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Chromatographic and electrophoretic studies of circulating immune complexes in plasma

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ABSTRACT

The protein nature of soluble immune complexes from fresh plasma was studied by combining several analytical biochemical techniques. Free immunoglobulins (Ig) G were separated from larger immune complexes by gel permeation chromatography. In a second step, immune complexes, free IgA and IgM were isolated by protein-A and protein-G affinity chromatography and analysed by two-dimensional gel electrophoresis. Sixteen plasma samples from healthy donors were analysed and evaluated visually. Their protein profiles on the gels turned out to be similar, showing only slight quantitative differences. In one case, additional proteins were detected. To prove the ability of the method, immune complexes were analysed from four plasma samples that showed macro creatine kinase type 1, a complex formation between creatine kinase BB and IgG. This methodology can be used for the examination of immune complexes of unknown protein composition in serum or plasma.

INTRODUCTION

In recent years, great interest has been expressed in the detection of soluble immune complexes (IC) circulating in the bloodstream of patients with infectious, neoplastic or autoimmune diseases [1]. For a better comprehension of the immunopathology of such diseases, a variety of methods have been developed for the detection and quantitation of circulating IC. Most of these techniques show the presence of elevated levels of IC and some provide semi-quantitative estimates, but they do not identify the constituent antigen in the complexes. If these antigens could be identified, some indication of the nature of the disease might be obtained.

The main purpose of this report is to present a way of isolation and analysis of IC in plasma or serum by combining several analytical biochemical methods. In a first step, gel permeation is used to separate the small free immunoglobulins G (IgG) from IC. By binding the IC to protein-A Sepharose (PAS) and protein-G Sepharose (PGS) in a second step, the high-molecular-mass proteins are eliminated. Finally, the IC are analysed by two-dimensional gel electrophoresis (2-DE). The antigen and antibody components are then separated, based on differences in isoelectric points and molecular masses. The aim of this methodology is the ex-

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amination of IC of unknown composition in normal sera or plasma. As a model we studied the plasma from patients that showed macro creatine kinase (CK, EC 2.7.3.2) type 1, a complex formed between CK-BB and IgG [2].

EXPERIMENTAL

Plasma

Sixteen plasma samples from normal subjects (20–50 years old, including both sexes) were collected in tubes containing Na_2EDTA as anticoagulant. All donors were considered healthy on the basis of clinical and biochemical criteria and were not taking any drugs known to influence the immune system. The blood was obtained by venipuncture and centrifuged without delay, and the plasma was immediately used for the analysis of IC, without storing at 4°C or freezing. As an example of the clinical usefulness of 2-DE in the protein separation of IC, we selected cases with macro CK type 1. Total serum CK activity and non-CK-M subunit activity were determined with Monotest CK NAC and Monotest CK-MB, respectively, at 37°C on a Hitachi 705 autoanalyser. Samples with CK-MB values higher than total CK were further investigated for the occurrence of macro CK by agarose gel electrophoresis with the 'Paragon' system (Beckman Instruments, Brea, CA, U.S.A.). The CK isoenzymes were detected with a colorimetric detection kit from Sigma (St. Louis, MO, U.S.A.), based on the reduction of tetranitroblue tetrazolium.

Gel permeation chromatography of plasma proteins

For the separation of IC from free IgG, we used the fast protein liquid chromatographic system (FPLC) from Pharmacia-LKB (Pharmacia-LKB Biotechnology, Bromma, Sweden), consisting of a liquid chromatographic controller LCC-2152, an HPLC pump P2150, an automatic switching valve 2154 and a variable-wavelength monitor 2151. Plasma samples were diluted with phosphate-buffered saline (PBS) (0.15 M NaCl, 0.018 M K_2HPO_4, 0.01 M KH_2PO_4, 0.02% NaN_3; pH 7.5) in a ratio of three parts of sample to one part of PBS buffer. The mixture was filtered through 0.2-μm Acro filters LC13 (Gelman Sciences, Ann Arbor, MI, U.S.A.) before use. The samples (2 $\times$ 200 μl) were injected onto the Superose 12 column (300 mm $\times$ 10 mm I.D.; particle size 10 μm) using a 200-μl loop. The proteins were eluted isocratically according to their molecular masses with PBS at a flow-rate of 0.25 ml/min. After 70 min the flow-rate was increased to 0.5 ml/min. The proteins were detected by their UV absorption at 280 nm. The absorbance range selector was set at 2.56. Fractions of 1 ml were collected. The high-molecular-mass fractions were pooled and applied to a PAS–PGS column.

Isolation of immune complexes

An equal amount of swollen PAS (Pharmacia) was mixed with an equal amount of swollen PGS (Pharmacia), and packed in the tip of a Pasteur pipette.

The gel bed was always 40 μl. Prior to affinity chromatography the gel was washed extensively with PBS. The fractions from the column chromatography containing the probable IC were applied to the microcolumn. IC, IgM and polymeric IgA were adsorbed on the microcolumn. Unbound proteins were eluted with a twenty-fold column volume of PBS. Afterwards the column was transferred to an Eppendorf tube and equilibrated for 30 min in 60 μl of dissociation buffer containing 30 g/l glycerol, 30 g/l sodium dodecyl sulphate (SDS) and 90 g/l 2-mercaptoethanol, then heated to 95°C for 5 min and centrifuged briefly at 12 000 *g*. The supernatant was carefully collected. Each PAS–PGS column was used only once.

Two-dimensional gel electrophoresis and silver staining

2-DE was performed by a modification of the method of O'Farrell [3] as described previously [4]. The first dimension was carried out in tubes of 14 cm × 0.15 cm. I.D. The collected sample was applied to the IEF gel containing 4% acrylamide, 2% Nonidet P40, 8 *M* urea, 4% 3.5–10 and 1% 5–7 carrier ampholytes (LKB, Bromma, Sweden). IEF was carried out overnight for 10 500 V h. The second dimension was performed in a slab gel (14.5 cm long, 14 cm wide, 1.5 mm thick) consisting of 12% acrylamide. 2-DE gels were stained with ammoniacal silver nitrate and formaldehyde citrate [5] with some modifications [6]. Stained gels were photographed and evaluated visually.

Protein identification

Fibrinogen, cleaved products of haptoglobin β chain and CK-BB were identified by western blot and immunostaining. Proteins resolved by 2-DE were electrophoretically transferred from the gels onto nitrocellulose sheets, as originally described by Towbin *et al.*[7] The transfer was performed in a Trans-Blot Cell (Bio-Rad, Richmond, CA, U.S.A.) in 25 m*M* Tris, 192 m*M* glycine (pH 8.3), 20% (v/v) methanol at 60 V for 3 h. After transfer the nitrocellulose membrane was blocked for 1 h in Tris-buffered saline (TBS) (0.02 *M* Tris and 0.5 *M* NaCl) (pH 7.5) containing 0.3% Tween 20. Then the sheets were incubated overnight in a TBS-Tween solution as above, containing the antibody at a concentration of 1:400. The antibodies against fibrinogen and haptoglobin raised in rabbits were from Dakopatts (Glostrup, Denmark), and the antibody against CK-BB raised in a goat was from Jackson Immunoresearch (West Grove, PA, U.S.A.). After washing, the sheets were incubated with an anti-rabbit antibody conjugated with horseradish peroxidase (Bio-Rad) at a concentration of 1:1000. After washing, specific proteins were visualized by the addition of 4-chloro-1-naphthol and hydrogen peroxide. For overall staining, the nitrocellulose was treated with a supplement of 20-nm gold particles (anionic) with Tween 20 at low pH, according to Moeremans *et al.* [8]. The sheets were incubated overnight in a solution containing 0.01% tetrachloroauric acid trihydrate, 0.1% sodium citrate and 0.2% Tween 20. CK-BB was also identified by co-migration with purified human CK-BB, obtained from Calbiochem (Calbiochem, La Jolla, CA, U.S.A.).

RESULTS

Isolation of IC from blood samples

Complexed Ig were first separated from free Ig by size-exclusion FPLC in PBS at pH 7.5, to minimize subsequent binding of non-complexed IgG to PAS–PGS. Fig. 1 shows a typical run of plasma with four peaks of absorbance at 280 nm. The shapes of the profiles obtained from column chromatography of different samples are highly reproducible. The first two peaks (I and II) contain high-molecular-mass proteins, including possible IC. Very large proteins, including IgM, are eluted in peak I and correspond to the void volume. Peak II mainly contains IgA, fibrinogen and other large proteins. The non-complexed IgG are found in peak III, and albumin and α_1-antitrypsin in peak IV. Peak I and peak II (fractions 9–11) were pooled (total 3 ml) and subjected to PAS–PGS affinity chromatography. Fraction 12, a transition fraction between peak II and peak III, was analysed separately. IC, IgM and polymeric IgA are bound to the PAS–PGS matrix and other proteins are washed out.

2-DE analysis of IC from normal plasma samples

Fig. 2 shows a typical 2-DE pattern of normal plasma IC. The gels of sixteen IC

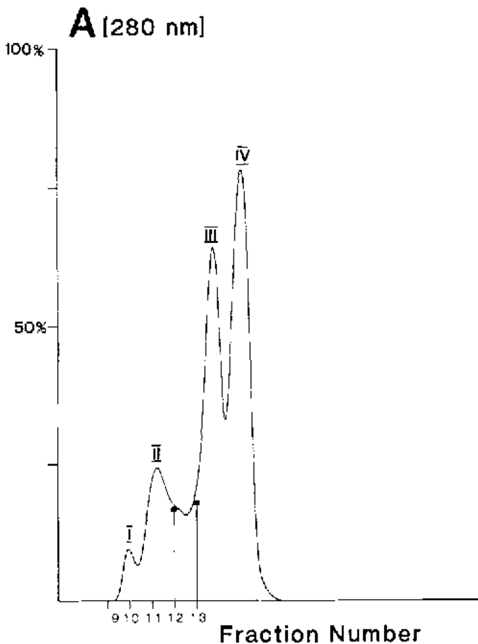

Fig. 1. Size-exclusion FPLC elution profile of plasma proteins. For each run, *ca.* 10 mg of proteins were injected into a Superose 12 column. Chromatography was performed at a flow-rate of 0.25 ml/min with PBS (pH 7.5). Peak I and peak II (fractions 9–11), containing possible IC, were pooled and further analysed. Fraction 12 was analysed separately.

from healthy individuals are very similar, showing that the technique is reproducible. In one case we found two additional protein fractions at molecular mass 30 kDa. They reveal very intense spots on the gel. The positions of the IgA, IgM and IgG heavy chains have been established earlier [9,10] and are well known. The origin of the set of larger proteins at molecular mass of 48 kDa is unknown, but they probably represent fragments of the μ-heavy chain, because commercially available polyclonal μ-chain-specific antibodies react with this set of proteins [9]. In contrast to the heavy chains, which are largely unresolved owing to their carbohydrate microheterogeneity and their substantial sequence variability, the light chains contain little if any carbohydrate and yield a great number of distinghuisable spots, arranged as a band at 27–31 kDa on the SDS gel (based on standards). Fig. 3 shows two patterns of light chains of plasma IC from a healthy

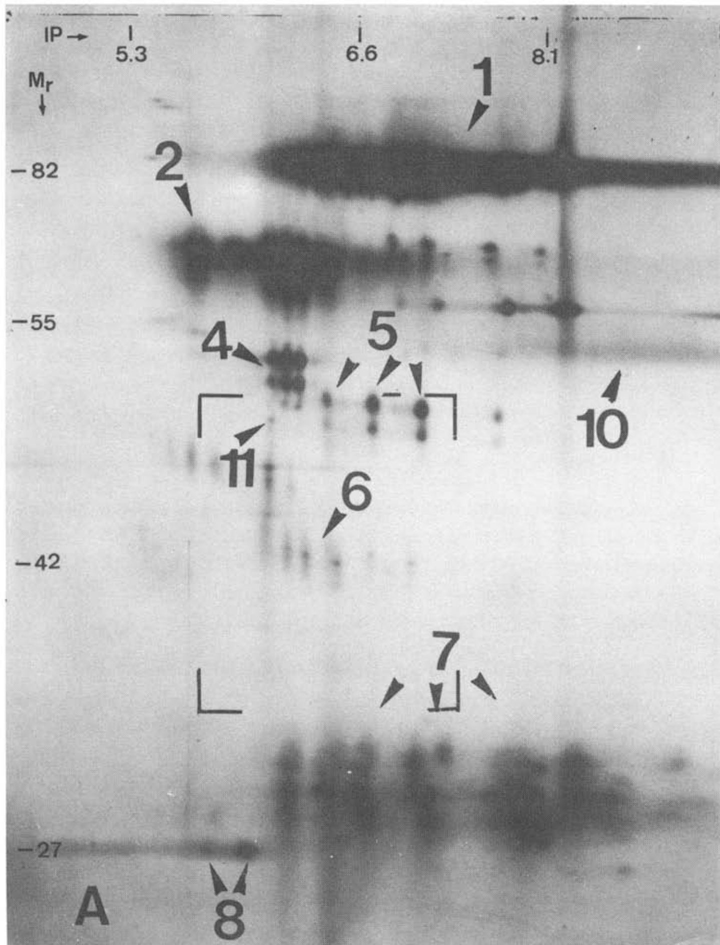

Fig. 2.

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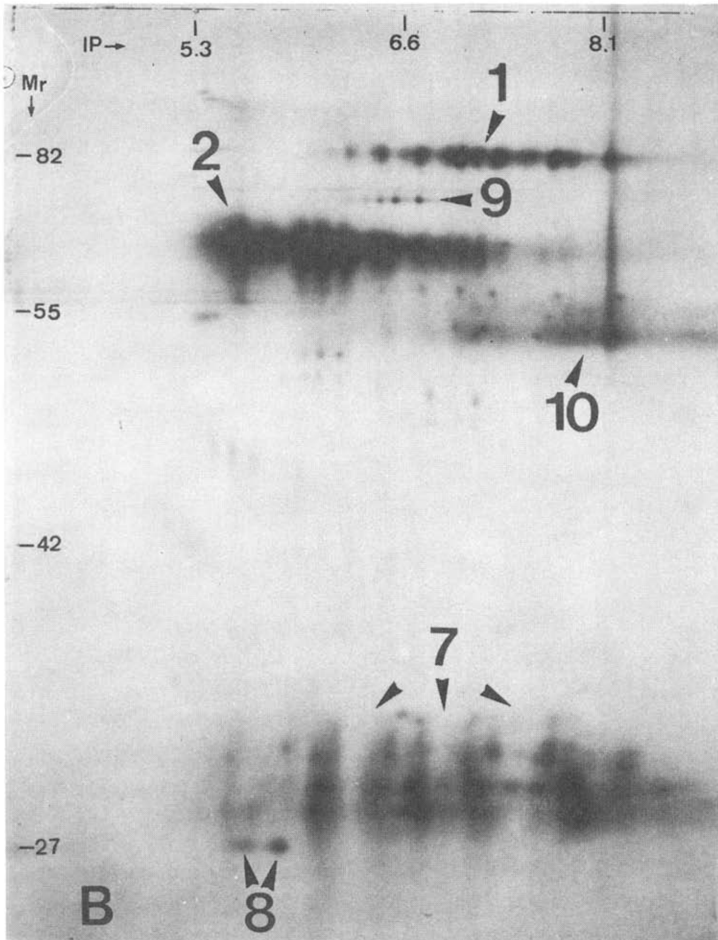

Fig. 2. Silver-stained 2-DE gels of IC, unloaded IgM and unloaded IgA fractions, isolated from plasma by size-exclusion FPLC and PAS-PGS affinity chromatography. (A) 2-DE pattern of peak I and peak II (fractions 9–11); (B) 2-DE pattern of fraction 12. The numbered arrows point to known plasma proteins: 1 = IgM heavy chains; 2 = IgA heavy chains; 3 = fibrinogen β-chain; 4 = fibrinogen γ-chain; 5 = constant fragments of IgM; 6 = haptoglobin β-chain, breakdown products; 7 = Ig light chains; 8 = apolipoprotein A-I; 9 = albumin; 10 = IgG heavy chains; 11 = actin. The molecular masses are shown in kilodaltons (kDa). The corners of the region that are shown in Fig. 4 are marked.

man. The plasma samples were obtained at an interval of four months. The charges and the molecular masses of the light chains seem to remain constant. A careful comparison shows that almost all the spots seen in one pattern are also found in other pattern. There is at most a slight variation in their intensity. In two other cases of healthy women, we also found a light chain pattern constant over weeks.

Besides the Ig fractions, some components are invariably found in low concen-

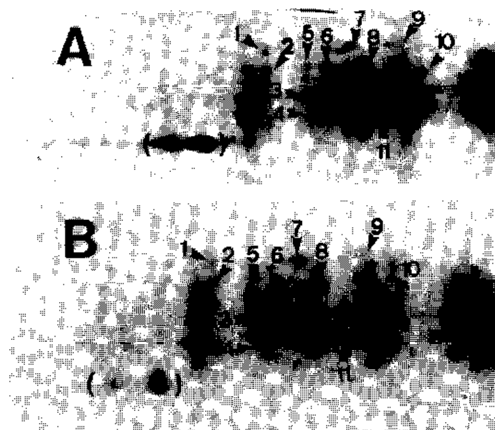

Fig. 3. Silver-stained 2-DE patterns of the Ig light-chain region of fractions 9 11. The IgM, IgA and IC were isolated at an interval of 4 months from the plasma of a healthy man. (A) First isolation, (B) second isolation four months later. The arrows point to clones of Ig light chains. For a better orientation they are numbered. Apolipoprotein A-I is indicated by parentheses.

trations in all investigated samples, such as albumin, apoliproprotein A–I, actin and fibrinogen (Fig. 2). They could be identified by comparison with other 2-DE patterns of human plasma [9,11] In all preparations, a protein group at 40 kDa appears, although concentration varies. It has been identified as a cleaved product of the haptoglobin β chain by Anderson and Anderson [12]. We confirmed these findings by immunoblotting with the corresponding antibodies, and gold staining.

2-DE analysis of IC from plasma samples with macro CK type 1

To test the capability of the presented method for isolating antigen–antibody complexes, experiments were done with plasma containing IC of known antigen composition (CK-BB). We selected four plasma samples from patients admitted to the University Hospital with myocardial diseases or carcinoma.

TABLE I

TOTAL CK ACTIVITIES AND CK-MB ACTIVITIES IN PLASMA FROM FOUR HOSPITALIZED PATIENTS SHOWING MACRO CK TYPE 1 BY AGAROSE GEL ELECTROPHORESIS

Adult upper reference limits: men, 270 U/l for CK and 25 U/l for CK-MB; women, 150 U/l for CK and 25 U/l for CK-MB.

Patient	Sex	Total CK (U/l)	CK-MB
M.A.	F	320	431
K.S.	F	252	272
G.W.	M	331	648
M.J.	M	112	266

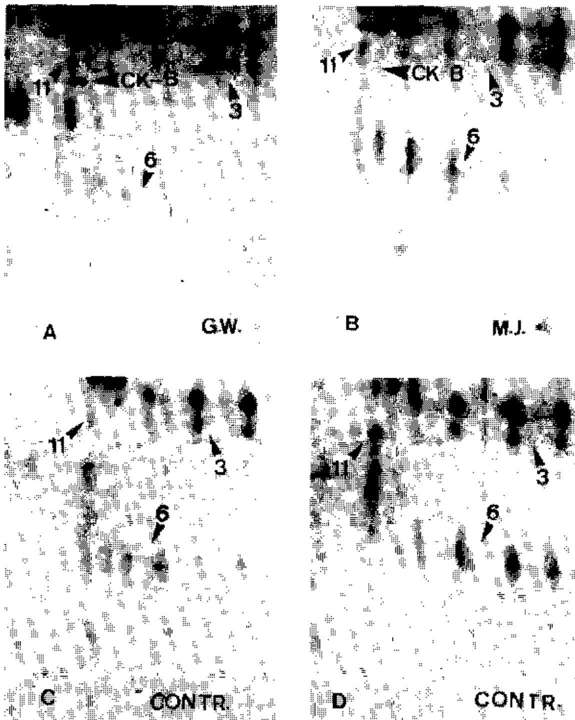

Fig. 4. Photographic enlargement of the macro CK region from Fig. 2, where the corners of the section are marked. The arrow indicates the CK-B subunit. The depicted region includes proteins with apparent molecular mass from 50 kDa to 35 kDa, and isoelectric points of pH 5.9–7.1. (A) Patient G. W. and (B) patient M. J. both showing macro CK type 1. (C) and (D) control cases without macro CK type 1.

Table I shows total CK and CK-MB activities as measured by immunoinhibition. The presence of macro CK type 1 was confirmed by agarose gel electrophoresis. They show abnormally migrating electrophoretic bands. Fig. 4 shows the detail of the 2-DE profile from a macro CK IC. The CK-B subunit migrates as a unique protein with an apparent molecular mass of *ca.* 42 kDa and an isoelectric point of *ca.* 5.9. The position of the CK-B subunit was confirmed by co-migration experiments with pure human CK-BB, and by immunoblotting with polyclonal antibodies against CK-BB.

DISCUSSION

Our strategy for detecting antigenic components of IC in human plasma or serum comprised precleaning of most other protein components from IC material by size-exclusion, incubating the high-molecular-mass proteins with PAS–PGS under conditions in which complexes are preferentially bound, eluting IC materi-

al from PAS–PGS in SDS-denaturating buffer and analysing the protein fractions by 2-DE. Resolved proteins were then stained with silver diamine. Probable antigenic components are made visible.

For protein removal, gel permeation is preferred to precipitation with polyethylene glycol (PEG) or ammonium sulphate. One hazard of these precipitation techniques is an eventual release of antigens from the insolubilized complex. For example, 3.5% PEG yielded only 34% of a preparation of preformed radiolabelled complexes, compared with a yield of more than 90% after gel permeation [14]. IgM and polymeric IgA are not removed by initial gel permeation, and they also interact with PAS. However, contamination with these proteins seems not to disturb the evaluation of the 2-DE pattern. Compared with conventional gel permeation columns with Bio-Gel or Sephadex, size-exclusion FPLC offers the advantage of much higher resolution in less than 90 min, and a greater recovery of individual proteins.

Protein A and protein G are bacterial proteins that specifically bind to the Fc region of Ig by a non-immune reaction. In contrast to protein A, which binds IgG1, IgG2, IgG4, IgA2 and IgM, protein G interacts with all IgG subfractions. This may be of importance because of the high incidence of IgG3-IC in cases such as macro enzymes [15] or infectious diseases [16]. Protein A and protein G were used in a second step to separate IC from other high-molecular-mass non-Ig fractions. The constituents of IC were analysed by 2-DE and silver staining. Besides the Ig fractions, some components are invariably found in all investigated samples, such as low amounts of albumin, liproproteins, fibrinogen, actin and breakdown products of haptoglobin. In addition, trace amounts of unidentified components are detectable. It is well known that contaminants and constituents other than the relevant antigen and antibody may be bound in low concentrations to Sepharose [17]. Experiments in our laboratory with purified fibrinogen demonstrated that fibrinogen interacts slightly with PAS–PGS. On the other hand, several studies have shown that many plasma proteins bind to circulating IC, albeit in low concentrations [18–20]. The question is: how could non-Ig become associated with circulating IC? One possibility is that there is a specific linkage between the Fab region of the Ig and the plasma proteins, or a non-specific interaction between the Fc part of Ig with plasma proteins. Another possibility is that these proteins are altered by free radicals or enzyme damage, and then become non-specifically adherent [21]. In one case of a healthy donor, we found striking protein spots on the gel, which were absent in the other cases. It is known that elevated amounts of IC can also be detected in normal serum samples. The reported values range between 3% [22] and 19% [23].

Although it is easy to demonstrate the presence of an antigen and/or an antibody in model complexes, attempts to identify suspected constituents in clinical material pose considerable problems, even when the same methods are employed [17]. For this purpose we tested the suitability of our method with clinical material. We isolated IC from plasma of adult patients in which we noted macro CK

type 1. The binding of enzymes to antibodies is a well known phenomenon and has also been described for lactate dehydrogenase [24], amylase [25], alkaline phosphatase [26], acid phosphatase [27] and alanine aminotransferase [28]. The molecular mass of macro CK type 1 has been reported between 240 and 350 kDa [29], but complexes of 650 kDa have also been reported [30]. In any case, the IC are well separated from the free IgG (158 kDa) by the size-exclusion FPLC system. The Ig content of the IC is usually IgG [30], but IgA has also been described [31]. The binding site was localized on the Fab or $F(ab')_2$ fragment of the Ig molecule [32]. The antigen component of the IC, in this case CK-BB, can be demonstrated as a well defined spot with the 2-DE system, a technique of high efficiency in disrupting antigen–antibody complexes. The denaturing effect of 2-DE can be a drawback, but on the other hand it is a potent tool for resolving complex protein mixtures. Thus, it could theoretically be the method of choice for analysing the protein nature of IC. Attempts to analyse IC in clinical situations, such as certain rheumatic diseases and multiple sclerosis, are currently in progress.

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