell interactions. The dye binding observed at the cell-cell interfaces of actively interacting monocytes and cancer cells is believed to be associated with adhesion receptors having standard immunoglobulin fold domains (76).

A possible explanation of the above phenomena is that the V domains, expected to fully decouple from C domains upon dye complexation, are liberated from antigen binding-associated distortion and may seize antigenic determinants much easier. Such a mechanism would well explain the observed enhancement of antigen binding. The complete liberation of the N-terminal chain fragment observed in the Congo red-protein complex in immunoglobulin L λ chain does not seem to occur in the absence of dye, as may be judged from its lack of proteolytic susceptibility (61,62). Its increased flexibility is clearly sufficient to make the domain susceptible for dye binding. The noticeably higher flexibility of the N-terminal polypeptide chain fragment in many proteins and its particular role in domain stability is often observed (75-81). Here the role of clutch seems to elucidate the function of the N-terminal fragment in the V domain of immunoglobulin. It could allow partly independent rotation of the V and C domains, facilitating binding antigen determinants, but also help to keep the conjunction of these domains sufficiently strong to enable generation of signal transduction.

Since the susceptibility of the VH and VL domains to torsion-derived constraints is expected to differ, full symmetry of the rotational movements seems unlikely. The different elbow angles observed in non-complexed Fab and Fab-antigen complex are perhaps the best evidence of that (82). It may induce relative rotation of the V and C domains, generating signal transduction.

CONCLUSIONS

The complexity of intramolecular signaling generated by antigen binding has escaped complete analysis, and despite many studies confirming it the problem is
still subject to controversy (26,31,83). The use of self-assembling dyes with a specific affinity for antibodies altered upon antigen binding, and simultaneously affecting their functional properties, has supplied new evidence supporting intramolecular signal transduction. Supramolecular ligands are helpful reagents in studies of intramolecular signaling, and also interesting in view of their possible application in therapy.

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