Rapid optimization of immunoadsorbent characteristics

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Immunoaffinity chromatography is employed in many research areas. We have developed an assay system that overcomes some of the tediousness and uncertainty in dealing with immunoadsorbents (IA). The preparation of IA and the effect of various procedures on the dissociation of antigen–antibody complex and the regeneration of IA are rapidly screened by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. Non-covalently bound proteins are dissociated and separated during the electrophoresis from IA beads. A wide variety of associative and dissociative conditions can be tested on small amounts of IA. Information about bio-specifically and non-biospecifically adsorbed proteins can also be obtained. By using relatively small volumes of media containing either an antigen or another biospecific molecule, the optimal parameters for affinity chromatography (specificity, binding capacity, efficiency of solvents in dissociation of the complex and their effect on the adsorbent), or even for ion-exchange chromatography, can be determined without first performing several time- and material-consuming chromatographic experiments.

An enormous number of studies have utilized affinity chromatography during the past two decades (Wilchek & Jakoby, 1974; Hoffmann-Ostenhof *et al.*, 1978; Lowe, 1979). This technique is based on specific interactions between matrix-bound ligands and soluble biochemicals and it enables purification of many biochemical entities on the basis of their biological function or individual chemical structure (Cuatrecasas, 1972).

Abbreviations used: IgG, immunoglobulin G; IA, immunoadsorbent(s); IgG fraction from antiserum immobilized to CNBr-activated Sepharose 4B; IA beads, the bead form of the immunoadsorbent gel; F(ab')₂, bivalent pepsin-generated N-terminal fragment of IgG; Fc, papain-generated C-terminal fragment of IgG; L and H, light and heavy chains of IgG; SDS, sodium dodecyl sulphate; PMSF, phenylmethanesulphonyl fluoride; GdmCl, guanidinium chloride; C1, C3, C4 etc., first, third, fourth (etc.) protein components of the serum complement system; anti-C3 etc., anti-C3 antiserum (etc.); SpA, protein A of *Staphylococcus aureus*; HSA, human serum albumin; NHS, normal human serum; NGS, normal goat serum; EQB (equilibrating buffer for IA), 50 mM-sodium phosphate/250 mM-NaCl/5 mM-EDTA, pH 7.4 (at standard temperature and pressure); QAE, quaternary aminoethyl; CM, carboxymethyl; SP, sulphopropyl.

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Frequently, a rare biological molecule can be purified to homogeneity by use of a complementary ligand or specific antibody.

Immunoaffinity chromatography employing covalent immobilization of either antigen or antibody to water-insoluble carrier is in fact a predecessor of affinity chromatography. The first well-characterized IA was prepared by coupling ovalbumin to diazotized *p*-aminobenzyl-cellulose (Campbell *et al.*, 1951) and was used as an immobilized antigen for the purification of antibody molecules (for a review, see Silman & Katchalski, 1966). The concept of using an immobilized antibody for specific detection of radioactive antigens was introduced about two decades ago (Gurvich & Drizlikh, 1964).

IA are usually prepared by allowing CNBr-activated Sepharose 4B to react with an IgG fraction from immune sera (Robbins & Schneerson, 1974; Porath & Kristiansen, 1975; Kristiansen, 1978). As a rule, the high affinity of the antibody–antigen complex precludes elution of the antigen in the solvent of application. Very often, only non-specific desorption methods employing deforming solvents are necessary; most of them, however, can be detrimental to the antigen, and possibly to the antibody molecule as well. Optimal conditions for dissociation of the antigen–antibody

complex, which are necessary for isolation of intact antigen and for regeneration of immunoadsorbent, have been principally determined by an empirical approach. This may involve many successive chromatographic procedures integrated with the analyses of each fraction. In the present paper we describe a simple approach that, in a few steps, analyses and defines the appropriate conditions for affinity chromatography. A preliminary account of part of this work has already been presented (Janatova & Gobel, 1983).

Experimental

Materials

Reagents for polyacrylamide-gel electrophoresis, including Coomassie Brilliant Blue R-250, high- and low- M_r protein standards were purchased from Bio-Rad Laboratories. SDS was from BDH. GdmCl and β -mercaptoethanol were from Pierce Chemical Co. CNBr-activated Sepharose 4B, protein A-Sepharose CL-4B, Trizma Base, EDTA, glycine (crystalline, NH_3 -free), QAE-Sephadex A-50, DEAE- and CM-Sepharose CL-6B and pepsin were obtained through Sigma.

IgG fractions from goat antisera raised against human C3, C4, C5 and NGS were purchased from Cappel Laboratories. NHS was obtained from normal, healthy donors. Blood, freshly drawn into Vacutainers (Becton-Dickinson, no. 6432), was allowed to clot for 60 min at 22°C, followed by another 60 min on ice. Serum, free of haemolysate, was separated by centrifugation at 685g [1800 rev./min (rotating radius 18.9 cm)] for 15 min at 4°C. All other chemicals and reagents used were of the highest grade available.

Preparation of IA

The IA beads were prepared by allowing 2 vol. of the dialysed IgG fraction of the appropriate antiserum to react with 1 vol. of CNBr-activated Sepharose 4B, which was pretreated with 1 mM-HCl, followed by 0.1 M- NaHCO_3 , pH 8.3, containing 0.5 M-NaCl, as recommended by the manufacturer (Pharmacia Fine Chemicals AB, 1979). Coupling of IgG was performed at pH 6.8 in 50 mM-sodium phosphate buffer, containing 150 mM-NaCl, for 16 to 20 h at 4°C, and residual active sites were blocked with 0.2 M-glycine, pH 8.2. IA beads were washed alternately three times with 50 ml of 0.1 M- NaHCO_3 , pH 8.3, and 0.1 M-sodium acetate, pH 5.0, both of which contained 250 mM-NaCl. Finally, the IA beads were equilibrated with EQB. The IA beads were stored suspended (1:1, v/v) in EQB containing 0.02% NaN_3 at +4°C, and treated with 1 mM-PMSF after every use with serum or other system containing proteinases.

Characterization and analysis of IA by SDS/polyacrylamide-gel electrophoresis

Unless stated otherwise, 10–15 μ l portions of settled IA beads were suspended in an equal volume of EQB and the slurry was diluted 2:1 with Tris/glycine buffer, pH 6.8, containing 9% (w/v) SDS and 30% (v/v) glycerol before application to individual wells (6 mm wide and 1.5 mm thick). In some instances, when the complete dissociation of heavy (H) chains in the presence of 5% (v/v) β -mercaptoethanol was required, the samples were heated to 95°C for 5 min before analysis. The electrophoretic separation was carried out in a 10% (w/v)-polyacrylamide gel in the presence of 0.1% (w/v) SDS as described by Laemmli (1970). Gels were stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 in methanol/acetic acid/water (5:1:4, by vol.).

Conversion of IgG- into $F(ab')_2$ -IA

Immunoadsorbent beads with covalently bound IgG were equilibrated with, and then suspended (1:1, v/v) in, 0.4 M-sodium acetate, pH 4.3. The slurry was incubated with 1 part of pepsin per 100 parts (w/w) of immobilized IgG in a shaker bath for 2 h at 37°C. The action of pepsin was stopped by adjusting the reaction mixture to pH 8.0 with solid Tris base (0.22 g/ml). IA beads were placed into a small column and washed sequentially with 10 vol. of 50% (v/v) ethylene glycol in EQB, 3 M-NaCl in EQB, 0.1% (w/v) SDS in EQB, 1 M-acetic acid, and finally with EQB. Beads were stored as a 1:1 (v/v) suspension in EQB containing 0.02% NaN_3 at 4°C. A sample of every new batch of $F(ab')_2$ -IA was analysed by SDS/polyacrylamide-gel electrophoresis.

Determination of IA binding capacity

In addition to SDS/polyacrylamide-gel electrophoretic analysis, one of the most convenient approaches was to measure a decrease in the content of functional activity of medium containing the appropriate antigen before and after the addition of IA. Portions (10 μ l) of NHS were evaluated for C3 content by haemolytic titration of 1 ml before and after addition of 100 μ l of anti-(C3,IgG)-IA or anti-[C3, $F(ab')_2$]-IA beads.

Identification of the most efficient solvent for regeneration of a given batch of IA

To determine the optimal conditions for dissociation of antigen-antibody complex, the IA beads were first saturated with the antigen from an appropriate medium placed in a 5 ml Jelco disposable syringe fitted with a porous polypropylene disc at the bottom. In the experiments presented in Figs. 3 and 4 below, NHS containing 5 mM-EDTA

was used. The IA/NHS (v/v) ratios were dependent on the IA binding capacity and on the concentration of the antigen in NHS. As a rule, the portion of NHS contained a double amount of the antigen necessary to saturate a given portion of IA beads. For example, 0.5 ml of anti C3-IA beads (3 mg of C3/ml) were treated with 3 ml of NHS (1 mg of C3/ml). In all instances IA beads were maintained suspended during the 15 min incubation in NHS at standard temperature and pressure and then drained. After a thorough wash with EQB, IA beads were divided into several 50 μ l aliquots. Each aliquot was placed into a 1 ml tuberculin syringe (Becton-Dickinson, no. 5602) fitted with a porous disc at the bottom, washed with 1 ml of solvent, and then incubated with 1 ml of the same solvent for 1 h at standard temperature and pressure. After two washes with 1 ml of EQB, each aliquot of IA beads was treated with 5% (v/v) β -mercaptoethanol and evaluated for residual, unstripped antigen by SDS/polyacrylamide-gel electrophoresis. In many instances the analysis of unreduced samples was carried out as well.

Results and discussion

In the course of our research, it has been necessary to be able to control the adsorption of specific proteins or their fragments from serum. For this and other reasons we sought a way to analyse and characterize efficiently certain IA in order to answer the following questions: (i) what ratio of IgG to Sepharose beads should be chosen to obtain high binding capacity? (ii) is all uncoupled protein ligand removed? (iii) what, if any, are the non-biospecifically bound proteins? (iv) which of many possible eluents (chaotropes, low-pH or high-pH reagents, high-ionic-strength buffers etc.) should be used for isolation of the antigen and regeneration of IA? (v) how effective is regeneration? We have used and analysed several antigen-antibody systems (e.g., C4, C5, factor H, factor I) with results similar to those which are illustrated below for C3.

Preparation of IA

The immobilization of ligands containing primary amino groups to CNBr-activated Sepharose 4B can be accomplished over a wide pH range, with yields improving with increasing pH. When the protein-agarose adsorbents are prepared, the protein ligand, in order to retain its biological activity, should be attached to the matrix by the fewest possible bonds (Cuatrecasas, 1970). Consequently, we have carried out the covalent coupling of IgG at pH 6.8 to enhance protonization of ϵ -amino groups, and thus to reduce the probability of multipoint attachment of the same antibody

molecule (Cuatrecasas, 1970; Parikh *et al.*, 1974; Porath & Kristiansen, 1975). In general, about 10 mg of protein ligand was reported to be immobilized on 1 ml of Sepharose gel, and even lower levels have been recommended (Eveleigh & Levy, 1977). For the purposes of our other studies, however, we needed to prepare IA beads with higher binding capacity (up to 4 mg of antigen/ml of IA). Therefore for most experiments, including those presented in Fig. 3 (below), total protein from 2 ml of reconstituted IgG fraction containing 20 mg of monospecific antibody was coupled to 1 ml of CNBr-activated Sepharose 4B. The quantity of total protein immobilized to 1 ml of IA beads was calculated from the change in A_{280} and the absorption coefficient of IgG [$A_{280}^{1\%, 1\text{ cm}} = 13.8$; Schultze & Heremans (1966), Table 41 in section 2, pp. 222-223]. Saturation of the adsorbent was observed to be dependent on the length of time CNBr-activated Sepharose 4B was exposed to air. Up to 55 mg (see Fig. 4 below) of total protein could be coupled/ml of the Sepharose beads. Antigen binding was directly proportional to the monospecific-antibody concentration, over the range ~ 1 -4 mg of antigen/ml of IA beads containing from 5 to 20 mg of the antibody.

Characterization and analysis of IA

Our initial problems in controlling IA led us to employ SDS/polyacrylamide-gel electrophoresis for their characterization and analysis, as described in the Experimental section and demonstrated below. First, we monitored the immobilization of IgG on CNBr-activated Sepharose as illustrated in Fig. 1. Either very minor, or no, protein bands were present after fresh IA beads were electrophoresed in the absence of β -mercaptoethanol. This indicated that uncoupled proteins had been removed. Both the H- and L-chains, which serve as a convenient internal standard for a given batch of IA, were detected after the suspension of unused IA beads was mixed (2:1, v/v) with a sample buffer containing 15% (v/v) β -mercaptoethanol. Unless the samples were heated before SDS/polyacrylamide-gel electrophoresis, certain fractions of H-chains remained undissociated and could be detected as two bands with mobilities corresponding to M_r values of ~ 94000 and 82000 respectively. The comparison of lanes 2 and 2' suggests that heat treatment, however, may disrupt some covalent bonds between IgG and the Sepharose matrix.

Conversion of IgG- into $F(ab')_2$ -IA

The presence of the bands corresponding to dissociated ($M_r \sim 50000$) and undissociated ($M_r \sim 94000$ and 82000) H-chains on electrophoretograms are often inconvenient, as they may

Fig. 1. Preparation and characterization of immunoadsorbents

The antibody-containing IgG fraction (lanes 1 and 1') was immobilized on Sepharose as described in the Experimental section. In this instance, 10 mg of monospecific antibody and 13 mg of other IgG molecules were coupled/ml of settled Sepharose beads. After a thorough washing procedure, the IgG-IA beads (lanes 2 and 2') were treated with pepsin in order to convert IgG- into $F(ab')_2$ -IA (lanes 3 and 3'). No attempt was made to remove Fc peptides before the analysis of this $F(ab')_2$ -IA (lanes 3 and 3'). The SDS/polyacrylamide-gel-electrophoretic analysis was carried out both in the absence (a) and the presence (b) of β -mercaptoethanol. Only samples in lanes 1', 2' and 3' were heated before the analysis. High- and low- M_r standards (lane S) were those as supplied by Bio-Rad Laboratories. Abbreviations used: Ig, immunoglobulins; H, heavy chain of Ig; L/Fd', a mixture of L (light) chains and Fd' (pepsin-generated N-terminal fragments of H); Fcp., pepsin-generated peptides from Fc (C-terminal fragment of H).

obscure detection of certain antigens. During preparation of serum depleted of a particular protein (e.g., Cheson *et al.*, 1984), the presence of the intact Fc portion of IgG molecule could, moreover, lead to binding of the C1q subcomponent of C1, and thus to the activation of complement cascade (Reid & Porter, 1981). Therefore we investigated the possibility of converting immobilized IgG into its immobilized $F(ab')_2$ fragment. In doing so, IgG-IA beads were incubated with pepsin at various substrate/enzyme (w/w) ratios, while pH, buffer composition and temperature were maintained as in the protocol for generation of $F(ab')_2$ from IgG in solution (Stanworth & Turner, 1973). Complete conversion was achieved with ratios as high as 200:1. At a ratio of 100:1, the results were found to be the same between 2 and 24 h incubation at 37°C and pH 4.3. Occasionally, small amounts of H-chain remained undigested.

An example of one of 17 conversions of immobilized IgG into its $F(ab')_2$ fragment under standard conditions is shown in Fig. 1. Under non-reducing conditions only small Fc peptides, which migrated close to the dye front, were observed. In the presence of β -mercaptoethanol, reduced $F(ab')_2$ fragments (i.e., L-chains and pepsin-generated Fd' portions of H-chains) were detected

as well. It is rather remarkable that the treatment of IgG-IA beads with pepsin results in only ~15% loss of the original binding capacity of IgG-IA. As follows from Figs. 1, 3, and 4, and from the electrophoretograms of similar unreduced samples (results not shown), small Fc peptides are strongly associated with IA, but most of them are not disulphide-bonded to the immunoadsorbent. This observation would also suggest that very few antibody molecules are attached via the Fc region. The presence of Fc peptides did not seem to interfere with binding or eluting of the antigen, unless the latter or its fragments were in the same M_r range as the Fc peptides. If necessary, Fc peptides could be removed electrophoretically in the presence of SDS or by a procedure described in the legend to Fig. 4.

How extensive is non-biospecific adsorption to Sepharose IA?

Many researchers, including ourselves, were aware of the possibility of non-biospecific protein adsorption to bioaffinity media (O'Carra *et al.*, 1974). For example, IgG molecules other than monospecific antibody can bind to carrier with an immobilized antigen (Zoller & Matzku, 1976). It was also noted previously that antigens recovered

from antibody-immobilized IA were often contaminated with albumin (Eveleigh & Levy, 1977). Both the Sepharose matrix and the immobilized protein might act as weak ion-exchange media. It has also been suggested that increased ligand concentration might result in increased non-biospecific adsorption (Pharmacia Fine Chemicals AB, 1979).

To determine whether non-biospecific protein adsorption would be dependent on the whole IgG molecule, its $F(ab')_2$ fragment, and the presence of bound antigen, a series of samples was analysed by SDS/polyacrylamide-gel electrophoresis in the absence or presence of a reducing agent (Fig. 2). All adsorbents tested in these experiments were prepared from commercial CNBr-activated Sepharose 4B as described in the Experimental section for preparation of immunoadsorbents. In a control sample, the adsorbent with immobilized glycine did not give any positive reaction with protein stain (lanes 1 and 1'); after treatment with NHS, only small amounts of γ -globulin and albumin could be seen (lanes 2 and 2'). The adsorbent with an immobilized IgG fraction from non-immune NGS (lane 3') did not contain any non-covalently bound proteins (lane 3). After treatment with NHS (lanes 4 and 4'), slightly larger quantities of γ -globulin in comparison with plain Sepharose (lanes 2 and 2') were present. The increased amount of non-immunologically bound γ -globulin was observed when IA beads containing anti-human C3 (lanes 5 and 5'; 10 and 10') were treated with the antigen containing NHS (lanes 8 and 8'; 13 and 13'). It would then follow that the major non-biospecific contaminant from serum is γ -globulin. As can be seen in lanes 7 and 7', 9 and 9', 12 and 12' and 14 and 14', only albumin was eluted by 3M-NaCl in EQB, whereas the C3 antigen-antibody complex remained undissociated. In any case the amounts of non-immunologically bound γ -globulin and/or albumin were small in comparison with the bound antigen, especially considering that C3 represents approx. 1.5%, whereas γ -globulin represents about 20% and albumin about 50%, of total serum proteins. The results we have obtained with five different batches of IA are very similar. They are not, however, conclusive enough to answer the question as to whether or not the presence of the Fc portion of the IgG molecule and/or the presence of bound antigen enhances non-biospecific binding.

Determination of conditions for dissociation of antigen-antibody complex

To determine the optimal conditions for dissociation of antigen-antibody complex, the IA beads were first saturated with antigen and then divided into several portions, which were washed with

Fig. 2. Analysis of non-biospecific protein binding to Sepharose-based immunoadsorbents

Both the IgG- and $F(ab')_2$ -containing IA characterized in Fig. 1 are compared here with each other and with the controls with respect to immunospecific (C3) and non-immunospecific (serum albumin, γ -globulin) binding. After incubation with a 10-fold volume of an appropriate serum for 15 min at standard temperature and pressure, each of the adsorbent samples was washed twice with a 10-fold volume of EQB. The order of samples ($\sim 12.5 \mu\text{l}$ of IA beads each) analysed by SDS/polyacrylamide-gel electrophoresis with indication of their treatment is as follows: plain Sepharose with glycine before (lanes 1 and 1') and after (lanes 2 and 2') NHS; non-immune IgG-Sepharose before (lanes 3 and 3') and after (lanes 4 and 4') NHS; anti-(C3,IgG)-IA before (lanes 5 and 5') and after either NGS (lanes 6 and 6') or NHS (lanes 8 and 8'); anti-[C3, $F(ab')_2$]-IA before (lanes 10 and 10') and after either NGS (lanes 11 and 11') or NHS (lanes 13 and 13'). Washing of either NGS (lanes 7 and 7'; 12 and 12') or NHS (lanes 9 and 9'; 14 and 14')-treated IgG-IA and $F(ab')_2$ -IA respectively with 3M-NaCl in EQB removed only albumin. The analysis of reduced samples is shown in the lower panel ('+ β -Mercaptoethanol'). Similar results were obtained with another four batches of the immunoadsorbents which contained antibodies against C3, C4 or C5. The amount of albumin bound seemed to increase slightly with the higher concentration of the IgG ligand. Abbreviations used: C3, the third component of complement; Ig, immunoglobulins; SA, serum albumin; b.b., Bromophenol Blue; Fcp., pepsin-generated peptides from Fc; C3 α , α -chain of C3; H, heavy chain of Ig; C3 β , β -chain of C3; L, light chain of Ig; Fd', pepsin-generated N-terminal fragment of H.

various solvents, followed by EQB, as described in the Experimental section. IA beads were then analysed for a residual, unstripped antigen by SDS/polyacrylamide-gel electrophoresis. Tests with a large array of solvents, which had been reported to be used in the immunoaffinity chromatography, were carried out with several antigen-antibody systems using several batches of both the IgG- or F(ab')₂-IA beads. Examples of such analyses are illustrated in Figs. 3 and 4. Our results altogether indicated that the ease of dissociation may vary from IgG to its F(ab')₂ fragment and from one batch of antiserum to another. The determination of the effectiveness of various solvents should be based on the ratio of protein antigen to antibody under reduced conditions, on the assumption that every time IA beads are treated with a reducing agent in the presence of SDS, the same amount of L-chain and H-chain or pepsin-generated Fd' portion of H-chain is released from a given batch of IA beads. For scanning purposes the absence of H-chains from the electrophoretogram is advisable. Mere inspection of the stained gels, even without scanning, clearly reveals which of the tested eluents is the

most powerful one. Results presented in Fig. 3 also demonstrate the effect of various solvents on the dissociation of Fc peptides from IA beads. Although most of our anti-C3-IA beads were similar in terms of antigen-binding and dissociating properties, an example of dissociating C4 from its immunoabsorbent (Fig. 4) indicates that the conditions for the dissociation of antigen-antibody complex may be dependent on the antigen as well. These results also stress the importance of assaying each batch of antisera, and each antigen-antibody system must be evaluated independently. Only polyclonal antibodies with relatively high affinities have been studied. Their affinity may account for difficulties in the regeneration step, since much less influence could be attributed to higher concentrations of antibodies coupled to beads.

It is difficult to apply exactly the same volumes into wells, differences being reflected in the amount of reduced F(ab')₂, the internal standard. It should be mentioned at this point that the mobility of antigens, their constitutive chains, as well as that of immunoglobulins, was the same whether they were bound to IA beads or whether free in solution at the time of subjection to SDS/

Fig. 3. Study of the effect of high ionic strength, chaotropes, detergents and low- and high-pH solvents on the dissociation of the antigen from another batch of immunoabsorbent, anti-[C3,F(ab')₂]-IA

The C3 protein was extracted from NHS (lane 1). The most effective regeneration of IA beads was achieved with 1 M-acetic acid (lane 7). The other solvents tested were: 2, 50% (v/v) ethylene glycol in EQB; 3, 3 M-NaCl in EQB; 4, 3 M-MgCl₂ with 20% (w/v) sucrose; 5, 3 M-KSCN; 6, 0.1% SDS in EQB; 8, pH 10 buffer; 9, 1 M-NH₃; 10, 0.1 M-NH₃; 11, 1 M-acetic acid with 1% Nonidet P40; 12, 1 M-acetic acid with 1% Triton X-100; 13, 0.5 M-acetic acid; 14, 0.5 M-acetic acid with 1% Nonidet P40; and 15, 1% Chaps {3-[(3-cholamidopropyl)dimethylammonio]propanesulphonic acid}. Ratios, volumes and time intervals are described in the Experimental section. The ligand concentration in the parent immunoabsorbent was about 20 mg of monospecific and 23 mg of other IgG molecules/ml of Sepharose. About 8 μl of IA beads were applied per each lane. On analysis in the absence of a reducing agent (results not shown), only C3 (*M_r* ~ 185 000) and non-disulphide-bonded Fc peptides could be seen. Abbreviations: C3α, α-chain of C3; C3β, β-chain of C3; H, heavy chain of IgG; L, light chain of IgG; Fd', pepsin-generated N-terminal fragment of H; Fcp, pepsin-generated peptides from Fc (C-terminal fragment of H).

Fig. 4. Evaluation of the different antigen-antibody complexes by SDS/polyacrylamide-gel electrophoresis

The immunoadsorbent used in this study was prepared by pepsin treatment from the one which had 55 mg of IgG coupled to 1 ml of Sepharose beads. The concentration of monospecific anti-(human C4) in the IgG fraction was not tested by either Cappel Laboratories or ourselves. The unused immunoadsorbent anti-[C4,F(ab')₂]-IA as shown in lane 1 was incubated with NHS (lane 2). Samples of C4-saturated IA (lane 2) were tested for the ease with which the C4 antigen would dissociate from its immobilized antibody by employing the following solvents: lane 3, 50% ethylene glycol in EQB; 4, 3 M-NaCl in EQB; 5, 3 M-MgCl₂ in EQB; 6, 3 M-KSCN; 7, 0.1% SDS in EQB; 8, 1 M-acetic acid; 9, glycine buffer, pH 10; 10, 1 M-NH₃; or 11, 0.1 M-NH₃. The immunoadsorbent before the removal of Fc peptides is shown before (1°) and after (2°) saturation with C4. The washing of IA beads from (1°) with results shown in lane 1 was performed in the following order: (i) 0.1% SDS in EQB; (ii) EQB without EDTA; (iii) 50% (v/v) methanol in EQB without EDTA; (iv) EQB without EDTA; and (v) EQB. This procedure resulted in removal of non-disulphide-bonded Fc peptides as confirmed by SDS/polyacrylamide-gel electrophoresis in the absence of a reducing agent (results not shown). The binding capacity of the IA did not seem to be affected by the treatment. High-*M_r* standards were run in lane 12. Abbreviations used: C4α, C4β and C4γ, α-β- and γ chains of C4; H, heavy chain of IgG; L, light chain of IgG; Fd', pepsin-generated N-terminal fragment of H; Fcp, pepsin-generated peptides from Fc (C-terminal fragment of H); b.b., Bromophenol Blue.

polyacrylamide-gel electrophoresis. The C3 and C4 antigens tested in the present study may cause a special problem because of the possibility of covalent binding via transesterification to antibody molecules or to the polysaccharide matrix. This problem would only be limited to the protein containing a thioester bond (Janatova & Tack, 1981; Janatova, 1983).

Applications of SDS/polyacrylamide-gel electrophoresis to characterization and analysis of media for affinity chromatography

The applications of IA are numerous, as we believe, are the implications of our new approach. For instance, apart from (i) withdrawal of contaminants during isolation of proteins, or (ii) preparation of sera depleted of specific protein, we tested very successfully (iii) the possibility of extracting C3 or C4 from small volumes (30 or 100 μl respectively) of patients' sera with only 15 μl of IA beads and comparing the polypeptide structure of these molecules with that of C3 or C4 from healthy individuals; (iv) we were also able in the same way to analyse the state of C3 or C4 activation after the incubation of individual serum samples with, e.g., bacteria. In many cases the

antigen reversibly immobilized on IA beads exhibited its functional activity and could also serve as an immobilized substrate or enzyme respectively. If intended for antigen isolation, the antibody avidity and the binding capacity of IA should be much lower than those that were employed in the present study.

The specificity of protein binding as evaluated by SDS/polyacrylamide-gel electrophoresis can be extended to any Sepharose-based affinity media. The idea of separating the biospecific molecule from its affinity adsorbent in an electrical field has been suggested and has already been employed for preparative purposes by Dean *et al.* (1977) and Morgan *et al.* (1978a,b).

The binding of serum proteins to, and their elution from, different ion-exchange adsorbents as a result of different pH and/or ionic strength can also be evaluated by SDS/polyacrylamide-gel electrophoretic analyses (J. Janatova & R. J. Gobel, unpublished work). However, it is the proteins that remain bound to the adsorbent which would be detected in the resulting electrophoretogram. The presence of ion-exchangers such as CM-Sepharose CL-6B or DEAE-Sephacel do not interfere with electrophoretic separation of proteins, since their

charges are suppressed by the presence of SDS. There are some mechanical problems encountered when electrophoresing SP-Sephadex A-50, or QAE-Sephadex A-50, because their volume is a function of ionic strength.

The dissociation of IgG (human, rabbit, guinea pig) or immune complexes from protein A immobilized on Sepharose is as difficult as the dissociation of antibody-antigen complex, and is usually accomplished with 1M-acetic acid/0.1M-glycine/HCl, pH3, or with 3M-KSCN (MacSween & Eastwood, 1981). The immune complexes withdrawn with protein A-Sepharose can also be displaced by using 2% (w/v) SDS in 8M-urea, precipitated with ethanol, solubilized in SDS and finally analysed by SDS/polyacrylamide-gel electrophoresis (Pharmacia Fine Chemicals AB, 1979). The advantages of direct SDS/polyacrylamide-gel electrophoretic analysis of protein A-Sepharose containing immune complexes under the conditions described for IA beads are obvious.

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