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 Mini-Reviews in Medicinal Chemistry, 2014, *14*, 1104-1113

Intramolecular Immunological Signal Hypothesis Revived - Structural Background of Signalling Revealed by Using Congo Red as a Specific Tool

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Abstract: Micellar structures formed by self-assembling Congo red molecules bind to proteins penetrating into function-related unstable packing areas. Here, we have used Congo red - a supramolecular protein ligand to investigate how the intramolecular structural changes that take place in antibodies following antigen binding lead to complement activation. According to our findings, Congo red binding significantly enhances the formation of antigen-antibody complexes. As a result, even low-affinity transiently binding antibodies can participate in immune complexes in the presence of Congo red, although immune complexes formed by these antibodies fail to trigger the complement cascade. This indicates that binding of antibodies to the antigen may not, by itself, fulfill the necessary conditions to generate the signal which triggers effector activity. These findings, together with the results of molecular dynamics simulation studies, enable us to conclude that, apart from the necessary assembling of antibodies, intramolecular structural changes generated by strains which associate high- affinity bivalent antibody fitting to antigen determinants are also required to cross the complement activation threshold.

Keywords: C1q binding, complement activation, Congo red, immune complexation, supramolecular protein ligand.

1. INTRODUCTION

Attempts to find evidence of intra-molecular immunological signalling associated with antigen binding and complement activation have a long history [1]. The signal is usually postulated to be structural in character, initiated by antibody-antigen binding and transmitted by sequential structural changes in the Fc fragment, enabling it to initiate effector activity. The peculiar domain arrangement of immunoglobulins which facilitates intramolecular movement, makes this idea highly plausible; however formally proving the hypothesis by identifying signal- derived specific structures is not an easy task. Failure to obtain conclusive evidence is primarily caused by the non-homogeneity of immune complexes, which in their complement- activating form are typically represented by polyclonal population of bivalent antibodies bound to large insoluble antigens – mostly cell surfaces [2-4]. Moreover it appears that information obtained from X-crystallography studies of Fab-hapten complexation cannot be directly compared to that of bivalent antibody binding due to different structural constrains generated in both cases [5-10]. This indicates that commonly used techniques are insufficient to resolve the problem. The question therefore remains: what is the nature of the signal (if it is indeed intramolecular)?

Two hypothetical models, based on torsional and allosteric mechanisms, were initially proposed to identify intramolecular signal transduction. Since neither yielded conclusive evidence, another idea – based on the so-called associative model – was suggested. This idea assumes that simple aggregation of antibodies on the antigen surface is sufficient to trigger effector activity, rendering intramolecular changes unnecessary [11]. Aggregation and suitable arrangement of antibodies is a required step in matching the structural and C1q binding requirements [12]; however accurate biological control over the system seems implausible if assembly of antibodies is the only factor determining complement activation [13, 14]. This observation has led to further investigations and the search for an independent controlling mechanism [15, 16].

An entirely unexpected breakthrough in studies of intramolecular structure-dependent signalling induced by antigen complexation was obtained by using Congo red as a specific supramolecular ligand and indicator of structural changes [17-20]. The choice of this particular dye was motivated by its unique ability to self-assemble and penetrate (in the aggregated form) into protein molecules, which "open up" as a result of local or global structural alteration.

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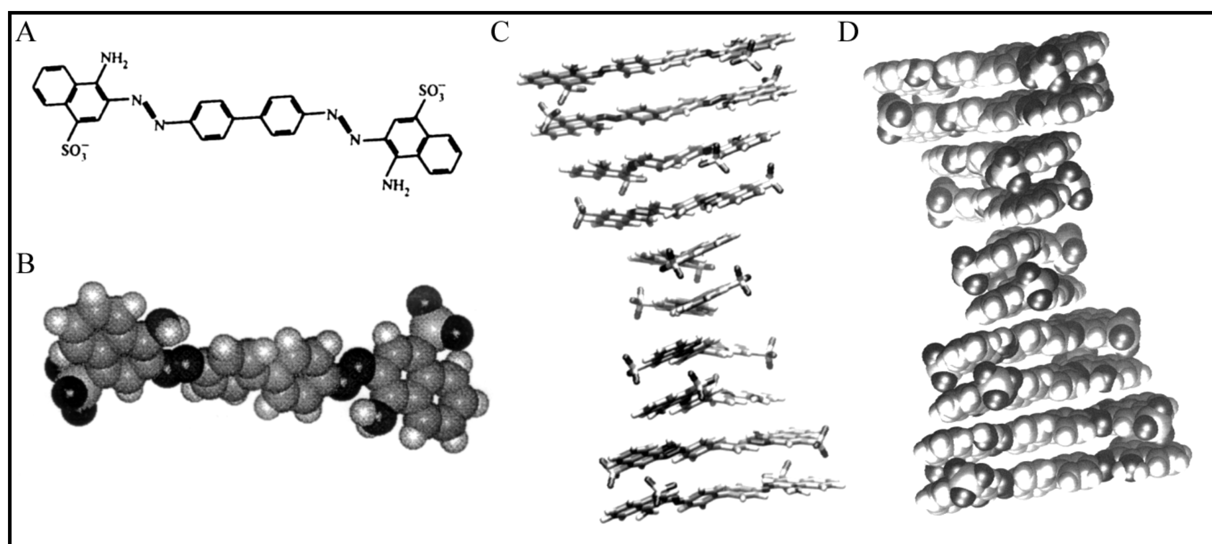

Fig. (1). Congo red. Single molecule and an MD simulation-derived model of its supramolecular organization. (**A**): Congo red molecule – structural formula. (**B**): Congo red molecule – space filling model. (**C**): Arrangement of molecules in supramolecular Congo red organization in water. (**D**): arrangement of molecules of supramolecular Congo red – space-filling model.

Congo red is often used for staining and for interaction with amyloids – (Fig. **1**) [21, 22].

Although the self-assembling tendency of this dye has been known for years, its binding form is typically modeled as a pool of individual molecules [23-26]. The fact that Congo red may interact with proteins as a supramolecular ligand fundamentally changes the interpretation of its character as well as the effects of its interaction. Proteins become susceptible to Congo red penetration in unfolding conditions, but also as a result of local, structural instabilities associated with biological function [27-29]. As a rule, complexation of supramolecular ligands happens outside of the active site; however it may significantly affect the protein's biological properties. This is due to the fact that – by penetrating into the protein in its active state – the ligand effectively stabilizes it and enhances its function. Such enhancement is observed when the protein complex formed represents the final output of a biological process. When applied to intermediate, transient forms – such as enzyme-substrate complexes – uncompetitive inhibition should be expected instead since the ligand prevents the complex from dissociating (by arresting the protein structure in its transient state). Antibodies which form complexes with antigens appear capable of binding Congo red [30]. This ability results from structural changes generated by strains associated with interaction between the antibody and the antigen.

2. THE CONGO RED-INDUCED ENHANCEMENT EFFECT

Congo red-induced stabilization of the antibody-antigen complex manifests itself as enhancement of the complexation process under increased concentrations of Congo red. This effect is easily measurable via agglutination using the standard SRBC-antiSRBC immune complex. An increase in agglutination (and therefore in formation of antibody-antigen complexes) is observed, with more antibodies participating in such complexes. Fig. (**2**) presents the binding of radio-iodinated IgG antibodies (^{125}I) from the polyclonal anti-SRBC serum labeled in a standard way [31] to red cells as well as the corresponding agglutination induced by increasing concentration of Congo red. The enhancement is expressed as an increase in the quantity of antigen-binding antibodies caused by the engagement of low- affinity antibody fraction, which is removable by washing in the absence of Congo red.

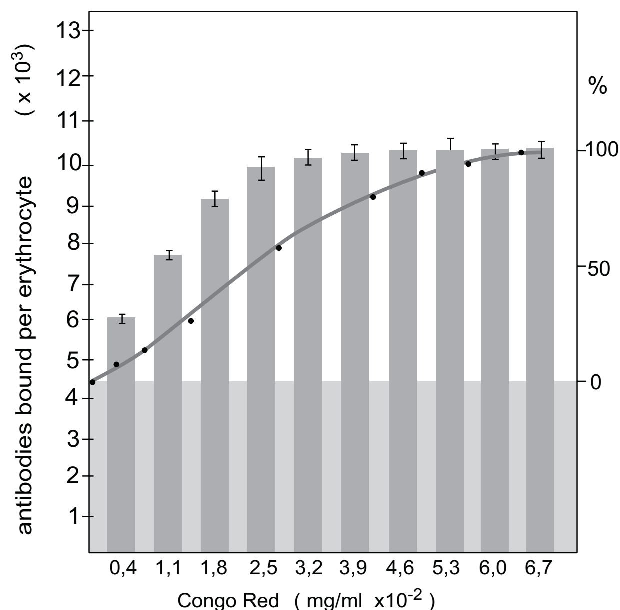

Fig. (2). Enhancement of immune complexation (SRBC-antiSRBC) by Congo red studied using radio-iodinated antibodies [31] with the Congo red effect expressed as the involvement of low-affinity antiSRBC antibodies from the polyclonal IgG fraction (white background), together with a curve presenting the corresponding increase of agglutination (%) above the level produced by high-affinity antibodies which do not require dye to undergo complexation (gray background).

Immune complex formation enhanced by increasing concentrations of Congo red was assessed using an agglutination assay with sheep red blood cells (SRBC), and also binding of radio-labeled (^{125}I) anti-SRBC-specific polyclonal IgG to red cells [31]. The average quantity of SRBC-specific antibody molecules bound to erythrocytes was determined on the basis of radioactivity measurements. Shaded background: high affinity antibody binding (agglutination in the absence of Congo red); white background: binding of low affinity antibodies stabilized in the immune complex by Congo red (removable by washing in the absence of dye).

The superimposed curve represents the corresponding measure of the enhancement effect expressed by increased agglutination (in percentage points), assuming that the agglutination level without Congo red is 0% and that the observed plateau represents a value of 100%. All calculations were based on a known number of erythrocytes and concentrations of antibodies used.

The presented fraction participates in agglutination which is enhanced by Congo red. The polyclonal antiserum contains a population of antibodies with various degrees of antigen affinity. This is due to the antibody synthesis mechanism, which produces antibodies capable of generating highly stable complexes, but also a large quantity of antibodies which only weakly interact with the antigen. The presence of Congo red enables a greater fraction of these short-lived complexes to persist. As the dye concentration increases, more antibodies bind to the antigen – they are literally "caught in the act" of complexation. When Congo red is not present, low-affinity antibodies do not form stable complexes and are easily washed out. While Congo red reinforces all complexes (including stable ones), the effect is most readily visible and detectable in the case of weakly interacting antibodies whose affinity for the antigen is too low to ensure formation of a stable complex under normal conditions (*i.e.* without the dye).

Not all supramolecular structures are capable of binding to proteins and interacting in this manner. Only those ligands which consist of strongly self-assembling molecules with a ribbon-like micellar structure can form stable complexes. This is due to the exposure of hydrophobic fragments, large interaction surface and high plasticity of the ligand (caused by noncovalent stabilization of associated molecules) [32]. This type of complexation does not cause denaturation and allows the protein to retain its function [27, 28, 33].

Protein binding of Congo red as a supramolecular ligand has been confirmed by applying the dye as a rhodamine B carrier in a system where pure rhodamine B does not bind to the target protein (*e.g.* non-native light chain of IgG). Rhodamine B molecules intercalated into the carrier dye (micellar Congo red structure) penetrate into target proteins and form cumulative complexes (Fig. **3**). The formerly observed correlation between the self-assembling tendency

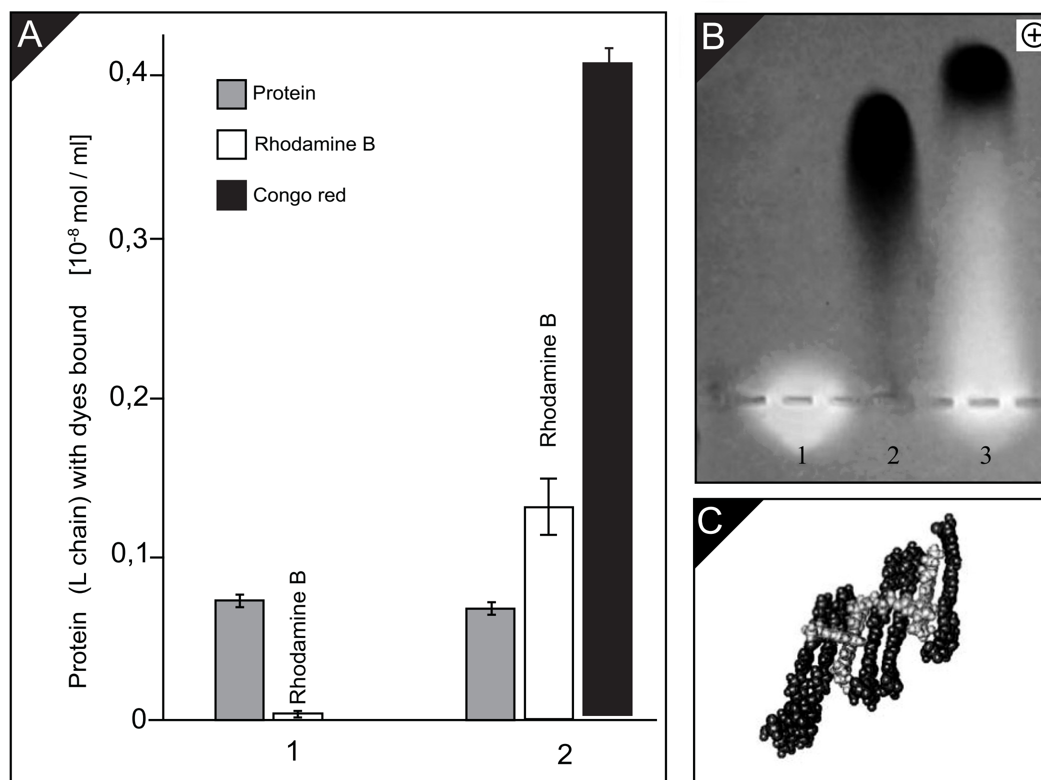

Fig. (3). Supramolecular ligation character of Congo red revealed by using it as a carrier of intercalated non-bonding rhodamine B. (**A**): Differing outcomes of rhodamine B incorporation without (1) and with (2) Congo red. (**B**): Electrophoretic migration of individual dyes registered in UV. 1: rhodamine B, 2: Congo red, 3: co-assembled dyes (1+2). Veronal buffer; pH=8.6; agarose bed. (**C**): Structural model of co-assembled dyes.

of different dyes and their ability to participate in protein complexation also independently confirms the supramolecular ligation mechanism [28, 29, 34].

Congo red only appears to react with immune complexes formed by complete bivalent antibody molecules. No reaction with the dye and no enhancement effects are observed when antibodies are replaced by Fab or even $(Fab)_2$ fragments obtained by digestion, indicating that in the absence of Fc the extent of strain-derived structural alterations is not sufficient to render antibodies capable of binding the dye [35-38]. The fitting of bivalent antibodies to randomly distributed antigenic determinants seems to be the main cause of strains in natural conditions, triggering structural changes responsible for binding the dye. This strain generation mechanism differs from that, which is caused by adjustment and fitting within the binding site: it is independent of the specificity of interaction and therefore more prevalent [2, 39-44]. Nevertheless, while the described mechanism appears preferable, the involvement of strong targeted interactions in strain generation cannot be entirely discounted [7].

2.1. Localization of Dye Ligands in Immunoglobulin Domains

Studies concerning the localization of complexed Congo red dye in immunoglobulin domains have been performed using monoclonal L chains purified from the urine of patients suffering from multiple myeloma. Two electrophoretically differentiated complexes (representing slow- and fast-migrating fractions) were distinguished by heating L λ chain dimers in the presence of Congo red, or incubating them under increasing dye concentrations (Fig. **4**). Structural analysis of protein-Congo red complexes enabled us to localize Congo red ligands in V domains.

The binding site was identified following analysis of trypsine digestion products of the slow- migrating complex. Digestion revealed that – under gentle heating – the N-terminal polypeptide chain packing area becomes the least stable structural element in the domain, rendering it susceptible to dye penetration. The supramolecular dye ligand occupies a cavity formed by removal of the N-terminal fragment of the polypetide chain. This exposed fragment may be easily cleaved by trypsine digestion. PAGE-SDS electrophoresis and amino acid sequencing of proteolysis products enable precise identification of the dye binding site [19, 20, 45].

A four-molecule dye ligand occupying the above mentioned cavity produces the slow- migrating complex. This initial complexation creates conditions which favor further dye penetration. In contrast, an eight-molecule ligand is involved in the fast-migrating complex. Dye penetration in this phase is gradual and the complex may also be represented by L chain dimers containing ligands with 5, 6 or 7 assembled dye molecules per chain. This results in a streaky electrophoretic pattern as the migration rate of complexes depends on their dye- derived charged load. Dye complexation is reversible and the initial form of the light chain may be reconstituted following removal of dye by long incubation with a material which strongly binds Congo red (*e.g*. Sephadex G25) – see Fig. (**4**) [46].

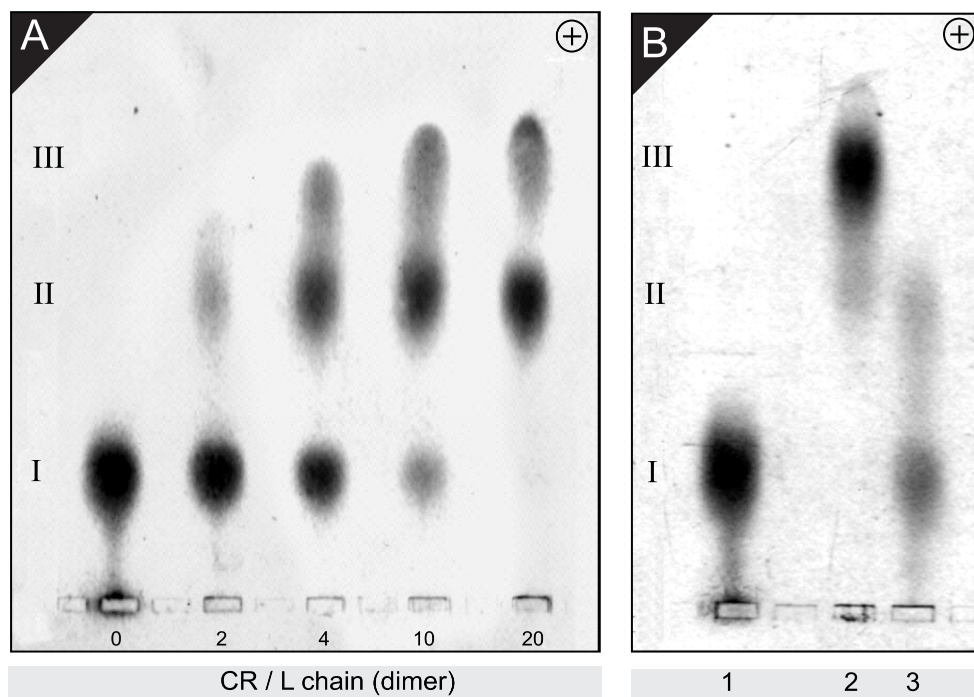

Fig. (4). Agarose gel electrophoresis of L-lambda chain and its complexes with Congo red. (**A**): L-lambda chain (I) and L-lambda chain – Congo red complexes (II and III) formed under increasing molar excess of dye molecules. (**B**): Reversal of the complexation process (III → II and I) obtained after the removal of Congo red from the isolated complex III by strong adsorption using Sephadex G25. **1**. L-lambda chain (control sample) (I). **2**. isolated complex (III). **3**. complex (II) and the Congo red-deprived L-lambda chain (I) obtained by reversal.

2.2. Effects of Congo Red on Signal Transduction and Complement Activation

Complement activation was used as a model effector process in immunological signalling studies with particular focus on C1q-antibody binding. In order to avoid the inhibitory effect of Congo red upon complement enzymes [36], IgG-C1q complexation was performed gradually using C1q (purchased from QUIDEL USA) and C1q-depleted human serum (C1qDHS) as independent components. Sheep red blood cells (SRBC) and polyclonal anti- SRBC IgG obtained from immunized rabbits were used as the model immune system. In line with expectations, Congo red was found to enhance complexation, strengthening antigen- antibody complexes while also promoting C1q-IgG binding. The effect was registered by hemolysis after addition of C1qDHS.

The use of Congo red for independent characterization of low- and high-affinity antibodies by enhancing their capacity for immune complexation and complement activation was an important element of studies aiming to verify the intramolecular signalling hypothesis. Analysis of complexation of this supramolecular ligand and, in particular, of the resulting enhancement of antigen-antibody binding effects reveals that immune complexes formed by high- and low-affinity antibodies differ with respect to their complement activation capacity. As dye concentrations increase, low-affinity antibodies derived from the polyclonal serum become increasingly engaged in immune complexes and agglutination. Even so, they remain incapable of triggering hemolysis by complement activation. This lack of activity indicates that some particular strain-induced structure must be produced for signalling to occur (Fig. **5**).

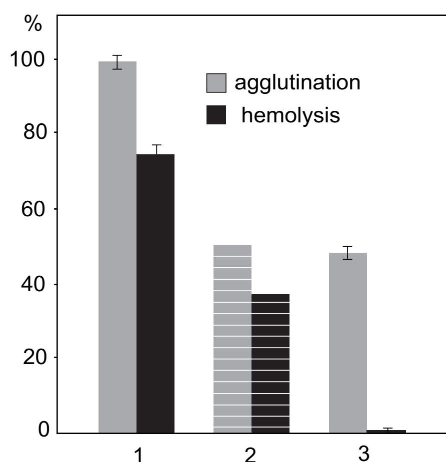

Fig. (5). Use of isolated low-affinity antiSRBC antibody fraction agglutinating red cells in the presence of Congo red to reveal its inability to induce complement-driven hemolysis of red cells (in contrast to high-affinity antibodies) as indicated by agglutination (gray bars) and hemolysis (black bars). **1**. Activity of antibodies (IgG – polyclonal population) containing both high- and low-affinity antiSRBC IgG molecules studied in the presence of Congo red – control sample. **2**. Activity of agglutination and hemolysis predicted for the isolated low-affinity antibody fraction assuming the same mutual relation of both activities as in the control sample. **3**. Failure of low-affinity antibodies to trigger hemolysis despite preserved agglutination activity.

In trying to confirm the presented hypothesis we focused on the effects of Congo red complexation on high-affinity antibodies, *i.e.* those which remain complexed when their low- affinity counterparts are washed out. Surprisingly, despite the lack of additional antibodies which might potentially form immune complexes (see Fig. **2**), Congo red complexation was found to enhance hemolysis (Fig. **6**).

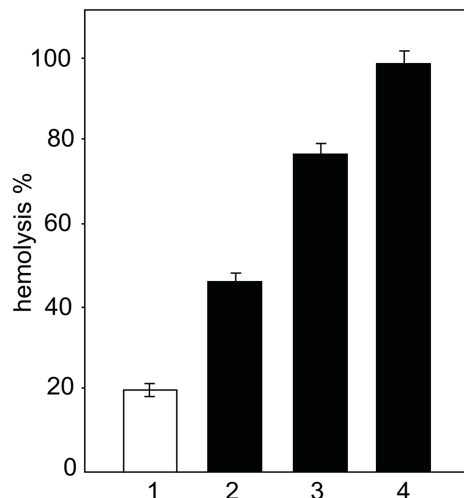

Fig. (6). Application of Congo red to reveal the signalling threshold for initiation of complement-driven hemolysis through enhancement of immune complexation and C1q binding. The effect is evidenced by using combinations of the following components of the signalling system as independent reagents.
RCA – agglutinated red cells – the immune complex (depleted by washing out the low- affinity antibody fraction).
C1q – initial component of the complement system.
C1qDHS – C1q-depleted human serum containing the remaining complement components.
1 - (RCA) ↓ + C1q ↓ + C1qDHS (without Congo red added).
2 - RCA ↓ + Congo red ↓ + C1q ↓ + C1q DHS.
3 - RCA ↓ + (C1q + Congo red) ↓ + C1q DHS.
4 - RCA ↓ + Congo red ↓ + (C1q + Congo red) ↓ + C1q DHS.
Arrows indicate washing. No hemolysis was observed in the RCA + Congo red + C1qDHS system.

In this case enhancement cannot be explained as a function of the quantity of antibodies engaged in immune complexes. Rather, it depends on structural changes required for complement activation, which are induced by Congo red. This means that even after removal of low-affinity antibodies the surviving complexes retain some antibodies which still cannot activate the complement system in the absence of Congo red. Their strain-dependent activation state can be described as intermediate: it is close to exceeding the activation threshold, and can do so as a result of Congo red complexation. The existence of intermediate-affinity antibodies (which may cross the activation threshold in the presence of Congo red) serves as definite proof that complement activation is conditioned upon encountering a suitable immunoglobulin structural pattern [47, 48].

This structural change is a manifestation of the immunological signal.

Strains associated with antigen complexation may therefore create suitable conditions for two separate activation thresholds to be exceeded:

1) Structural changes triggered by antibody/antigen binding (via dye-induced stabilization) further enhance the efficiency of immune complexation;

2) Changes which facilitate C1q binding and may also be enhanced by Congo red.

This two-step mechanism, made possible by the wide variety of structural strains generated by fitting bivalent antibodies to randomly distributed antigenic determinants, is clearly correlated with the observed two-phase complexation of Congo red with the light chain under increasing heating.

Forced rotation of V domains during antigen complexation destabilizes the packing of the N- terminal fragment of the chain, enabling Congo red penetration [19, 20]. This process is accompanied by loosening of the V domain structure, which initially results in partial melting of beta-structural fragments comprising the upper core. Increasing strains overcome the second threshold, expanding the area of instability which eventually engulfs the lower core (Fig. **7**). At this stage a fast-migrating complex of the L chain can be formed in the presence of Congo red. As concluded from MD simulation studies, melting of the lower core finally decouples the C-terminal fragment of the polypeptide chain of the V domain, increasing the rotational independence of V and C domains and enabling structural relaxation of the Fab fragment.

Asymmetry of the Fab fragment, caused by variations in the stability of V domains (H and L) in the polyclonal population and by their variable involvement in antigen binding, leads us to expect a similar variability in strain generation [49-57]. While in our model structural modifications focus on the light chain – likely due to the limited stability of its V domain and (usually) lower engagement in antigen complexation – the heavy chain remains the deciding factor in enabling relaxed-state rotational movements, producing favorable conditions for C1q complexation. MD simulations were used to verify that this mechanism is possible in practice. Relaxation was obtained using a Fab fragment with the L chain disconnected in the elbow region (L107, N108) (Fig. **8**).

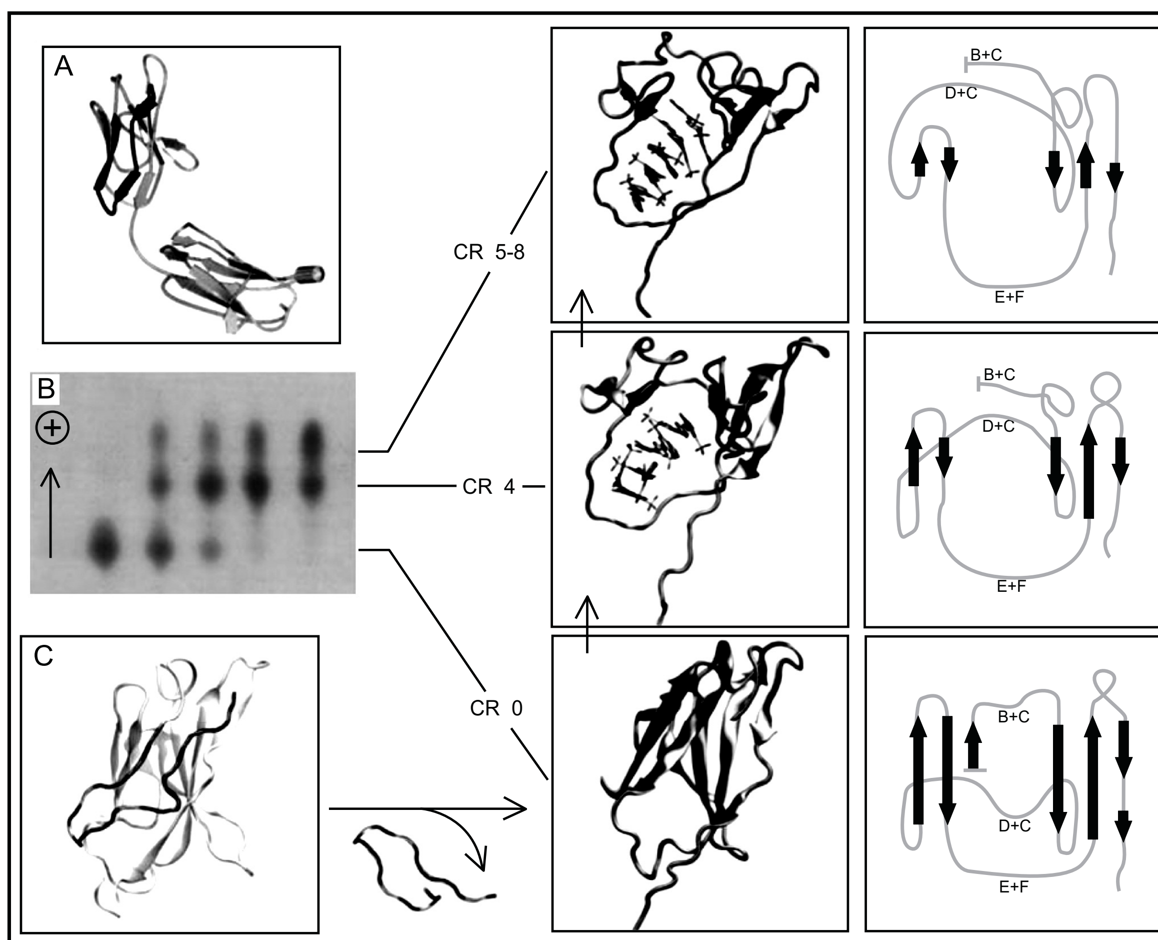

Fig. (7). MD simulation model explaining the formation of L λ chain Congo red complexes (with dye location in V domains) referred to as "slow" and "fast" depending on their migration speed under electrophoresis (picture B) and revealing the mechanism by which the binding locus is rendered susceptible (by displacing or cutting off its N- terminal polypeptide loop – diagrams A and C respectively). The figure also presents the subsequent structural effects of dye binding to the V domain of the L chain (right).

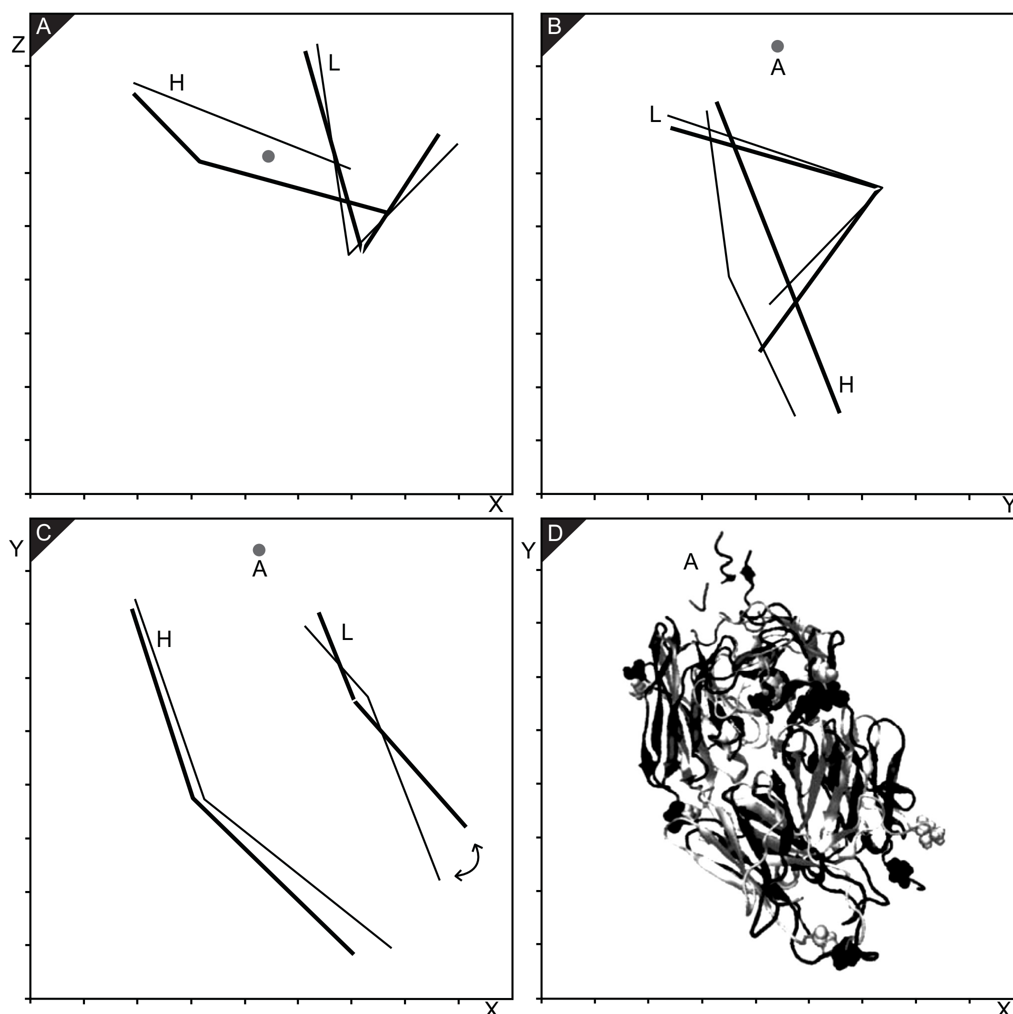

Fig. (8). MD simulation [63] approach to analyzing the signal generation mechanism. MD simulation results presenting the movement of Fab domains and revealing structural relaxation which results from asymmetric intervention in the L chain, disconnected in the elbow region. Lines connecting Ala75-Gly124-Gln202 in the H chain and Ser92- Leu107-Thr210 in the L chain denote domain position prior to (thin) and following (thick) removal of the L chain. Structural changes are depicted as XZ (**A**), YZ (**B**) and XY (**C**) projections. **D** – Ribbon-like presentation of superimposed Fab molecules to visualize the displacement of C domains.

Conformational changes were calculated according to molecular dynamics simulations (NA/MD) of the native and structurally modified Fab-antigen complex (PDB ID: 1ADQ) [58]. Antigen atoms were kept fixed and provided stable attachment points for Fab, which was allowed to move freely throughout the simulation.

The simulation confirmed that structural strains are generated in the Fab fragment, although the model characterizes monovalent Fab binding as producing lower exposure to strains than in the case of bivalent molecules.

Weakly interacting antibodies which bind to antigens only in the presence of Congo red do not trigger the complement cascade even when C1q is introduced (see Fig. **5**). This suggests that C1q does not bind to antibodies if their structure does not permit it (*e.g.* when C domains are insufficiently displaced by antigen binding). If, however, suitable conditions arise for C1q binding, the corresponding antigen-antibody interactions intensify [59]. This reversible mechanism can be taken as proof of the bidirectional, structural connection between C1q binding and the formation of immune complexes.

3. CONCLUSION

Function-related complexation of supramolecular Congo red with antibodies was applied as a nonstandard technique of validating longstanding theories related to intramolecular immunological signalling. This technique enables the complement activation process to be analyzed in natural conditions, using a standard, highly heterogeneous polyclonal pool of antibodies. Structural alterations generated in Fab fragments by binding of antibodies to the antigen initiate signalling which prepares Fc for C1q binding. Among the broad range of structural changes in V domains which may be induced by specific interactions and/or nonspecific strains derived from fitting of bivalent antibody molecules to randomly distributed antigenic determinants only some reach sufficient intensity to trigger effector activation.

Direct susceptibility of antibodies to C1q complexation as an effect of signalling can be achieved by making Fc fragments fully available for binding via steric unshielding [41, 60], or by ensuring higher exposure of Fc through increased rotational freedom and suitable positioning [12] and/or enlarging the binding site to accommodate the (suitably exposed) hinge region or C domains of the Fab fragment [61-63].

Our work suggests that supramolecular Congo red acts as a ligand and may interfere with signalling. In this study we have obtained evidence:

1) Confirming intramolecular signalling by antibodies triggering complement activation.
2) Indicating that, under natural conditions, only high-affinity bivalent antibodies may generate the signal.
3) Revealing that Congo red greatly enhances immune complexation and C1q binding.
4) Disclosing the mechanism of dye-protein complexation and the enhancement effect.
5) Positioning supramolecular Congo red as a model reagent which possesses nonstandard properties, representative of a whole group of self-assembling protein ligands – potential future drugs.

Recognition of the specific ribbon-like supramolecular structure of Congo red enables us to classify this dye as a new type of reagent which may form complexes with susceptible proteins in a non-standard way, penetrating outside the binding site. In particular, Congo red can bind to antibodies which form immune complexes due to structural alterations induced by interacting with an antigen, making antibodies susceptible to interactions with supramolecular ligands. As a consequence, ongoing research involves the use Congo red and/or related compounds in immune-targeting therapies (see Fig. **3**).

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

This work was financially supported by the Jagiellonian University Medical College grant system K/ZDS/001351. All authors contributed equally to the presented experimental work, interpretation of results and preparation of the paper.

LIST OF ABBREVIATIONS

AntiSRBC	=	Rabbit IgG antibodies antiSRBC
C1qDHS	=	C1q depleted human serum
RCA	=	Red cells (agglutinated by antibodies)
SRBC	=	Sheep red blood cells
MD simulation	=	Molecular dynamics simulation

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Received: April 30, 2014 Revised: November 10, 2014 Accepted: November 11, 2014