Chapter 2
Supramolecular Congo Red as Specific Ligand of Antibodies Engaged in Immune Complex

Anna Jagusiak, Joanna Rybarska, Barbara Piekarska, Barbara Stopa, and Leszek Konieczny

Abstract Supramolecular Congo red has been used to validate long-lasting theories regarding intramolecular signaling in antibodies and its relation to activation of the complement system. Strong enhancement of antigen-antibody complexation resulting from the binding of supramolecular ligands enables also polyclonal antibodies having intermediate affinity to trigger complement cascade apart of high affinity antibody fraction. This would not have been possible in the absence of Congo red. The property of antibodies provides specifically their ability to trigger the complement system allowed when sufficient structural strain is produced by antigen complexation provides an evidence of intramolecular signaling.

The selective complexation of supramolecular ligands with antibodies engaged in immune complexes enables their using as carriers of drugs in immunotargeting system.

Keywords Intramolecular immunological signal • Complement activation • IgG V domain stability • N-terminal fragment • Enhancement of antigen binding • Congo red as carrier of drugs • Immunotargeting system • Congo red selective complexation of antibodies in immune complexes

Self-associating organic molecules which form ribbonlike micellar structures may, owing to their structural characteristics, penetrate inside proteins and form stable complexes. Such penetration is possible in areas of the protein which have been destabilized, either temporarily or permanently – such as antibody/antigen complexes. Since Congo red (CR) has been used in research as the most typical

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supramolecular protein ligand, the presented experiments and analysis also focus on this particular dye. CR binds strongly to antibodies, enabling us to study (among others) intramolecular signaling related to complement system activation. What is more, the mutual affinity of CR and immune complexes paves the way towards immunotargeting, i.e. targeted delivery of drugs. This is due to the fact that supramolecular CR – a micelle-like structure – may intercalate foreign bodies, including drug molecules. Congo red does not react with free antibodies – it is only capable of binding to antibody/antigen complexes where structure of antibody undergoes some alteration due to interaction with the antigen. Any potential drugintercalated into the CR micelle can thus be delivered to an area where the antigen is plentiful, ensuring targeted action. This chapter discusses the presented topics in detail.

2.1 Looking for Evidence of Postulated Intra-molecular Immunological Signaling

Once the structure of immunoglobulins has been divined, it soon became clear that their Fab and Fc fragments play differing roles in the process of triggering immunological response. While the Fab fragment selectively binds to the antigen, the Fc fragment – separated by a hinge – appears to be involved in triggering complement system activation through complexation of the C1q subcomponent. Notably, the Fab-antigen interaction is independent of Fc and proceeds even when the Fc fragment has been removed by digestion [1, 2].

The complement system is a collection of proteins which attack and destroy cells recognized as alien by the immune complex. Strict control over this mechanism is critical for homeostasis and therefore represents an important study subject in medical research. In accordance with prevalent views, such control is maintained by intramolecular rearrangements which carry information from Fab to Fc, and then onwards to C1q (Fig. 2.1).

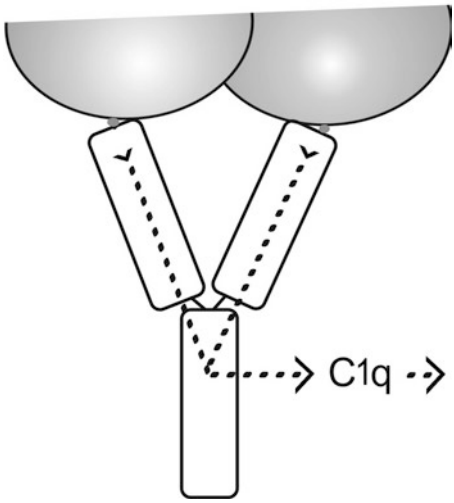

Fig. 2.1 Schematic depiction of the intramolecular signaling pathway inside the antibody (*dashed line*)

Nevertheless, despite significant effort by many leading researchers, the specifics of this mechanism have proven exceedingly difficult to elucidate and some uncertainties persist. In attempting to explain intramolecular signaling, analysts initially focused on the hinge region which links Fab and Fc. Experimental data indicates that subclasses of immunoglobulins which differ with respect to the composition of this hinge region also exhibit variable efficiency of Fab-to-Fc signal transmission, and moreover that reduction of the disulfide bond in the hinge region prevents successful activation of the complement system [3–5]. In turn, some attention was directed towards structural strain in the antibody molecule, produced by antigen binding and regarded as a possible signal carrier. This view is embodied in the so-called distortive mechanism theory. Another competing theory proposed an "all or none" switching mechanism, i.e. an allosteric model based on the assumption that immunoglobulins are, in fact, allosteric [6].

Since none of the presented models succeeded in providing a satisfactory explanation, further analysis was needed. Some researchers noted the fact that, under ordinary circumstances, the formation of an active immune complex involves many different antibodies, and that complement activation requires local concentration of Fc fragments. This so-called associative model appeared to explain the signaling puzzle to a sufficient degree, particularly given the lack of evidence favoring intramolecular signaling [7, 8]. Earlier theories were swept aside and the issue appeared solved. This situation persisted for many years, until scientists learned how to produce monoclonal antibodies via crystallization of Fab fragments cleaved from the IgG molecule, and formulated new analysis protocols based on the use of small antigens (haptens) [9–11]. Surprisingly, these studies produced little in the way of useful results. Structural changes appeared small, even negligible – again suggesting that intramolecular signaling must somehow involve torsional effects, which emerge only when the antigen is bound to a complete, two-arm antibody.

Spectacular progress in genetics achieved in the 1980s, particularly the ability to synthesize arbitrarily modified antibodies, brought new hope of understanding the purported intramolecular signaling mechanism. Still however, despite some focus on the interaction of CH_1 and CH_2 domains, the problem of signal remained practically unsolved [12–14].

Our research group decided to attack the problem through chemical recombination of antibodies by digestion, reduction and re-joining of immunoglobulin fragments solely by disulfide bonds. The goal was to determine whether this kind of modified structure would retain the ability to carry the signal to Fc, despite major alterations in the hinge region. This process is illustrated in Fig. 2.2.

While the "full" two-arm molecule constructed from free Fab and Fc fragments exhibited complement activation potential (to a limited degree), its one-arm equivalent proved entirely inert. This suggested that even a deficient antibody may transmit the signal, if only suitable conditions exist for structural strain to emerge. Nevertheless, the participation of the hinge region in signal transmission remained a mystery [3, 15, 16].

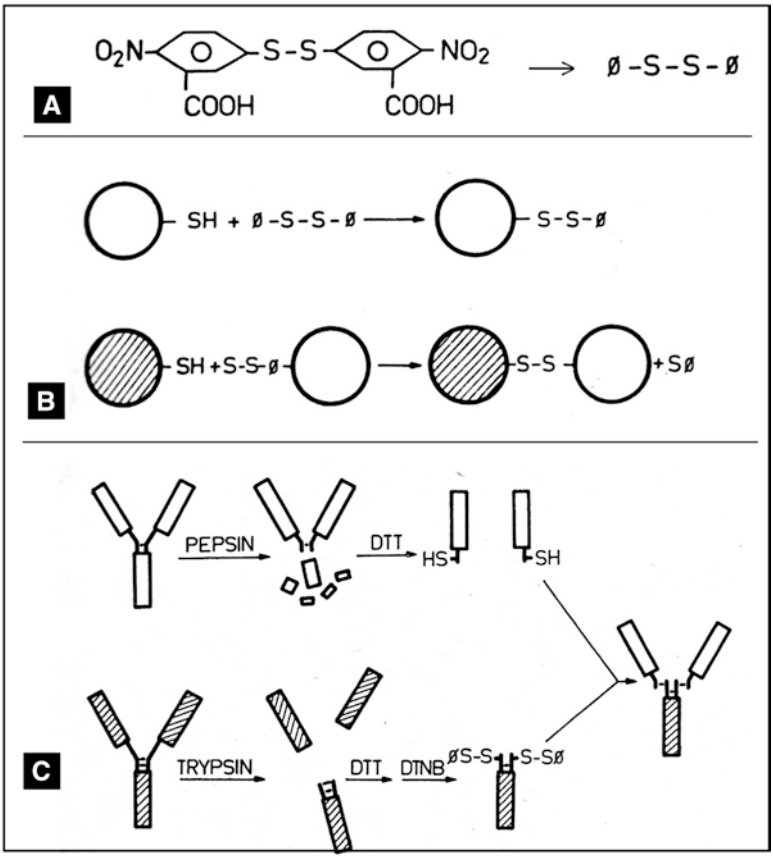

Fig. 2.2 Schematic view: (**A** and **B**) Controlled formation of linking disulfide bonds. (**C**) Production of recombinant IgG

2.2 Evidences of Intramolecular Signaling Supplied by Using Congo Red

A whole new approach to the problem was enabled by the use of CR, based on our team's original concept. While CR had long been known as a useful amyloid stain, its interaction with amyloids was explained as individual molecules attaching themselves to specific binding sites which recognize the dye. In contrast, our study revealed that CR may form complexes with a wide variety of proteins and that it does so as a supramolecular ligand – i.e. a distinct structure consisting of many associated dye molecules acting as a single unit [17–25].

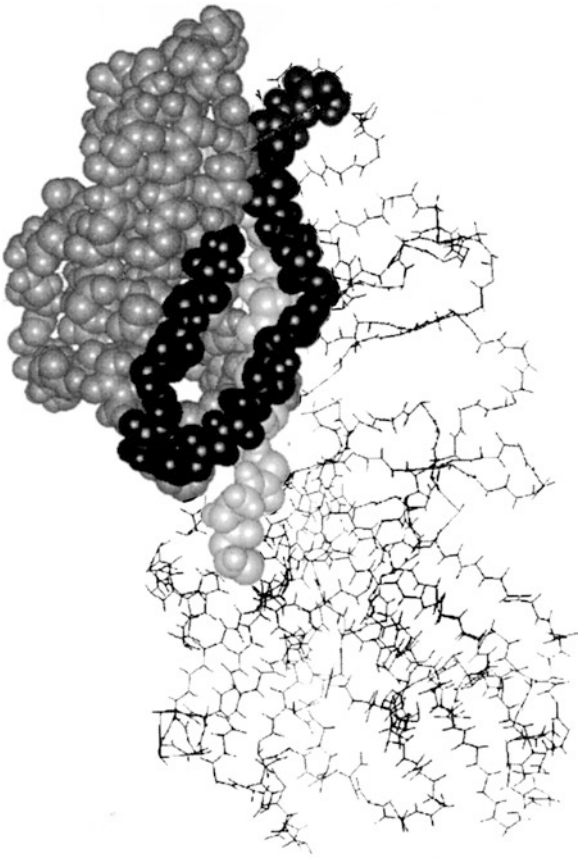

Fig. 2.3 V domain of the L chain lambda, with its hightlighted N-terminal fragment covering gap created by its removal. Space filling model

An important breakthrough occurred when CR was found to interact with immune complexes, but not with free antibodies. This phenomenon shed new light on the intramolecular signaling pathways leading to complement system activation. Such selective binding suggested that CR is capable of anchoring itself in the V domain of the antibody, which also happens to be the site of the greatest structural strain resulting from antigen complexation. Furthermore, the V domain also houses the N-terminal fragment, which, by default, is relatively unstable (Fig. 2.3) [26–28].

Confirmation of this theory was provided by analyzing the complexation potential of CR vs. light chain dimers progressively destabilized through heating or increased concentrations of the dye. The displacement of the N-terminal fragment from its packing locus "opens up" the V domain, enabling the supramolecular dye to penetrate and anchor itself in its interior. Under experimental conditions, the first complexes to emerge involve ligands composed of four molecules. As the dye concentration increases, the ligand may grow to include up to eight dye molecules (Fig. 2.4A, B respectively). This is evidenced by electrophoresis, where larger com-

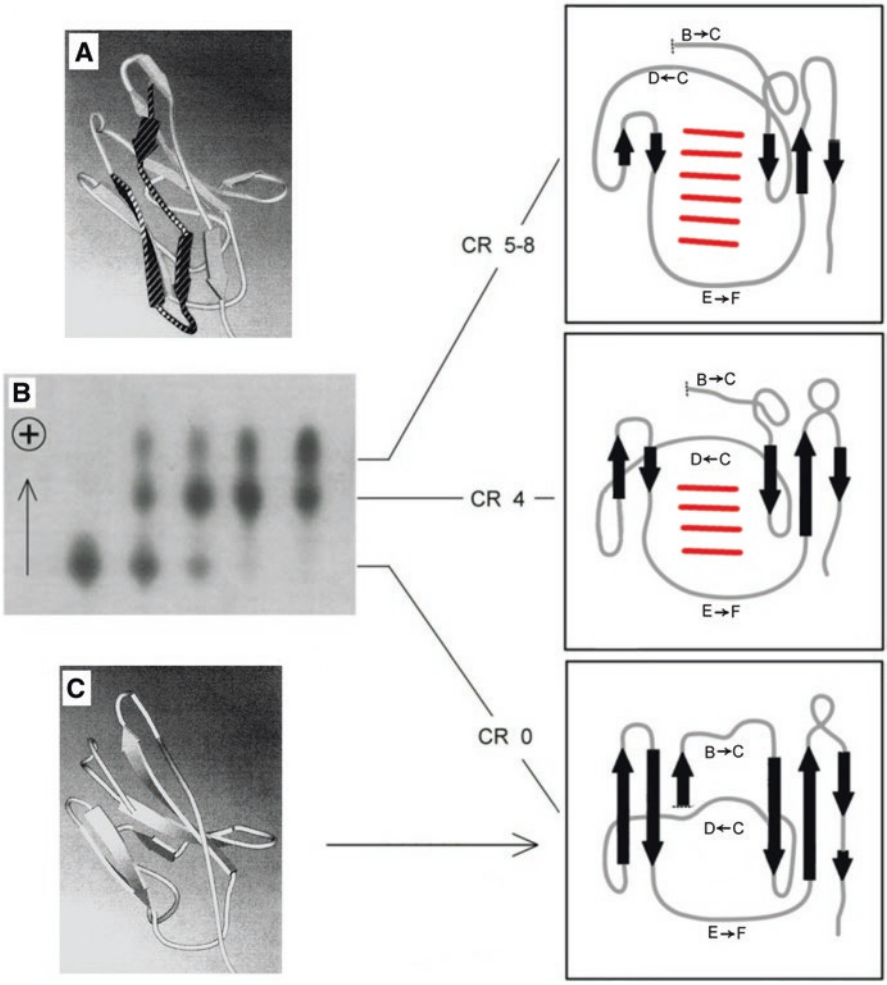

Fig. 2.4 Complexation of L chain dimer with CR (**A**) Light chain V domain. (**B**) Thermally generated complexes of CR with the L chain V domain presented by corresponding models. (**C**) V domain deprived of its N-terminal fragment through digestion. Agarose electrophoresis of L chains induced to form complexes with CR upon the stepwise increasing temperature

plexes migrate faster due to their greater charge (contributed by CR). In each case, the trigger for complexation appears to be the N-terminal fragment, which is displaced from its packing locus and replaced by the supramolecular ligand. The displaced N-terminal fragment subsequently becomes susceptible to digestion [26, 29–31].

CR/light chain complexation may be accelerated by heating, which further perturbs the N-terminal fragment. In contrast, when dealing with immune complexes, complexation appears to be induced by structural strain resulting from antigen bind-

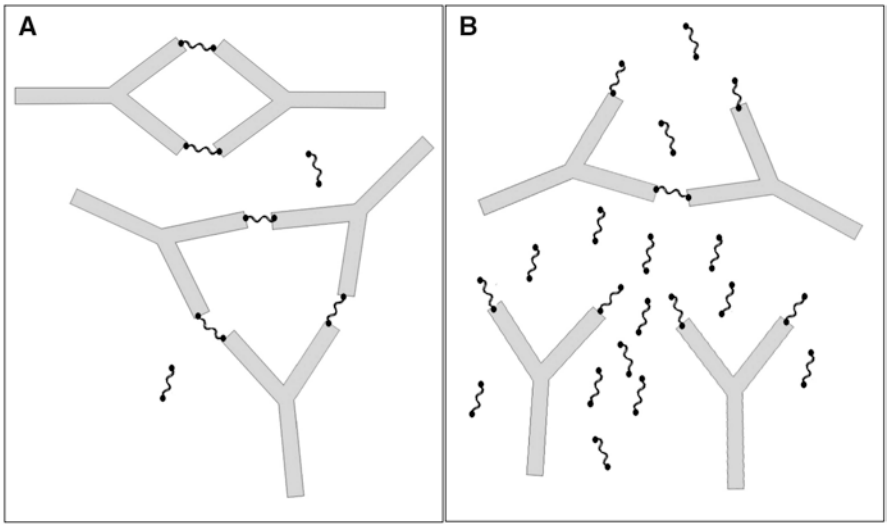

Fig. 2.5 Immune complexation of antibodies linked by binary haptens (**A**) and breakage of immune complexes caused by overabundance of hapten particles (**B**)

ing. This phenomenon, however, only emerges in complete two-arm antibodies, attaching themselves to antigen determinants located randomly on the cell surface. Neither isolated Fab fragments nor their dimers are capable of binding CR, even in their complexed state. This proves that the antibody-antigen reaction is not directly responsible for the affinity to CR, and that dye complexation requires structural strain in the antibody molecule [26, 29–31].

CR is complexed by whole antibodies in complex with haptens, but only when they are fixed on a solid surface, i.e. under conditions which lead to structural strain in bivalent antibodies.

CR also binds to anti-TNP antibodies linked by a hapten if the hapten itself is bivalent and creates strain in the antibodies it links (e.g. oxidized glutathione with amino groups substituted with TNP – Fig. 2.5).

This can be confirmed under electrophoresis, since the antibodies bound by the binary hapten form immune complexes, become soluble and migrate more rapidly when treated with CR. Their number grows as the concentration of the hapten increases, eventually reaching a maximum beyond which a falloff is expected (Fig. 2.6) – due to the fact that when the hapten is overly abundant, it becomes monovalent and therefore produces no strain in the attached antibodies (Fig. 2.5) [32].

The link between CR and immune complexes exhibits one more remarkable property: **it turns out that complexation of CRgreatly enhances theantigen/antibodycomplexation capabilities**. This is evidenced by a significant increase in the fraction of antibodies involved in immune complexes, especially in relation to low-affinity antibodies which are naturally present in the polyclonal serum. It should be noted that the serum contains many different types of antibodies with

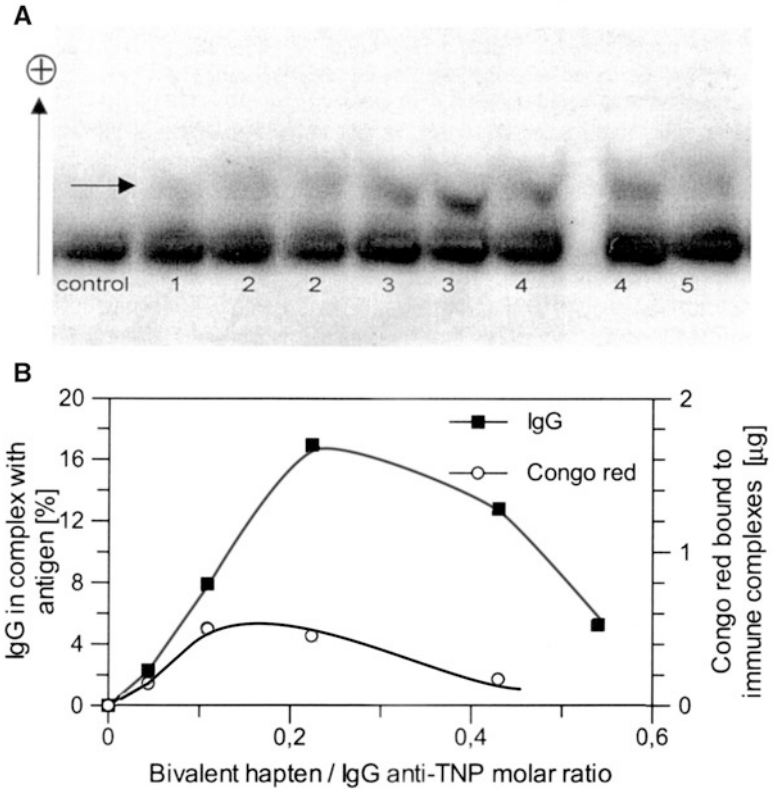

Fig. 2.6 Experimental evidence of formation soluble complexes with CR by binary hapten-linked antibodies (as depicted in Fig. 2.5). *Left to right*: increasing hapten concentrations. The mobile fraction (indicated by the *arrow*) comprises soluble immune complexes which have gained the ability to bind CR (Reproduction by permission – *J Physiology and Pharmacology*)

varying degrees of affinity – due to the inherent randomness in the antibody synthesis process. Low-affinity antibodies cannot form stable immune complexes and are washed out in the absence of CR. When the dye is present, their ability to bind antigens increases and the number of immune complexes per unit of volume grows (Fig. 2.7 **A**, **B**).

At this point it would be useful to determine the mechanism which drives increased complexation capabilities in the presence of CR, and also to find out whether such upregulation is accompanied by the corresponding increase in complement system activity (which would prove the existence of an intramolecular signal).

The assumption that, by binding its natural biological ligand, the protein undergoes structural rearrangement which favors penetration and complexation of supramolecular dye would explain why the ligand cannot be easily released once the protein-dye complex has formed. This phenomenon appears actual in situations

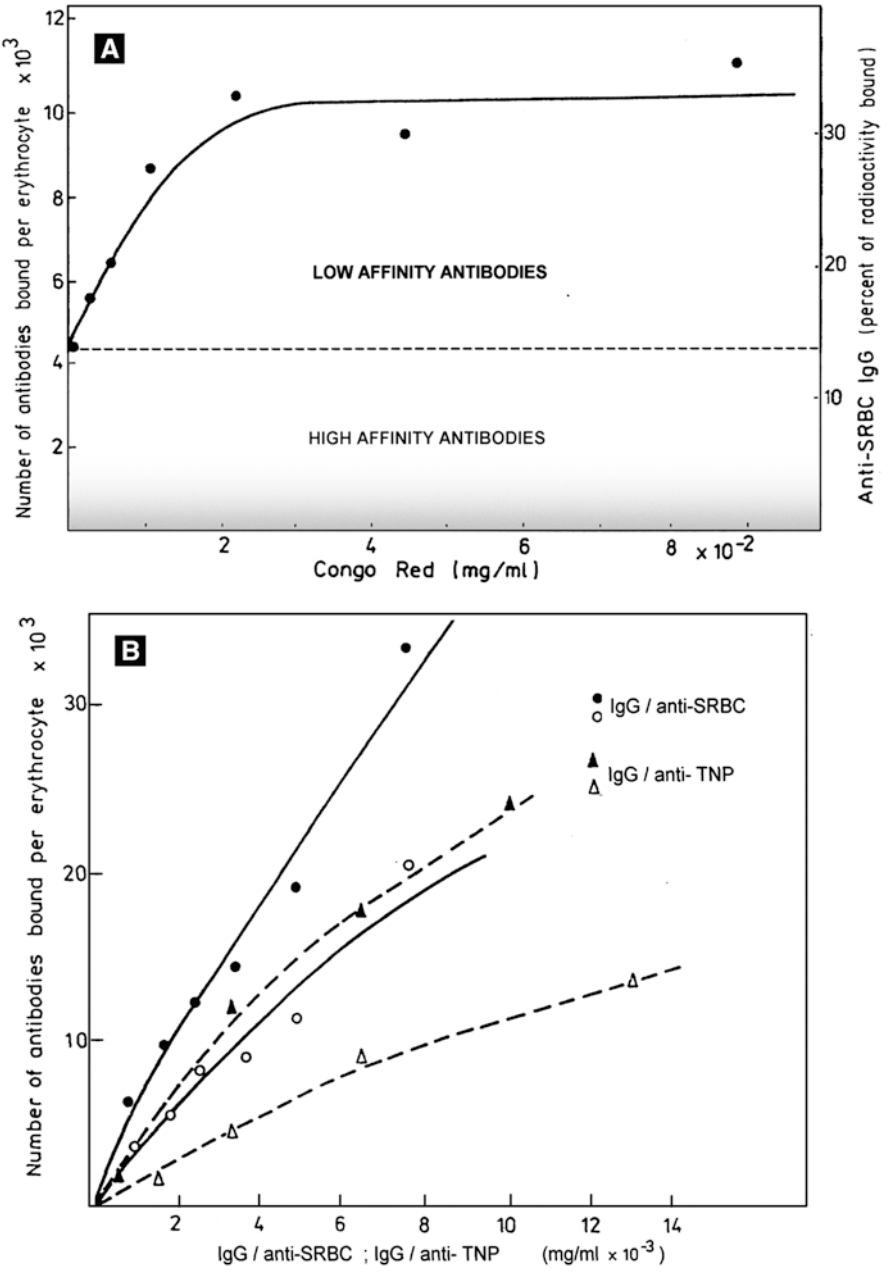

Fig. 2.7 Profiles presenting: (**A**) Increase in the quantity of erythrocyte-agglutinating antibodies under increasing concentrations of CR. High-affinity antibodies are capable of agglutinating cells in the absence of the dye. Anti-SRBC antibodies complex tagged by ^{125}I. High affinity antibodies – fraction non removable by washing. *Shadowed area* – fraction of highest affinity. (**B**) Enhancement of antibody/antigen interaction by CR, proving that the effect is independent of the type of antigen and specificity of the antibody (Reproduction by permission – *Archivum Immunologiae and Therapie Experimentalis*)

where irreversibility is finally expected – immune complexation, C1q binding etc. On the other hand, the same phenomenon would tend to inhibit the action of enzymes where the ligand must be released following catalysis. Indeed, such inhibition has been confirmed in the scope of complement activation which depends on the action of convertases [33].

The observed enhancement of antibody/antigen complexation capabilities may also be due to another factor: increased flexibility of the V domain, caused by penetration of a large noncovalently stabilized ligand to packing cavity of the replaced N-terminal fragment, bestowing greater internal mobility upon the domain (particularly its CDR loops) and therefore enabling them to align themselves to the antigen with greater accuracy [34].

The discovery and subsequent theoretical study of antigen complexation enhancement triggered by CR creates new possibilities with regard to analysis of intramolecular signal leading to complement system activation – assuming that such signal exists. Due to inhibition of convertase (and therefore of the complement system) by CR, measured as the efficiency of hemolysis, the signal transfer stage (immune complex/C1q) has been separated from the remainder of the activation cascade, including convertase. This reveals activation potential, since both the immune complex and the subsequent complex with C1q are insoluble and may therefore be separated from excess CR by washing, then combined with the remaining components of the complement system, thus preventing undesirable inhibition. To this end we have employed a commercial-grade C1q reagent (QUIDEL USA) and, separately, a serum containing complement system components but deprived of C1q (QUIDEL USA).

Once the excess dye has been washed out, the remaining insoluble complexes (immune complex and immune-C1q complex) prove capable of activating the complement system, triggering hemolysis in C1q-deprived serum. Figure 2.8 presents the results of this experiment. Confirmation of complement system activation reveals the role of CR in the process and confirms the presence of intramolecular signaling.

The immune complex (agglutinate) binds antibodies with varying affinity for red cells which participate in the immune response (SRBC/anti-SRBC). Weak (low-affinity) antibodies are quickly washed out in the absence of CR. The remaining complexes contain antibodies with strong or moderate affinity. The introduction of CR stabilizes the immune complexes formed by weak antibodies, allowing them to remain in the agglutinate. Nevertheless, such antibodies remain incapable of triggering hemolysis even when CR is present. Their properties may be studied by analyzing wash-out samples. Of course, antibodies which resist washing out are also characterized by variable affinity: the group includes strong (high-affinity) antibodies which do not require CR to form stable complexes and trigger hemolysis, but also weak (low-affinity) antibodies stabilized by CR but still unable to trigger hemolysis. The specific affinity threshold established by the wash-out procedure is somewhat arbitrary and depends on a number of conditions. It is assumed that CR account for approximately 50% of the washout-resistant pool. Interestingly, this group contains also antibodies which are unable to trigger complement activation,

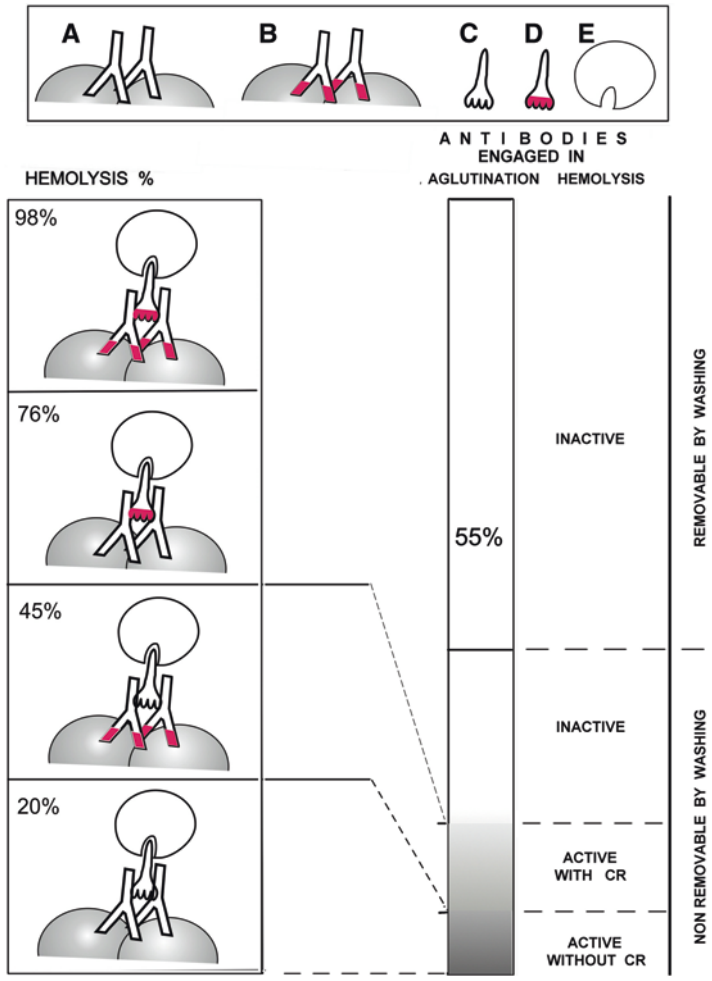

Fig. 2.8 Role of CR in amplifying the complement activation signal. Model view of the process (*left-hand column*). The efficiency of erythrocyte hemolysis by the complement system depends on CR activation of the signaling pathway. Selective action of CR upon successive components of the signal pathway has been marked in red. *A* – immune complex (anti-SRBC antibodies not treated with CR); *B* – antibodies selectively activated by CR; *C* – C1q; *D* – C1q selectively activated by CR; *E* – serum containing all components of the complement system except C1q. Participation of anti-SRBC polyclonal serum antibodies in agglutination and hemolysis is shown on the right

but which gain this ability by interacting with CR. This suggests that a sufficiently powerful intramolecular signal may only be generated by "strong" antibodies which incur significant structural strain when binding the antigen. This natural threshold appears evolutionarily conditioned to prevent accidental thus potentially dangerous activation of the complement system [35–38].

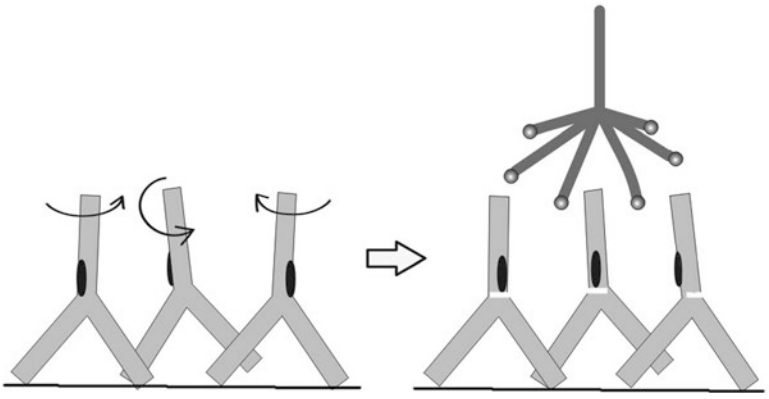

Fig. 2.9 Complexed antibodies showing random placement and orientation of Fc fragments. *Arrows* indicate the need for rotation, facilitating contact with C1q. This is enabled by intramolecular signaling which increases the mobility of Fc fragments

While CR helps explains the signaling mechanism, the origin of the signal itself remains an open question. Since it seems improbable for both V domains (VH and VL) to react to structural strain in the same way, antigen complexation induces torsional stress in the Fab fragment [39]. The resulting rotation uncouples Fc from Fab and allows the former to bind to C1q. This is important, since in the immune complex both the antibodies and their Fc fragments are oriented randomly, which would otherwise hamper complexation of C1q. The uncoupling provides Fc with rotational freedom and enables the link to be established easier (Fig. 2.9).

In this way, the use of a supramolecular dye approaches understanding both the function and the purpose of intramolecular signaling.

2.3 Application of Congo Red for Immunotargeting

The fact that CR selectively binds to antibody/antigen complexes creates an interesting opportunity with regard to targeted drug delivery. CR is not only capable of recognizing the immune complex, but – owing to its supramolecular nature – intercalate various drug particles, acting then as a carrier. Such intercalation should not be regarded as simple mixing of dye and drug molecules – it is more akin to solvation, which involves close interaction between the solvent and the solute. If the solute is structurally flat and presents a planar arrangement of aromatic rings, it can be easily intercalated into the CR micelle. This effect is further enhanced if the solute is positively charged (Fig. 2.10) [34, 40, 41].

The model presented in this chapter comprises CR and rhodamin B (Fig. 2.10). Interaction between both dyes is evidenced by tracking the release of rhodamine B from a dialysis bag in the absence of CR and in a system where both dyes are combined via intercalation. It is readily evident that rhodamine B – itself a supramolecu-

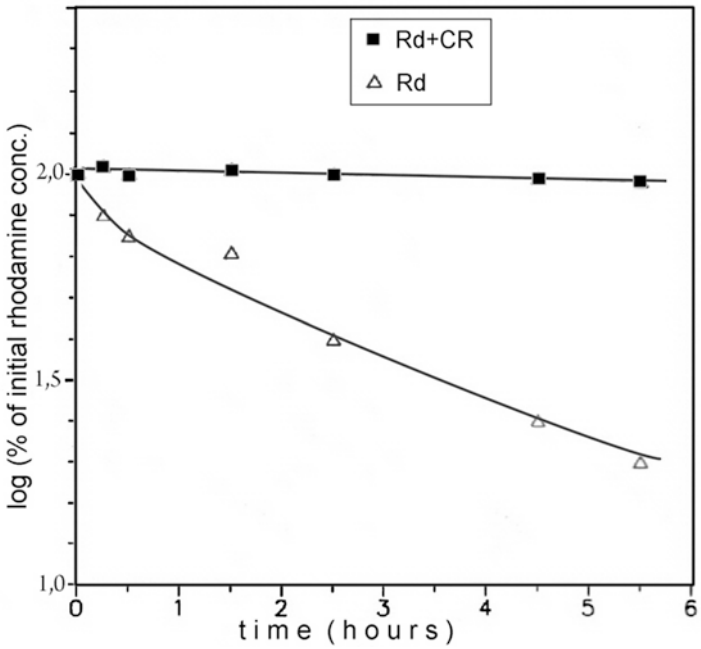

Fig. 2.10 Evidenced by dialysis arresting of rhodamine B (Rd) by supramolecular CR (intercalation), compared to progressive reduction in the concentration of free rhodamine B

lar system, but with weaker self-association tendencies – becomes nearly impervious to dialysis when CR is present. Additional complexes comprising supramolecular dyes and various foreign molecules, analyzed using the dialysis method, are listed in table shown in Fig. 2.11, and are compared to the model CR/rhodamine B complex. Where analysis suggests similar stability of both complexes, a value of 1 is listed. Figure 2.11 presents selected compounds which form co-micellar structures with CR, listing their stability [42]. It seems clear that both the spatial structure and electric charge play an important role.

To further confirm the carrier hypothesis, our analysis focused on a specific immune complex, i.e. agglutination of sheep erythrocytes capable of binding supramolecular ligands. Figure 2.12 presents a visualization of the agglutinate, following addition and subsequent washing out of CR with intercalated rhodamine B [43]. Strong fluorescence of rhodamine B overcomes CR absorption and can be clearly seen along boundaries of agglutinated erythrocytes. This proves that the antibodies involved in agglutination (immune complexes) are bound to the CR/rhodamine B aggregate. Notably, CR does not react with free antibodies – only antibodies engaged in immune complexation can bind the dye. *In vitro* analysis therefore provides evidence of the ability of supramolecular dye to serve as a vehicle for targeted delivery of drugs.

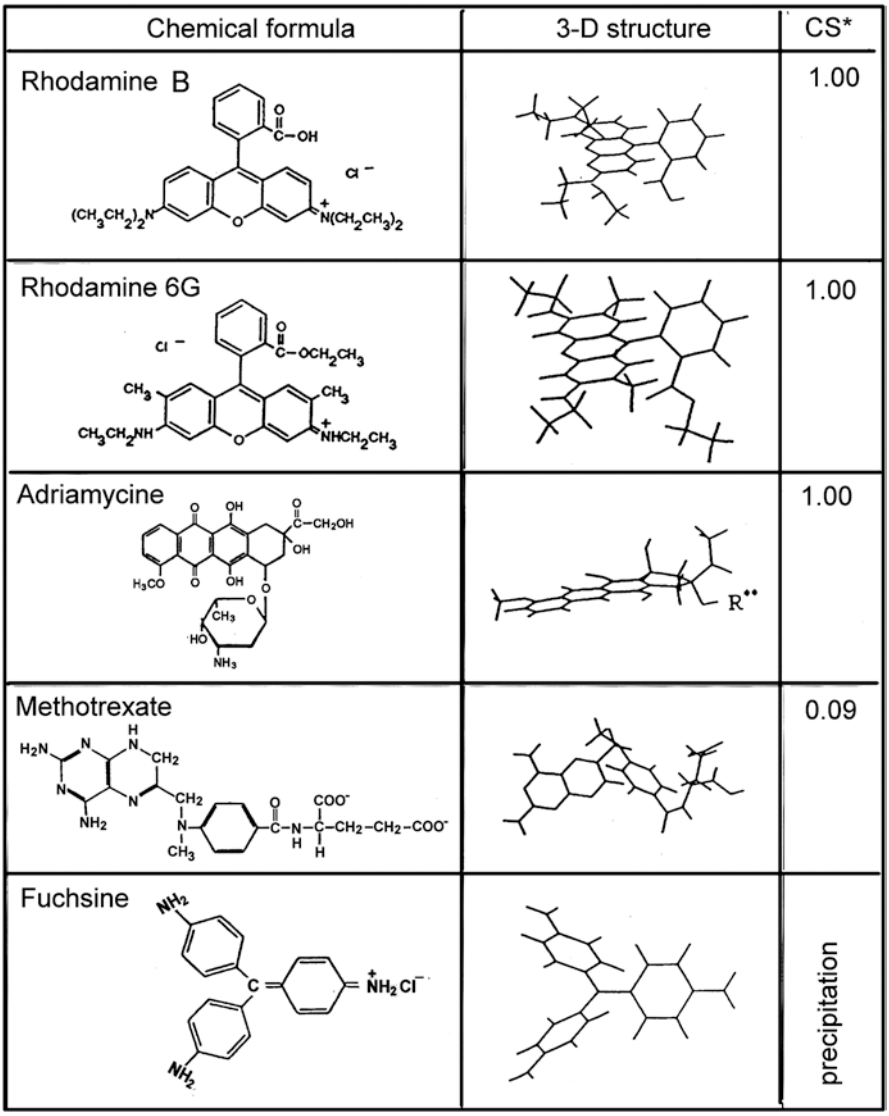

Fig. 2.11 Examples of structures readily intercalated by supramolecular CR due to their planar structure and/or positive charge. Complexation tendency ranged 0–1. (Reproduction by permission – *J Physiology and Pharmacology*)

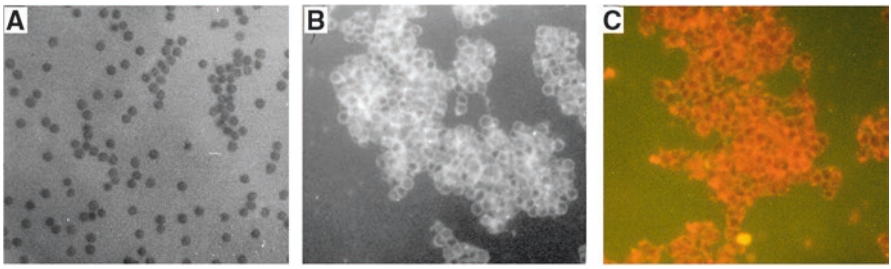

Fig. 2.12 Agglutination in the SRBC/anti-SRBC system produced by antibodies in complex with CR and rhodamine B intercalated. (**A**) not agglutinated red cells – control sample; (**B** and **C**) – agglutinated red cells – UV light (Reproduction by permission – *Folia Histochemica et Cytobiologica*)

It should be noted that *in vitro* test results do not always translate into similar outcomes *in vivo*. Successful use of supramolecular drug carriers in living organisms remains a complex problem due to undesirable reactions with serum proteins, particularly albumin. To determine whether the presented method is feasible, an Arthus reaction has been triggered in a rabbit host – i.e. the TNP antigen (ghosts of rabbit red blood cells conjugated with human IgG) was injected into the earlobe of an sensitized rabbit, producing local inflammation caused by aggregation of immune complexes. The other ear was subsequently injected with 2.5 ml 5 mg/ml) isotonic steryl CR solution. The ear where the Arthus reaction had originally been triggered was then backlit for photographic documentation (the rabbit's thin earlobe is easily penetrated by visible light, simplifying the process). Unfortunately, the unaided human eye is unable to distinguish between CR and hemoglobin – the blood present in the vessels in earlobe produces an color image similar to CR. Enhanced visibility of small blood vessels suggests that an inflammatory process is ongoing, which further hampers attempts to visualize dye accumulations. Effective analysis therefore required the use of specially prepared spectroscopic filters (the spectra of CR and hemoglobin differ somewhat).

Figure 2.13 presents the backlit tissue fragment with spectroscopic filters applied. The modified color scale enables us to easily distinguish the dye and hemoglobin (filter spectra are also presented, showing which wavelengths have been blocked). As expected, the dye is attracted to the antigen injection site, where the immune complex can be found. The experiment also highlights the kinetic characteristics of CR absorption and subsequent removal, showing how the proposed transport system may function in practice (Fig. 2.14).

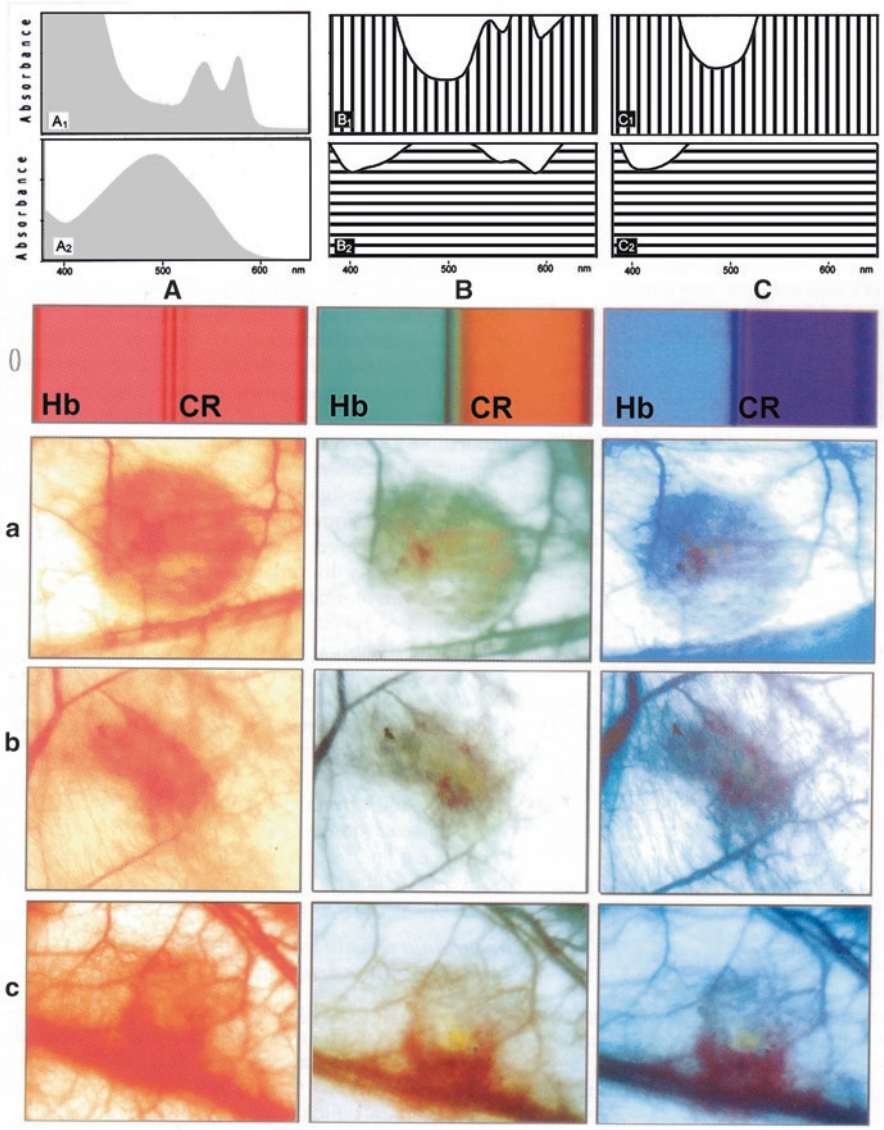

Fig. 2.13 Migration of CR to areas of immune complexation (Arthus reaction induced in rabbite-arlobe). Spectroscopic filters depicted above each column were used to differentiate CR and hemoglobin. Column *A* – spectra of hemoglobin and CR, column *B* and *C* – spectra with filters used (4–6) [43] a, b, c - three independent experiments. (Reproduced by permission – *Folia Histochemica et Cytobiologica*)

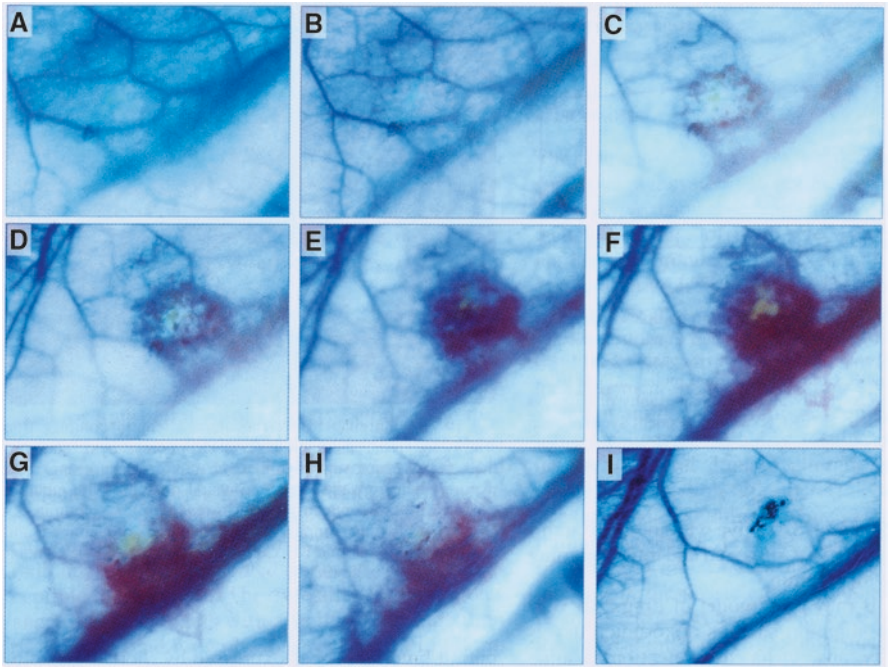

Fig. 2.14 CR accumulation kinetics in the Arthus reaction area: **A** – 1 h35′; **B** – 3 h30′; **C** – 4 h35′; **D** – 5 h; **E** – 9 h30′; **F** – 26 h30′; **G** – 52 h35′; **H** – 72 h20′; **I** – 14 days (Reproduced by permission – *Folia Histochemica et Cytobiologica*)

2.4 Toxicity of CR and Its Applicability for Immunotargeting

An important practical advantage of CR, as well as of other structurally similar supramolecular systems, is their relatively low toxicity. Supramolecular dye aggregations do not readily penetrate cellular membranes, and are easily excreted, along with the surplus of any intercalated substance (Fig. 2.15). Nevertheless, intestinal excretion of CR has been linked to carcinogenesis – most likely due to bacterial reduction of the dye, producing benzidine (a known carcinogen). This undesirable effect may be mitigated by administering a cellulose-rich diet, since cellulose eagerly binds CR and protects it from structural changes, including reduction.

In addition to its immune complexation potential, CR is also being studied in the context of amyloidaffinity, although the presented applications of supramolecular

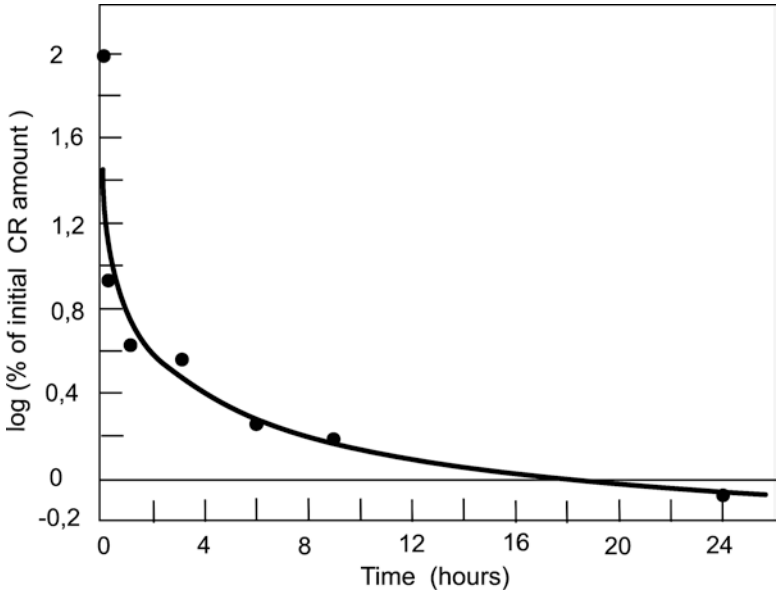

Fig. 2.15 Kinetics of CR clearance from the blood after its intravenous injection (2.5 ml of CR – 5 mg/ml) (Reproduction by permission – *Folia Histochemica et Cytobiologica*)

ligands are based on laboratory experiments. Practical medical applications would require further, independent research.

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