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In vivo accumulation of self-assembling dye Congo red in an area marked by specific immune complexes: possible relevance to chemotherapy

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Abstract: Supramolecular micellar structures have been proposed as carriers in aim-oriented drug transportation to a target marked by specific immune complexes. In this study, the self-assembling dye Congo red was used as a model supramolecular carrier and its accumulation in the target was studied *in vivo*. The target was created *in vivo* as the local specific inflammation provoked by subcutaneous injection of antigen to the ear of a previously immunized rabbit. The color caused by accumulation of Congo red after its intravenous injection was registered by pictures of the ear with suitably filtered visible light shining through it to distinguish Congo red against the background color of hemoglobin. The results confirmed the expected accumulation and retention of Congo red in the inflammation area marked by deposits of specific immune complexes. The role of albumin and its possible interference with transportation of drugs through the blood by supramolecular carriers was also subjected to preliminary examination. The results revealed that albumin collaborates rather than interferes with drug transportation; this is another factor making the use of supramolecular carriers for aim-oriented chemotherapy highly promising.

Key words: Congo red - Supramolecular drug carrier - Immunotargeting - Chemotherapy

Introduction

A powerful aim-directed drug transportation technique is impatiently awaited in medicine for effective therapy with toxic drugs. Many thresholds have to be crossed before a satisfactory result is achieved: the need for a clearly defined target, the need to decrease toxicity by entirely removing the drug excess from the organism, the need for efficient delivery of the transported drug to target cells, *etc*. The characteristics and properties of the drug carrier system are at the top of the factors that will determine an effective solution of the problem. Many attempts to solve this problem have been made [21, 25 , 38, 43, 45], but none have proved fully satisfactory. The use of liposomes as carriers seems to be the object of the most widespread expectations [37].

In this paper, a step in development of a new technique is described, based on the use of supramolecular systems as possible drug carriers. Supramolecular systems of ribbon-like micellar organization represented by Congo red or other compounds of similar structure seem very promising; they may adhere in their micellar form to polypeptide chains of beta conformation, becoming available for interaction with proteins [29, 32, 33, 36, 39, 46, 47]. In this respect, the most useful property of supramolecular compounds seems to be their capability to selectively bind antibodies engaged in immune complexes [35]. Additionally, the binding of the dye strongly enhances the complexation of antibodies with the antigen, significantly increasing the stability of the complex [40, 41, 42]. Essential for the technique is the low toxicity of supramolecular dyes, confirmed by their use for different medical purposes [3, 24]. It results from the limited capability of supramolecular dye to penetrate living cells, and its relatively easy removal from the organism. Many planar rigid molecules, including drugs, may be intercalated to dye micellar entities and carried in this form to the target [14]. The mechanism of immune-directed transportation of some compounds by Congo red has been studied in a model system *in vitro* [16].

In this work, selective dye accumulation at a locus marked by specific antibodies was tested *in vivo*. The

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formation of specific immune complexes in local inflammation was provoked by subcutaneous injection of antigen to the ear of an immunized rabbit. Changes of color in the inflamed area were observed and registered in strong visible light with suitable color filtration to differentiate Congo red from hemoglobin, in this way registering tissue infiltration by the dye. The role of albumin, which may affect supramolecular carrier and drug transportation, was also considered.

Materials and methods

Reagents and equipment. All reagents used were of analytical grade. Bovine albumin and the dyes rhodamine B and Congo red were purchased from Sigma Aldrich Chemical Co., Inc.

The other supramolecular dye, 4,4'-bis[1-hydroxy-4-sulfophenylo-2-azo]biphenyl, called HSB in this work, was synthesized by diazotization of benzidine and subsequent coupling with 4-hydroxybenzenesulfonic acid [23]. Its purity was tested by chromatographic analysis. The applied molar absorption value (ε_{400} = 25.15 $mM^{-1}cm^{-1}$) was determined by weighing. The self-assembling property of HSB was tested by dynamic light scattering using a DynaPro800 apparatus**.** The hydrodynamic radius of HSB was found to be 2.6 nm *versus* 1.6 nm of Congo red. The effect of enhancing immune complexation also confirms its supramolecular nature [35, 42]. The susceptibility of HSB to iodination was estimated using an ELAN6100 (Perkin Elmer) apparatus. An HBO50 (OSRAM) lamp was the source of visible light, with accessory equipment (PZO) containing the power supply unit and housing for the lamp with a 24.5 cm tube which allowed the studied living object to be kept at the necessary distance from the source of light. The combined photographic filters were used to obtain the designed transparency for the source of light. Their spectra may be revealed by subtraction of the spectra in Figure 1: B1 - A1 and B2-A2 for the blue-green filter, and C1-A1 and C2-A2 for the double blue filter. The fluorescence effects were studied using a transilluminator (UVP San Gabriel U.S.A.).

Congo red and HSB binding competition with albumin. Albumin was incubated at 37˚C for 15 min with the combined micellar form of the HSB-Congo red ratio increased step-wise. The resulting complexes were separated by agarose electrophoresis in veronal buffer (pH 8.6). Albumin isolated from the corresponding spots was tested for protein, Congo red and HSB content by spectrophotometric analysis. The combined micellar form of HSB and Congo red was obtained by incubating the solution of both dyes at a suitable proportion in a boiling bath for 15 min and gradually cooling it to room temperature.

Antigens. TNP-conjugated human IgG used for immunization of rabbits was prepared according to Little and Eisen [22]. The Arthus reaction was induced by injection of TNP-ized human IgG suspended in heat-aggregated IgG (to slow the diffusion) or using rabbit red blood cells conjugated with TNP-ized human IgG [13]. Red blood cells were hemolyzed by repeated freezing and thawing before use. Isotonic solutions of Congo red used for injection (2.5 ml, 5 mg/ml) were sterilized before administration by heating in a boiling bath for 10 min and then cooled.

Animals. Four young white New Zealand rabbits (about 3 kg) immunized with TNP-ized IgG (human) were used for the experiments twice, with a two-month interval. The total volume of rabbit plasma was assumed to be 106 mL [12].

Estimation of Congo red concentration in rabbit blood. Changes of Congo red concentration in the blood after injection of the dye were registered by spectrophotometric analysis of serum samples obtained from the rabbit blood at designed intervals.

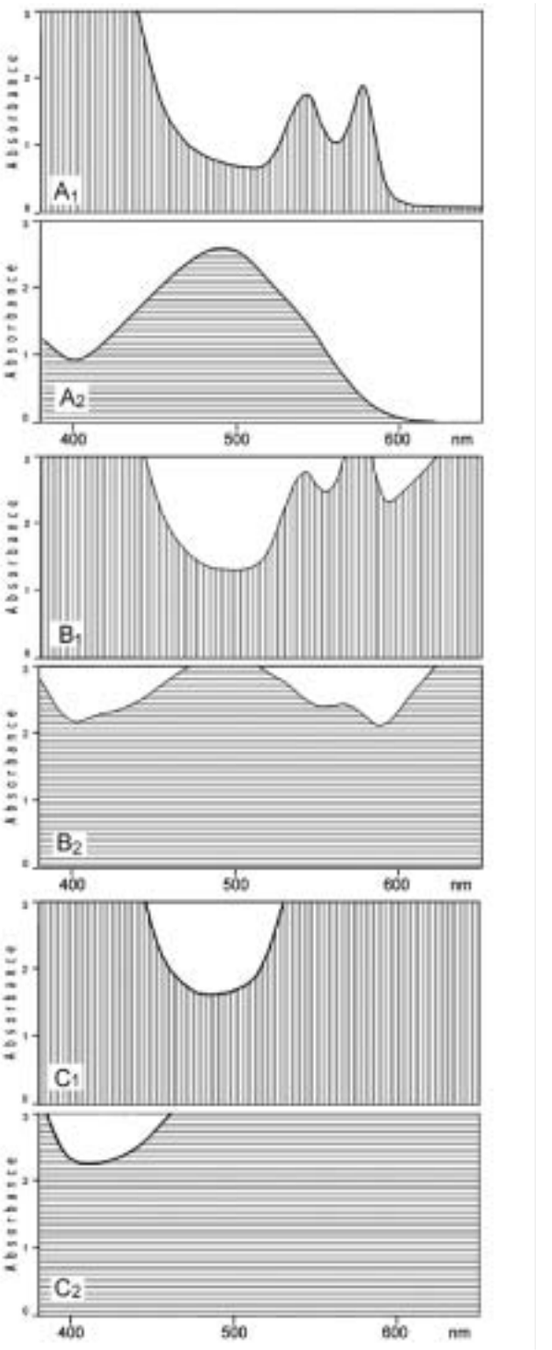

Fig. 1. Spectra of hemoglobin and Congo red (complexed with heat-aggregated IgG to obtain the spectral shift associated with complexation) shown at the dye concentration producing comparable color intensity. Dark areas represent the light absorbed, white areas - the light transmitted. The range of transmitted light on the wavelength scale and its intensity determine the color. **A1, A2** - absorption spectra of hemoglobin and Congo red, respectively; **B1, B2** - spectra of hemoglobin and Congo red superimposed on the spectrum of the blue-green filter; **C1, C2** - spectra of hemoglobin and Congo red superimposed on the spectrum of the blue filter (doubled). The color effects of hemoglobin (left sample) and Congo red (right sample) solutions corresponding to the presented spectra are shown in Fig. 2.A0, 2.B0 and 2.C0, respectively.

***In vivo* demonstration of Congo red in the area targeted by the Arthus reaction.** Migration of the supramolecular protein ligand Congo red to a locus marked by specific immune complexes was studied *in vivo*, following their accumulation at a site of localized immune complex-mediated vasculitis provoked by subcutaneous injection of antigen into a previously immunized rabbit and followed by intravenous injection of the dye (Arthus reaction).

Rabbit ear was chosen as the most convenient object to observe and document the development of inflammation and the persistence of the dye in the studied area. The relative transparency of rabbit ears to visible light made it possible to directly observe changes in the area of inflammation. Blue and blue-green filters were used to differentiate the similar red colors of the two dyestuffs present in the observed area: hemoglobin and Congo red. Figure 1 shows the spectra of both dyes and the resulting spectra obtained by superimposition of the dyes on the filter absorbancies which form the basis of color differentiation. The color effects corresponding to spectra are seen in a model system (Fig. 2.0) and in the tissue (Figs. 2-4). Pictures of the inflamed area were taken just before and then after antigen injection at suitable increasing time intervals. Pictures were taken by the camera at the designed time intervals.

Results

Congo red accumulation in the tissue area marked by immune complexes

Local inflammation was provoked in the ear of immunized rabbits by subcutaneous injection of the antigen. It is seen in the photographs (Figs. 2-7) as a network of dilatated blood vessels in the neighborhood of the injection locus.

Congo red was introduced intravenously through the marginal vein of the other ear of the rabbit about two hours after antigen injection, and the changes, now involving Congo red-derived color effects, were registered until the signs of the dye's presence disappeared, that is, for two to three days. The maximum accumulation of Congo red at the inflammation locus was usually reached a few hours after dye injection (6-8 h). The amount of the dye in the inflamed area gradually decreased with time, but its presence was often noted on the third and sometimes even the fourth day (Figs. 6, 7).

The Congo red accumulating in the inflamed area was revealed as a result of suitable light filtration as brown, violet or reddish stains, allowing it to be distinguished particularly at higher dye content against the blue or blue-green background (including the vessels not engaged in the reaction) (Figs. 2-6). The dye introduced intravenously was fully distributed all over the organism within minutes and then was gradually cleared from the blood (Fig. 8).

Supramolecular dyes have been observed to accumulate partly on the arterial intimal surface over a period of several hours, in this way influencing the kinetics of their clearance from the blood [9].

The dye accumulation may be driven by the expected specific complexation of the dye at the inflammation locus or may be the result of simple leakage from blood vessels due to increased permeability. As seen in the photographs (Figs. 6, 7), the dye distribution does not correspond exactly to the area of inflammation, indicating that its accumulation cannot be simply the effect of nonspecific outflow from the blood. The dye accumulates in the direct vicinity of the injected antigen, mostly at the walls of the blood vessels adjacent to the injection site. Its localization is thus typical for accumulation of immune complexes in the Arthus reaction. This is well seen in Figures 3, 4 and 6. The kinetics of dye accumulation at the inflammation locus (Figs. 6, 7) and the kinetics of the changes of dye concentration in the blood (Fig. 8) are clearly discordant, again confirming that the dye binds with high affinity to some insoluble material which appears and precipitates in the inflammation area and which probably consists predominantly of immune complexes. The relation of dye collection to the formation of immune complexes was additionally checked using deficient antigen (TNP-ized rabbit red cells instead of TNP-ized human IgG) for immunization to provoke the Arthus reaction. A very weak inflammation and Congo red accumulation observed in this case (Fig. 5) confirms that immune complexes drive the inflammation process and form deposits. They most likely bind the dye in a manner similar to that observed *in vitro* [16].

Preliminary evaluation of albumin access to supramolecular drug carriage

The conditions of Congo red passage through the blood, including in particular an effect that may threaten the persistence of the supramolecular form of this carrier, were also considered. This concerned primarily the effect of albumin, which binds many dyes and drugs [1, 2, 17, 18, 27] and hence may interfere with the supramolecular organization of the carrier and the carriage process [4, 5, 7, 31, 40, 48].

Albumin is a protein with multiple binding sites [6, 19]. It is engaged mostly in bilirubin and fatty acid transportation in the blood. Surprisingly, in contrast to its fatty acid binding capacity, the maximum capacity of albumin to bind Congo red and other supramolecular dyes seems to be significantly higher. Thus, while five fatty acid binding sites were identified in the albumin molecule [28], the number of Congo red molecules found to associate with albumin was 8-10 when tested under strong competition of dye (adsorption to Sephadex G25) (Fig. 9) and 16-18 when albumin-dye complexation was studied by agarose gel electrophoresis. This is evidence that albumin may attach self-assembling dyes with their supramolecular form preserved. Hence, a supramolecular carrier and albumin might collaborate in transporting a drug in the blood rather than interfere with it. Two compounds readily coassembling with Congo red but greatly differing in their ability to complex with albumin were analyzed in preliminary studies: rhodamine, a basic dye with almost no affinity

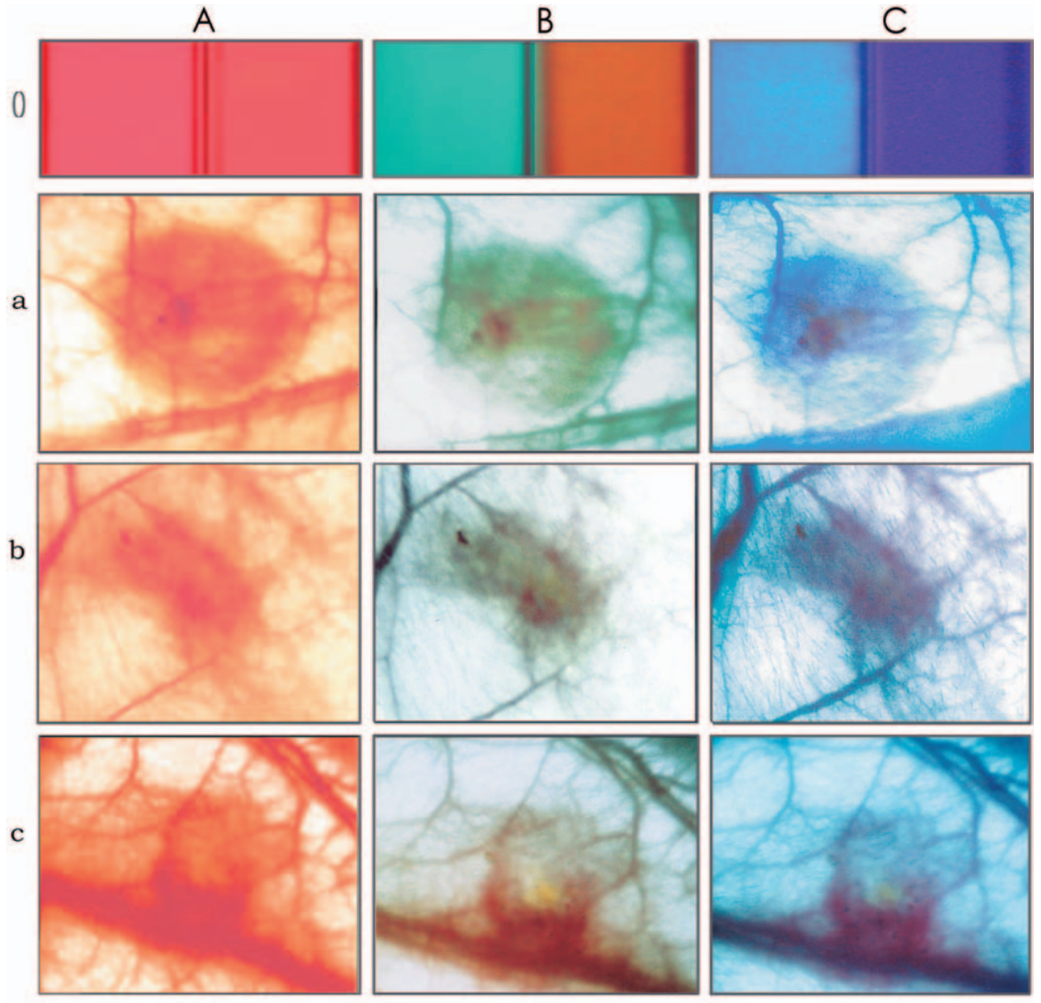

Fig. 2. Pictures of the Arthus reaction seen in the rabbit ear lit with visible light, selected from three independent experiments (**a, b, c**). Antigen - TNP-ised IgG (human). **A** - without filters (enlargement × 3); **B** - with blue-green filter (enlargement × 3.8); **C** - doubled blue filter (enlargement × 3). 0 - color test (see also Fig. 1).

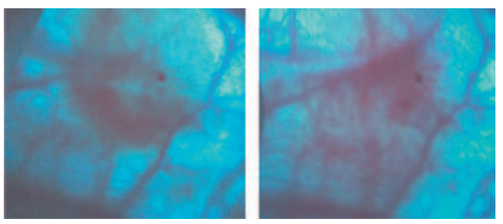

Fig. 3. Changes observed in the rabbit ear upon subcutaneous injection of TNP-ized red blood cells (hemolyzed) 5 min before Congo red injection (left) and 35 min after injection (right) (enlargement × 2.5).

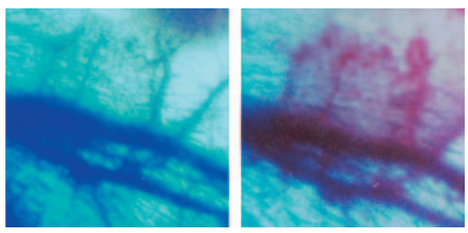

Fig. 4. Examples of changes involving blood vessels in the Arthus reaction area. Pictures from the experiment presented also in Fig. 3. (enlargement × 4).

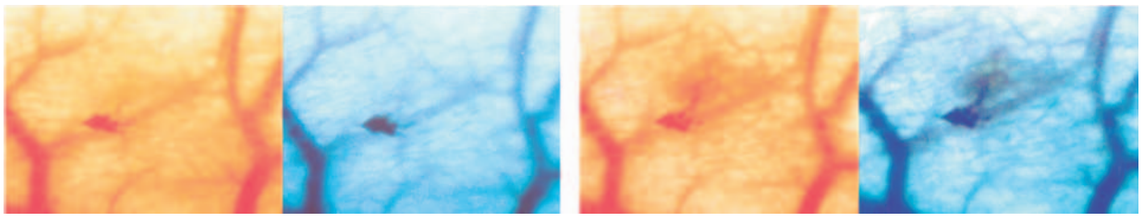

Fig. 5. A weak Congo red accumulation after injection of incomplete antigen (TNP-ized rabbit red cells instead of TNP-ized human IgG), presented in pairs: without filters and with blue-green filter used (pair on the left - before Congo red injection, pair on the right - six hours after Congo red injection, enlargement × 4.9).

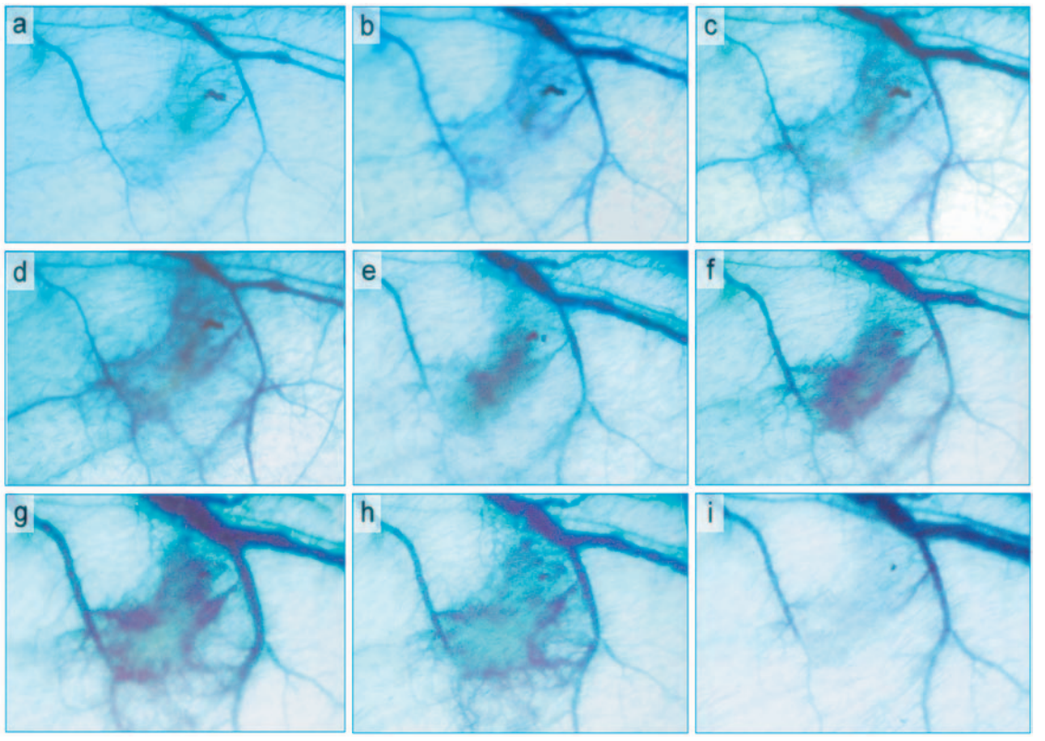

Fig. 6

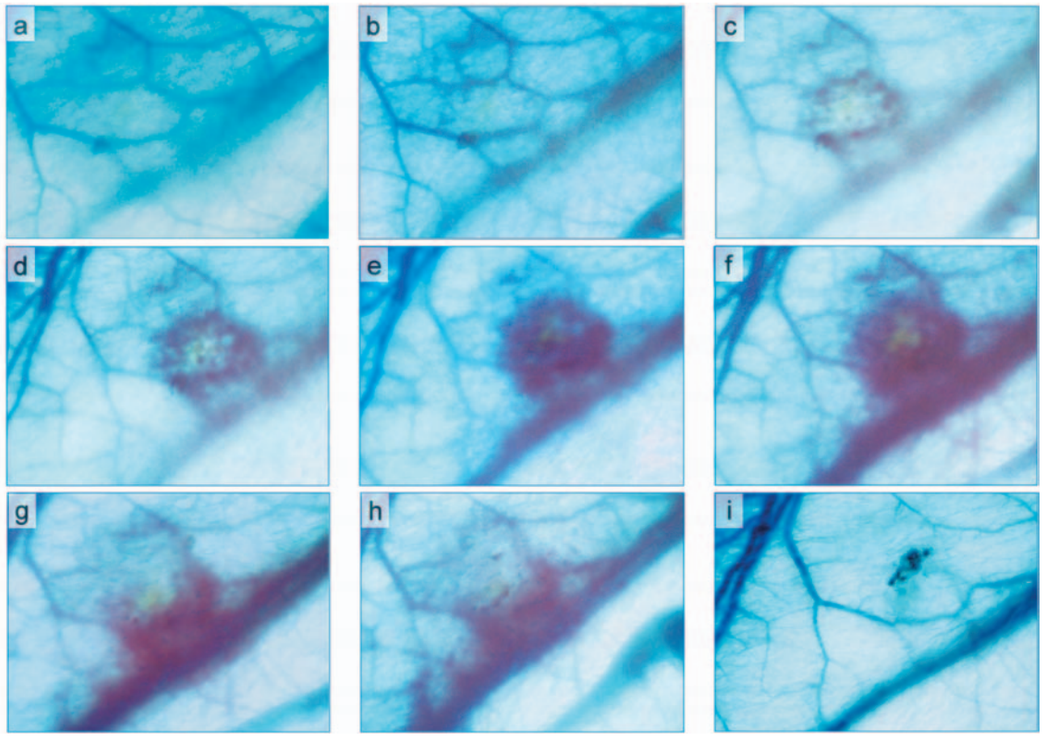

Fig. 7

Figs. 6, 7. Kinetics of Congo red accumulation in the Arthus reaction area and its diminution with time (registration using doubled blue filter). Measured times start at antigen injection (TNP-ised IgG) and then at Congo red injection (in brackets). **Fig. 6. a** - 1 h, **b** - 2 h 20 min (15 min), **c** - 3h 45 min (1 h 35 min), **d** - 4 h 5 min (2 h 40 min), **e** - 8 h 45 min (7 h 20 min), **f** - 27 h 55 min (26 h 30 min), **g** - 51 h 10 min (49 h 35 min), **h** - 75 h 45 min (74 h 10 min), **i** - 9-th day (enlargement × 2.7). **Fig. 7. a** - 1h 35 min , **b** - 3 h 30 min (1 h 30 min), **c** - 4 h 35 min (2 h 35 min), **d** - 5 h (3 h), **e** - 9 h 30 min (7 h 30 min), **f** - 26 h 30 min (24 h 30 min), **g** - 52 h 35 min (50 h 35 min), **h** - 72 h 20 min (70 h 30 min), **i** - 14-th day (enlargement × 3).

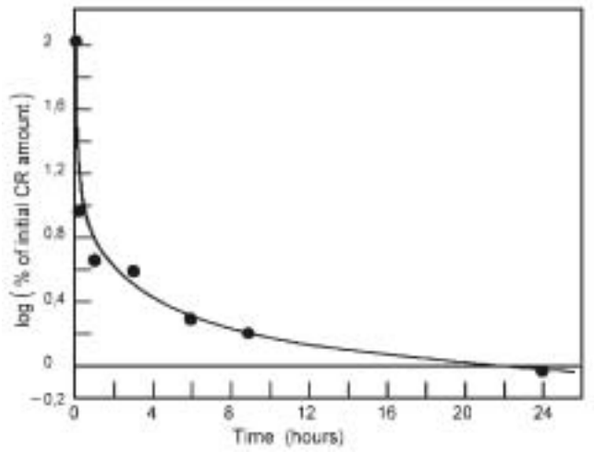

Fig. 8. Kinetics of Congo red clearance from the blood after its intravenous injection (2.5 mL of Congo red - 5mg/mL).

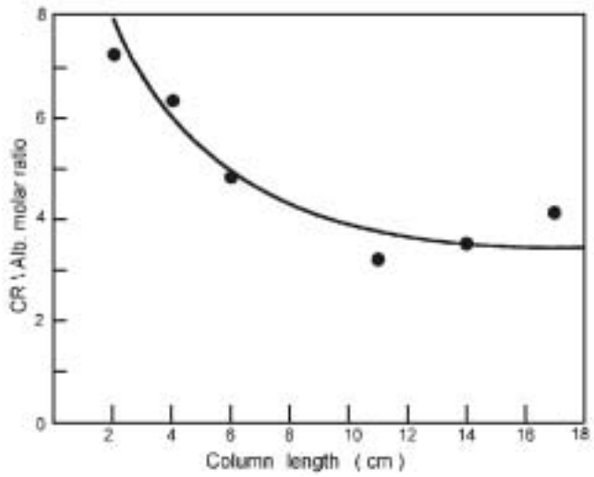

Fig. 9. The stability of Congo red-albumin complex (initial molar ratio 20:1) tested by filtration of albumin-Congo red incubation mixture through dye-adsorbing columns (Sephadex G25) of stepwise increasing length. Congo red-albumin molar ratio was determined spectrophotometrically after chromatography of eluates on TLG Silica Gel 60 plates (MERCK) for independent dye and protein estimation.

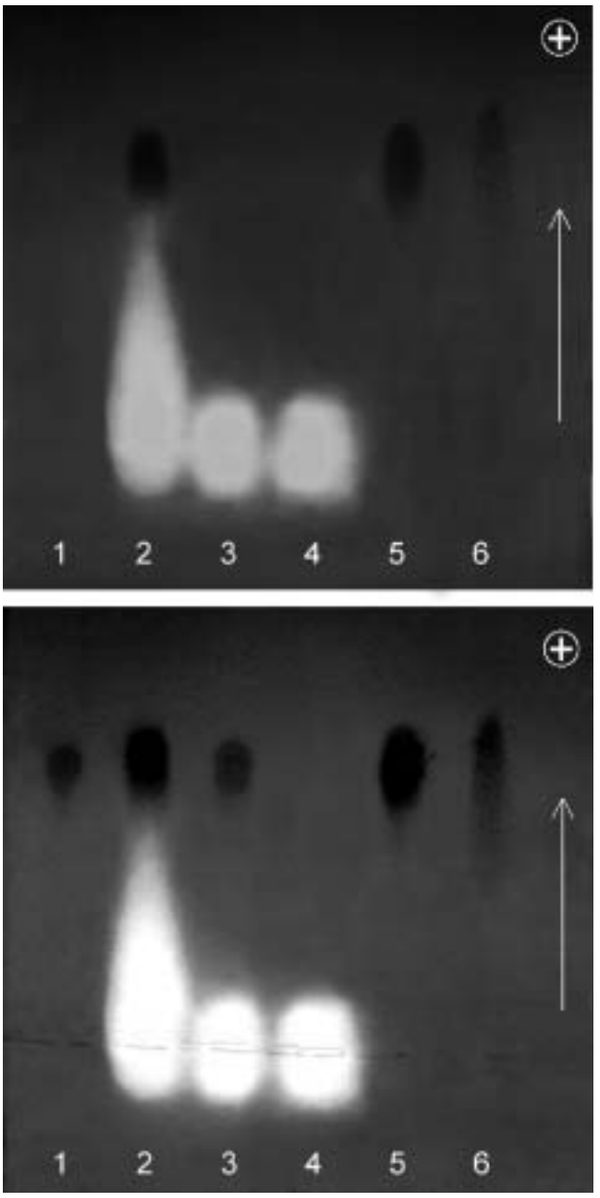

Fig. 10. Albumin enabled to bind rhodamine after prior complexation with Congo red, seen in agarose gel electrophoresis by detection of their fluorescence (upper figure), and after superimposing on a picture taken from the same experimental plate but with albumin stained with bromophenol blue dye (lower figure). 1 - albumin; 2 - Congo red-albumin complex and rhodamine B; 3 - rhodamine B and albumin; 4 - rhodamine B; 5 - Congo red-albumin complex (molar ratio 2:1); 6 - Congo red.

for albumin [36]; and HSB, a dye with molecular architecture closely related to Congo red and thus undergoing complexation with albumin.

Rhodamine B may be a model of drugs with very poor affinity to albumin, such as doxorubicin. It was chosen mostly because of its easy detection and some knowledge of its properties acquired during previous research on supramolecular ligation [16]. To verify preservation of the supramolecular character of Congo red complexed with albumin, the interaction of the rhodamine with free protein and with albumin-Congo red complex was compared. Figure 10 reveals the new albumin-binding property expressed by rhodamine complexation in the presence of Congo red. Only a two-molar excess of Congo red *versus* albumin was used in this experiment, to ensure that there was no free dye in the solution. Agarose electrophoresis was performed in conditions allowing separate migration of the studied dyes and albumin. Figure 10 shows that the albumin-

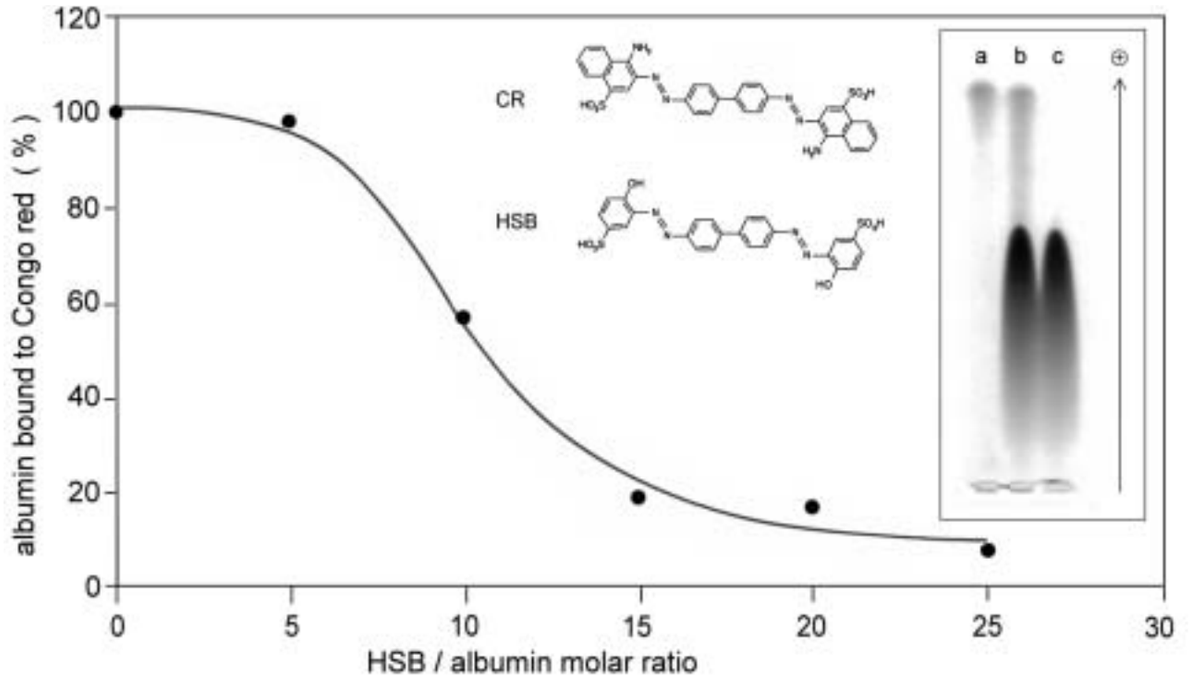

Fig. 11. Elimination of Congo red from complexation with albumin under increasing HSB to Congo red ratio in the mixed micellar form of both dyes used for the reaction. Insert shows the electrophoretic separation of individual dyes HSB (**a**) and Congo red (**c**) and the mixture (**b**), which reveals the fraction of the intermediate migration rate indicating some mutual interaction.

Congo red complex drives rhodamine migration in electrophoresis while free albumin remains completely inert, confirming the preservation of the supramolecular properties of Congo red. The observed reversibility of albumin complexes *in vivo* indicates that supramolecular carriers and albumin may form in the blood a dynamic equilibrium system with very interesting properties [26, 44].

The second example compound shows another possible model structure suitable for supramolecular carrying. It is represented by a molecule that may play the role of carrier for drugs and simultaneously may be used for transportation of radioactive atoms. The idea was to synthesize compounds preserving the fundamental Congo red architecture, with a symmetrical rigid molecule, a hydrophobic central fragment, and negatively charged ends which, however, can be chemically modified to make them accept the desired radionuclide. The presented compound (HSB) is susceptible to iodination. The dye readily self-assembles and strongly interacts with albumin. The competition of albumin binding with Congo red is seen in Figure 11. The insert shows that the tendency for HSB and Congo red to coassemble revealed by electrophoretic separation of individual dyes and the mixture (position b, where HSB is seen driving some Congo red portion as an effect of the formation of mixed micellar entities).

Discussion

The self-assembling dye Congo red and/or other related supramolecular systems of ribbon-like micellar entities are attractive candidate carriers in immunotargeting technique because of their selective interaction with antibodies in immune complexes and, independently, easy incorporation of planar rigid compounds (including many drugs) into their micellar structures (Fig. 12). Worthy of note is the noncorpuscular character of such liquid crystalline carriers which may help them to escape unwanted macrophage recognition [11]. The specific affinity of antibodies in immune complexes for supramolecular ligands probably results from distortion-derived structural domain alterations generated by antigen complexation [20, 30, 47]. The attachment of dye to antibodies significantly increases the stability of immune complexes, favoring engagement of low-affinity antibody molecules in the complex [35]. Also very important in the possible use of supramolecular compounds as carriers is their low toxicity, due basically to the limited ability of such colloidal material to penetrate living cells and their easy excretion. A portion of the dye does undergo some temporary association, mostly to large blood vessels [9, 10], and its excretion is slower. Some described cancerogenicity of Congo red is caused by benzidine liberated from it upon reduction by bacteria after oral administration [34]. The low toxicity of Congo

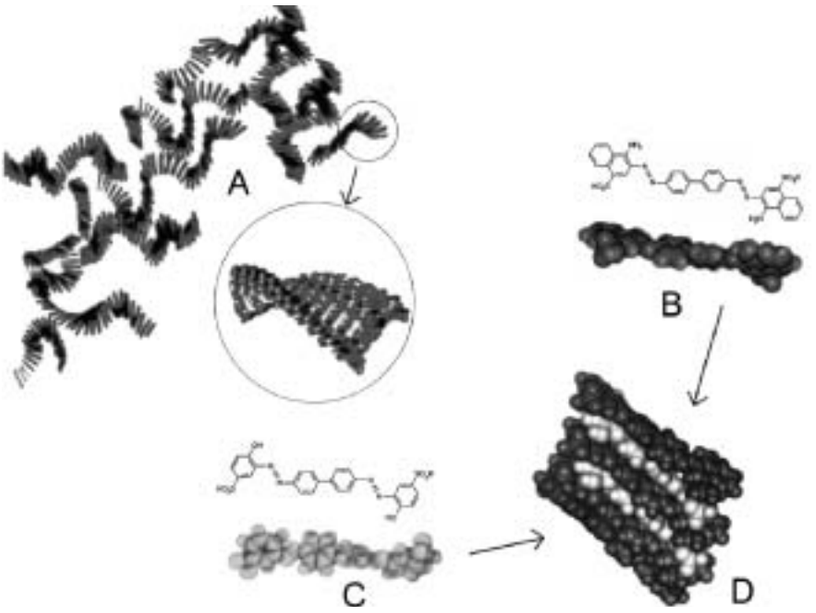

Fig. 12. The principle of supramolecular carriage organization: **A** - image of Congo red ribbon-like micellar structures; **B** - chemical formula of Congo red and its 3-D presentation; **C** - chemical formula of HSB and its 3-D presentation; **D** - formation of the combined micelle.

red, used for years in medicine, allowed this dye to be designed for the present work.

The self-assembling properties of Congo red favoring its use as a carrier in immunotargeting technique were previously studied in model systems *in vitro* [14, 15]. The main task of the present study was to verify whether the supramolecular carrier Congo red really finds a target area marked by specific immune complexes *in vivo* as was predicted from former *in vitro* experiments [16]. The formation of deposits of immune complexes was induced by Arthus reaction-derived inflammation provoked in the ear of immunized rabbits. Dye accumulation at the site of inflammation was confirmed by the specific Congo red-derived color change and its localization mostly in the immediate vicinity of blood vessels, a highly characteristic localization of immune complexes in the Arthus reaction. The kinetics of dye accumulation at the target also appeared to agree with the known kinetics of immune complex accumulation in the Arthus reaction (reaching maximum at 6-8 hours). The dye accumulation-derived color effects were greater when the injected material contained more soluble antigen, again suggesting that the deposits of immune complexes represent the dye-binding object.

The finding that dye known to interact with antibodies accumulates in the area of inflammation provoked by immune complexes makes their deposits the probable target and simultaneously indicates that the supramolecular character of the dye, enabling it to bind, is preserved *in vivo*. Although the evidence indicating that Congo red interacts selectively with immune complexes is growing, the structural plasticity of this ligand derived from its liquid crystalline nature, which may change its manner of interaction with proteins, leaves some unknowns in interpretation. Also the attraction of the dye to protein motifs other than the suggested beta-structure cannot at the moment be excluded. An important problem for drug transport and distribution is the special reactivity of albumin. Albumin may possibly disturb the supramolecular character of the carrier dye by binding individual molecules. It was found to firmly associate 4-5 Congo red molecules, but up to 16-18 under decreased environmental competition. Some studies performed in the late 1960s also showed that the number of molecules of a Congo red-related dye (Evans blue) bound to albumin is higher than expected from the number of protein binding sites [8]. Now the manner of anchorage of this self-assembling dye that preserves its supramolecular form may be proposed as an explanation. Albumin is known, however, to lose its load rapidly in contact with tissue receptors or cell surfaces [2, 4, 5, 7, 31, 40, 48]. Such an effect in the case of Congo red allows regeneration of its supramolecular form, and as a result probably favors formation of a combined equilibrium system in the blood composed of albumin and Congo red.

The binding of supramolecular dye ligands by albumin is strongly confirmed by its increased affinity for rhodamine after Congo red complexation (Fig. 10). This effect found in electrophoresis, hence in conditions that favor dissociation of weakly bound protein ligands, indicates that both the carrier dye and albumin may collaborate in drug transportation in the blood. As a result, the albumin-supramolecular carrier interaction seems to create an interesting dynamic transport system that supports rather than disturbs transportation of drugs in the blood.

In summary, the results indicate that supramolecular protein ligands with ribbon-like micellar structure may *in vivo* accumulate at a target revealed by specific immune complexes, and although the problem needs further studies, it seems highly promising for the elaboration of an effective immunotargeting technique.

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