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The use of the Congo red-related dye DBACR to recognize the heavy chain-derived abnormality of myeloma immunoglobulins

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Abstract

Introduction: The aim of this study was to differentiate heavy and light chain-derived instability of monoclonal myeloma immunoglobulins by complexation of matched supramolecular dyes. These are composed of several micellar pieces of self-assembled dye molecules which may penetrate the protein interior of the binding locus with polypeptide chains. These dyes were used to elicit, by precipitation, the postulated higher aggregation tendency of the heavy chain derived from its higher hydrophobicity.
Materials and Methods: Agarose gel electrophoresis was used to create conditions for dye complexation and to reveal the precipitation.
Results: Congo red derivatives with aromatic ring substitutes, BACR and DBACR, of increased penetrating capability were chosen to provoke the precipitation of abnormal immunoglobulins by displacing association-prone polypeptide chains from the protein interior.
Conclusions: The results of this study confirm the heavy chain-related propensity of some monoclonal immunoglobulins to aggregate and precipitate. The simplicity of the technique may improve clinical diagnosis and facilitate predictions of disease complications.

Key words: multiple myeloma, immunoglobulin aggregation, monoclonal immunoglobulins, Congo red, bis-azo dyes.

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INTRODUCTION

Many abnormal myeloma-derived monoclonal immunoglobulins tend to aggregate, producing protein deposits in tissues, with fatal clinical consequences. The character and localization of these deposits differ, probably depending on the properties of the protein. Early recognition of protein abnormality and prediction of its behavior (*in vivo*) is an important clinical task. The protein instability that lies at the basis of the aggregation property is difficult to determine unequivocally in clinical conditions. Protein instability may be studied by comparing the kinetics of denaturation of different proteins. This is a time-consuming process, however, requiring isolation and purification of the studied protein [8, 18].

In searching for a solution to this problem, we used supramolecular dyes of the Congo red family, which have been found to form complexes with some proteins, including immunoglobulins in unfolding conditions [6, 7]. Myeloma-derived immunoglobulins of unstable structure also allow penetration of these large dye ligands, composed of self-assembled molecules, to the protein interior [11, 15]. In contrast to single-molecule ligands, self-assembled compounds form strong definite complexes, and hence their formation may be used as an indicator of protein instability. As shown in previous studies, the self-assembling of Congo red and related molecules produces

ribbon-like micellar structures [16]. Fragments of these micellar entities (a few molecules long) may interact with a protein as compact single ligands. The ligand adheres to β-structured polypeptides within the β-pleated sheet, competing for space with polypeptide fragments [6]. As a result, the replaced polypeptide loop becomes exposed and may be expected to interact with its neighboring protein, leading to aggregation if its hydrophobic character is sufficiently high. Previous studies based on monoclonal myeloma-derived immunoglobulins showed that these proteins often formed strong complexes with supramolecular dyes, indicating their instability, but only some of them appeared to precipitate [14, 15]. Assuming that this interpretation is correct, it could mean that in those cases the exposed polypeptide loops derive from the heavy chain, which is generally more hydrophobic and which is commonly indicated as responsible for aggregation and precipitation of immunoglobulins [3, 9, 13]. Most probably, only displaced polypeptide fragments derived from the heavy chain tend to produce precipitating complexes with supramolecular dyes.

MATERIALS AND MMETHODS

Reagents

All reagents used in this study were of analytical grade. Congo red was purchased from Aldrich (Milwaukee, WI, USA). The Congo red derivatives 2-N-benzoylamino-4,4'--bis(1-amino-5-sulfonaphtyl-2-azo)biphenyl (BACR) and 2-N-diphenylacetylamino-4,4'-bis(1-amino-5-sulfonaphtyl-2-azo)biphenyl (DBACR) were synthesized as described earlier [17]. Bence-Jones proteins (light chains λ: KOK, AN and κ: LOZ, WOJ, BRY) were isolated from the urine of patients with multiple myeloma, then purified and characterized as described elsewhere [11]. Lyophilized polyclonal human immunoglobulin G (Gammagard S/D) was purchased from Baxter (USA).

Preparation of immunoglobulins

The $F(ab)_2$ fragments were obtained by pepsin digestion of human immunoglobulin G and isolated on a Sephacryl S-300 column. Heat aggregation of immunoglobulins and their fragments $F(ab)_2$ used for electrophoresis was performed in a standard way (protein concentration of 10 mg/ml, 20 min at 63°C). Precipitation of the heated IgG performed in the solution was measured nephelometrically at 640 nm. Precipitation was provoked at room temperature by the addition of dye to the clear solution of heated IgG (2 mg/ml, 63°C, 20 min) in an amount yielding a 10-fold final concentration in relation to protein.

Electrophoresis of serum proteins with supramolecular dyes

Electrophoresis of proteins with the supramolecular dyes was performed by the standard method (1% agarose, 0.05 M sodium barbiturate buffer, pH 8.6). Proteins were applied to rubber wells placed at the starting points and left for 3 min for diffusion to the agarose gel. The protein and dye used in the method were, however, started separately to prevent weak dye attachment to the protein at the excess of dye. The fast-migrating dye starts below, meets the protein, and passes by it, permitting transitory contact in which only strong complexation can occur and arrest the dye. The starting DBACR and Congo red concentration was 5 mg/ml. After electrophoresis and protein fixation by picric acid, the plate was washed with methanol and the excess dye was removed by blotting with filter paper. The proteins were stained with bromophenol blue dye after prior reduction of the bound dye with sodium dithionite [14].

Theoretical analysis

Content of hydrophobic amino-acid residues in Fab fragments. The content of the hydrophobic amino-acid residues in the selected polypeptide fragments of Fab (PDB entries 7FAB, 2FB4, 2F5A, 1IGC, 1DFB) was calculated assuming the classification of amino-acid hydrophobicity according to Chothia et al. [2].

Light chain dimer postdynamics structure analysis. The root mean square fluctuation (RMS-F) for light chain λ LOC (PDB entry 4BJL) were calculated according to the procedure in the CHARMM program [1]. The RMS-F value represents the size of the fluctuations in atomic positions in 3D space averaged over the final 500 ps of a molecular dynamics simulation.

RESULTS AND DISCUSSION

The instability of myeloma immunoglobulins and the subsequent aggregation tendency of these proteins may result from heavy or light chain structural defects independently, producing different structural forms of deposits and, finally, different clinical manifestations. Despite the great diversity of both immunoglobulin chains, the aggregation tendency is commonly correlated with the instability of the heavy chain rather than the light chain. The poor solubility of the isolated heavy chain has been known for years [3, 13]. This property seems to be connected with its VH domain in particular [5]. The light chain's precipitation propensity is much lower, although free light chain may slowly produce deposits in tissues, mostly of amyloid character.

Large supramolecular dye ligands of the Congo red family have been proposed for use as indicators in a simple clinical test to predict the aggregation tendency of myeloma-associated proteins [14, 15]. These dyes may form complexes with immunoglobulins when their penetration into the domain is allowed by protein instability [11, 15]. The introduction of a large, ribbon-shaped, supramolecular dye ligand into the domain forces the replacement of some polypeptide chain fragments, emptying the locus for the dye ligand [6, 7]. The replaced

loop becomes flexible and exposed to its surroundings, favoring aggregation. The significantly facilitated proteolytic degradation of the Congo red-IgG complexes (obtained by heating) observed, which indicates the increased accessibility of the polypeptide loops to the enzyme, also argues for such a mechanism [10]. The attachment of a large amount of weakly binding dye that usually occurs at protein surfaces may increase protein

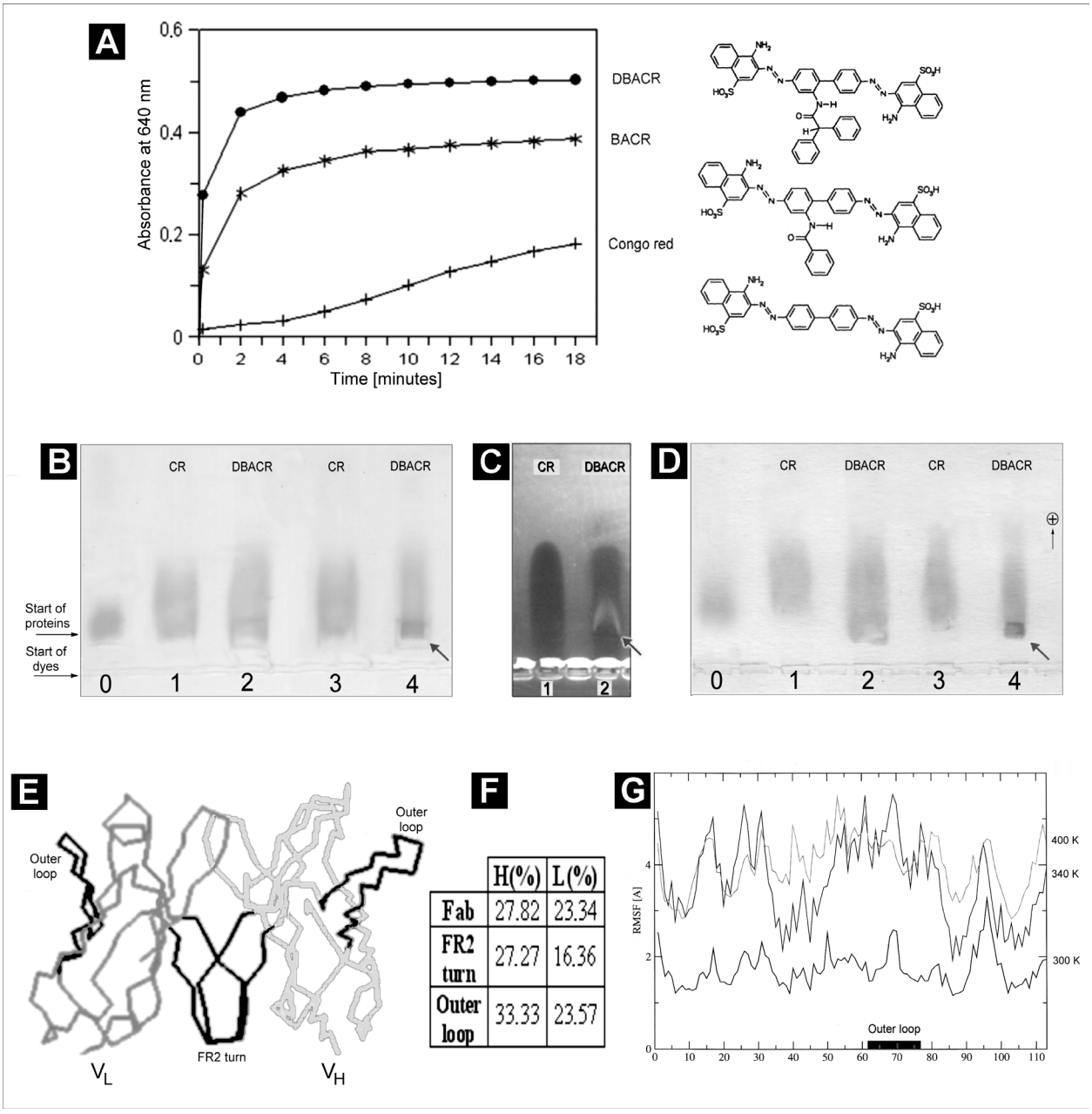

Fig. 1. (**A**) Precipitation of heated human IgG induced by Congo red and its analogs BACR and DBACR. Changes registered nephelometrically at 640 nm. Protein concentration: 2 mg/ml. (**B**) Studies of precipitation of native (lanes 1 and 2) and heated (lanes 3 and 4) human polyclonal IgG and (**D**) native (lanes 1 and 2) and heated (lanes 3 and 4) $F(ab)_2$ fragments (derived from human IgG) upon interaction with Congo red and DBACR, during transitory dye/protein contact in the course of electrophoretic migration. Protein concentration: 10 mg/ml. Immunoglobulins were stained with bromophenol blue after reduction of dyes with sodium dithionite. Arrows indicate the precipitated proteins directly at the square starting area. Control (position 0) – native proteins. (**C**) The precipitation of heat-destabilized IgG fraction is revealed by DBACR (brighter trail on the dark streaky spot – arrow) as seen directly in electrophoresis, before dye reduction and protein staining. (**E**) Polypeptide loops of the Fv model (PDB entry 7FAB): outer loop and FR2 turn (black) chosen for calculation of the mean content of hydrophobic amino acids. (**F**) Average content of hydrophobic amino-acid residues in heavy and light chains of Fab and its selected regions. (**G**) Fluctuations of polypeptide loops (RMS-F) of VL λ Loc (PDB entry 4BJL) at temperatures 300, 340, and 400 K. The fragment involving the outer loop is indicated on the abscissa by the black bar. The low-stability VL domain derived from light chain dimer instead of Fab was taken for simulation analysis to reveal the specific V domain fluctuation spectrum at the lowest possible temperature, thus exposing local fluctuation tendencies.

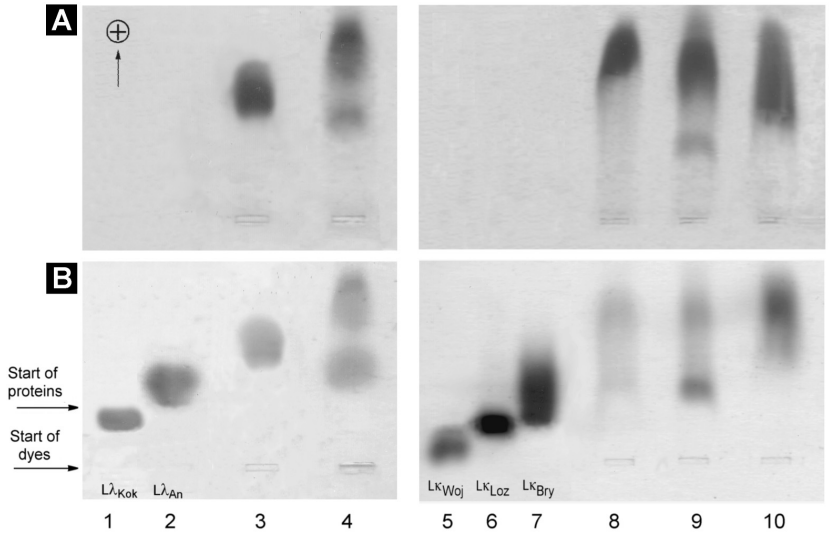

Fig. 2. Picture of the migration paths of different light chain-DBACR complexes in electrophoresis, lacking noticeable precipitation. **A** – protein-dye complexes, **B** – proteins stained with bromophenol blue after reduction of dyes with sodium dithionite, lanes 1, 2, 5, 6, 7 – proteins, lanes 3, 4, 8, 9, 10 – protein-dye complexes. Concentration of monoclonal light chains: 10 mg/ml.

solubility by electrostatic repulsion and may prevent precipitation. Some limitation of dye binding is thus necessary to reveal the aggregation propensity of the studied protein. Electrophoresis offers such conditions. The significantly different dye and protein migration rates allow removal of the dye excess from the complex together with the usually large amount of weakly attached dye molecules. Aggregation and precipitation may thus occur if the replaced polypeptide loop is sufficiently exposed and is rather hydrophobic, while only firmly bound dye remains in the emptied cavity.

For augmentation of this effect we used, instead of Congo red, its special derivative, DBACR, with a protein penetration capability stronger than that of Congo red [15, 17]. To test the mechanism of dye-induced immunoglobulin precipitation, polyclonal human IgG was used in this study. To this end, the solution of heat-destabilized IgG, diluted enough to avoid spontaneous aggregation, was triggered to be precipitated by dyes (Fig. 1A). The dyes that induce precipitation of IgG most efficiently, DBACR and Congo red, were chosen for electrophoretic tests. Figure 1B–D presents the precipitation of IgG and also $F(ab)_2$. According to this figure, only protein molecules that were destabilized by heating formed the precipitate. The traces of precipitation seen in native IgG derived most probably from the small portion of aggregated IgG which is usually present in commercial IgG.

Independently, the five different light chains (λ: KOK, AN and κ: LOZ, WOJ, BRY) isolated from the urine of patients with multiple myeloma (of which KOK and BRY were highly unstable [15]) were used in the studies as the control. In no case was precipitation with DBACR observed (Fig. 2). This again supports the suggestion that precipitation in the applied conditions is correlated with heavy chain-derived instability.

The "outer loop", which is clearly more hydrophobic in the heavy chain (Fig. 1E, F), is suggested to play a key role in heat-induced aggregation of immunoglobulins. Interestingly, it is also the receptor region for BiP [4]. It is located in the region of the polypeptide chain that becomes significantly flexible upon heating, again confirming its engagement in aggregation (Fig. 1G). The hydrophobicity of this loop was estimated to verify this suggestion. Fig. 1F presents the content of hydrophobic amino acids (according to the Chothia classification [2]) in selected polypeptide fragments, calculated as mean values of five Fabs.

Recently, studies of *camelidae* heavy chain antibodies, "camelized" human immunoglobulins, and other recombinant antibodies provided support for a generalization of these heavy chain features [5, 12]. This particular heavy chain property is likely connected with its dominant role in antigen recognition and binding. The unusually potent mechanism of immunoglobulin diversification burdens the attempt to generalize heavy chain properties with some uncertainty, however. Final verification of this assumption requires clinical studies. We believe that the simplicity of our proposed technique provides an approach to this problem.

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