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Separation of paraproteins from human plasma by membrane chromatography

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Abstract

Membrane chromatography using a commercially available blotting membrane was performed in a dead-end filtration mode to separate paraproteins from plasma of patients suffering from paraproteinemia. The affinity membrane was found to display distinct specificity to monoclonal IgG1. A dissociation constant (K_d) of 3.2 μM and a maximum binding capacity of 1.43 mg/cm^2 IgG1 paraprotein were obtained from the adsorption isotherm of the affinity membrane. The membrane was found to absorb immunoglobulins species-dependently because no binding of immunoglobulins from mouse, rat and rabbit could be observed. © 1997 Elsevier Science B.V.

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1. Introduction

The application of immunoglobulins and monoclonal antibodies in science, medical diagnostics and therapy is continuously increasing. This calls for effective methods of downstream processing of immunoglobulins from plasma or cell culture fluid. An effective purification of these complex fluids can be obtained by affinity filtration on microporous membranes. In comparison to conventional chromatographic matrices, the porosity and rigidity of the membrane structure allow high flow-rates without special equipment. Affinity membranes operate in convective mode, which results in reduced diffusion limitations and thus in higher throughputs. In affinity membrane technology complete saturation of binding sites can be attained in spite of short contact times between the membrane and the solute leading to faster processing times [1,2].

A series of recent publications have focused on chemical modification of commercially available filtration membranes to steer binding selectivity [3,4]. Filtration membranes substituted with antibodies or receptors were found to have high binding selectivity for recombinant proteins from cell culture fluids [5]. Pseudo-affinity ligands such as reactive dyes bound to membranes have been developed for application in downstream processing [6–8].

Recently, another group-specific affinity membrane using metal chelates has been successfully applied [9]. Protein separation by immobilised metal ions was shown to be based on the fine tuned interaction of chelated metal ions with surface-exposed histidine residues in proteins [10,11].

There have been numerous attempts to use ligand-substituted membranes in monoclonal antibody sepa-

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ration technology. Protein-A-coupled membranes were useful in capturing of monoclonal antibodies from serum-free cell culture supernatant [2,12]. Using membrane-based ion exchangers Lütkemeyer et al. [13] demonstrated the purification of antibodies from cell culture supernatant in gram quantities. Thiophilic membranes were successfully used to purify antibodies from cell culture media by Finger et al. [14].

In the present study a commercially available microporous membrane was found to display distinct affinity to immunoglobulins from human plasma. Using paraproteins obtained from plasma of patients suffering from paraproteinemia as model, the functional and kinetic properties of the membrane with respect to adsorption of human monoclonal immunoglobulins are described.

2. Experimental

2.1. Sample collection

Blood specimens of patients suffering from paraproteinemia were obtained from the outpatient department of the Clinic of Internal Medicine of the University of Leipzig. Plasma was obtained by centrifugation of freshly drawn citrated blood. Plasma samples were stored at −20°C. Animal blood was obtained by venipuncture from mouse, rat and rabbit.

2.2. Membrane chromatography of plasma

Nylon membranes, IMMUNODYNE (Pall, Glen Cove, NY, USA), were treated with 10 m*M* NaOH for 12 h at room temperature. After extensive washing with 10 m*M* sodium phosphate buffer, pH 7.0 (buffer I) three membrane discs (20 mm diameter, nominal pore size 0.45 μm) were mounted in a filtration module yielding a total filtration area of 9.4 cm^2 and a membrane volume (mvol) of 0.15 ml (module I). For scaling up experiments, filtration devices (module II) with five stacked membranes of 135 mm diameter having a filtration area of 715 cm^2 and a mvol of 11.4 ml were used. Filtration process was conducted in the dead-end mode at 4°C at different flow-rates. Between 0.05 and 0.5 ml plasma in small-scale and 20 ml in large-scale experiments, respectively, were applied. Prior to passing through the filtration module, plasma samples were equilibrated with 10 m*M* sodium phosphate, pH 7.0 (buffer I). The same buffer was used to wash out the unbound proteins. Elution of bound proteins was achieved with 0.5 *M* NaCl in buffer I. After regeneration with 0.01 *M* NaOH the membranes could be reused in new filtration cycles. The bound and unbound fractions were pooled, concentrated by ultrafiltration to original volume and analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunodiffusion.

2.3. Immunological analysis

Immunoglobulin classes and subclasses in the samples and pooled fractions were quantitated by ELISA test kits (Virotech System Diagnostica, Rüsselsheim, Germany). Paraproteins from eleven patients were typed by their heavy and light chain (κ,λ) composition by electroimmunodiffusion using specific antisera. Elevated concentrations of paraprotein IgA (3/1) were found in four plasma samples. Seven paraproteins belonged to the IgG (6/1) class. Their subclass specificity is given in Table 1.

2.4. Electrophoresis and Western Blot

For protein analysis samples were subjected to SDS–PAGE (8% polyacrylamide gels) under non-

Table 1
Concentration of IgG subclasses in paraproteinemia plasma samples compared with a control plasma of a healthy donor

Subclass	Concentration (mg/ml)			
	IgG1	IgG2	IgG3	IgG4
Control	6.9	3.5	0.6	0.6
Sample 1	63.0	0.1	0.0	0.1
Sample 2	0.4	12.5	0.1	0.1
Sample 3	23.3	2.0	0.2	0.1
Sample 4	41.6	0.5	0.3	0.1
Sample 5	117.7	0.7	0.6	0.1
Sample 6	91.8	0.2	0.1	0.0
Sample 7	116.6	1.2	0.4	0.3

reducing conditions according to Laemmli [15]. For Western Blotting the separated proteins were transferred to cellulose acetate membranes (Schleicher and Schuell) and the membranes were blocked with defatted-milk powder. After incubation of the membranes with goat anti-human IgG-(Fc)-antibody (1:1000) (Immunotech, Hamburg, Germany) and rabbit anti-human IgG-(λ)-chain antibody (1:1000) (Dako, Hamburg, Germany), respectively, the colour reaction was started by incubation with substrate solution (diaminobenzidine/H_2O_2).

2.5. Analysis of the binding kinetic

To establish adsorption isotherms of immunoglobulin binding to the membrane, the paraprotein IgG1(κ) was purified from patient's plasma (sample No. 5, Table 1) by large scale affinity filtration using the membrane module II. Briefly, 20 ml of the plasma containing 117.7 mg/ml IgG1(κ) were dialysed against buffer I and finally diluted 1:10 with the same buffer. The sample was loaded with a flow-rate of 0.44 mvol/min onto the filtration module. After washing with buffer I the bound paraprotein was eluted with 0.5 *M* NaCl and concentrated by ultrafiltration.

For determination of the adsorption isotherm the membrane module I was continuously fed with various paraprotein concentrations until the effluent concentration was identical to that of the feed stream concentration, respectively. After washing with buffer I, the elution was accomplished by 0.5 *M* NaCl. A linear flow-rate of 1.1 mvol/min was applied. The immunoglobulins were quantitated by spectralphotometrical analysis using *A*(280 nm; 10 mg/ml)=13.4.

2.6. Fragmentation of immunoglobulins

Purified monoclonal IgG1 paraprotein (50 mg/ml) (see Section 2.5) was dialysed against 0.1 *M* $NaHCO_3$, pH 7.0, and then incubated with 0.6 mg papain (Serva, Heidelberg, Germany), 10 m*M* cysteine, 2 m*M* EDTA at 37°C for 15 h. Then, the sample was dialysed against 10 m*M* sodium phosphate buffer, pH 7.0 for further processing.

3. Results and discussion

We already demonstrated that the nylon membrane, IMMUNODYNE, displays affinity to the immunoglobulins from human blood [16]. That membrane preferably adsorbed IgG with a distinct subclass specificity for IgG1 and IgG3. Immunoglobulins IgG2 and IgG4 were only weakly bound to the affinity membrane and appeared in the unbound protein fraction. These results prompted us to analyse the properties of this membrane with respect to human monoclonal immunoglobulin binding.

Human monoclonal immunoglobulins are present at high concentrations in plasma of patients suffering from paraproteinemia (Table 1). All plasma samples were subjected to membrane chromatography on the filtration module I at a flow-rate of 1.1 mvol/min. Fig. 1 shows a representative separation profile of an IgG1-type paraprotein. The affinity membrane was found to adsorb the paraprotein almost completely. It could be eluted from the membrane as a sharp peak

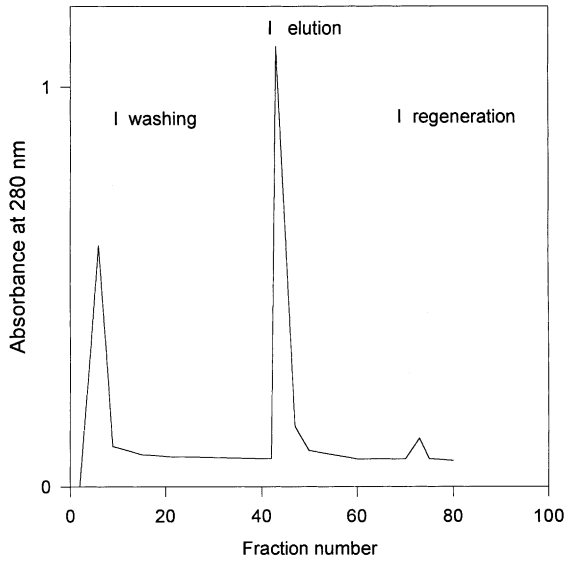

Fig. 1. Fractionation of human paraproteinemia plasma by membrane chromatography. 0.05 ml of a paraproteinemia plasma (sample 5) were chromatographed using the affinity membrane module I at a flow-rate of 1.1 mvol/min. The membranes were washed with buffer I and the bound proteins were desorbed by 0.5 *M* NaCl in buffer I. The protein distribution in the bound and unbound fractions was analysed by SDS–PAGE (8%) under non-reducing conditions.

by addition of salts. The analysis of the bound fractions after chromatographic separation of an IgG1(κ)-type and an IgG1(λ)-type paraprotein plasma, respectively, is shown in Fig. 2. Both paraproteins were effectively adsorbed on the membrane and recovered at greater than 90% purity. This indicates that adsorption of paraproteins of IgG1-type occurred irrespective of the type of light chain. Furthermore, all paraproteins of IgG1-type were bound to the membrane, whereas the IgG2 paraprotein did not adsorb at all. In case of monoclonal IgA, two paraproteins were bound and two did not bind. However, the possibility that this is due to a distinct selectivity of the affinity membrane for either IgA1 or IgA2 has to be experimentally proven. The binding of immunoglobulins to the membrane was found to be species-dependent because no adsorption of immunoglobulins from mice, rat and rabbit was found (results not shown).

To study the binding kinetics, the adsorption isotherm of membrane–IgG1-type paraprotein interaction was established (Fig. 3). The adsorption isotherm for the affinity membrane and the purified paraprotein of the IgG1-type fits the Langmuir-type

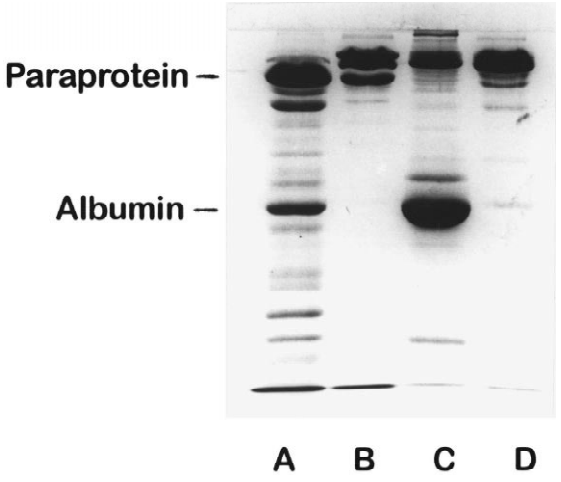

Fig. 2. SDS–PAGE analysis of desorbed fractions. Two paraproteinemic plasmas, subtyped as IgG1(κ) and IgG1(λ), respectively, were chromatographed using the affinity membrane as described in Fig. 1. The samples and the desorbed fractions were subjected to SDS–PAGE (8%) under non-reducing conditions. (A) IgG1(κ)-type paraproteinemia plasma (sample 5) (50 μg), (B) eluted fraction of A (30 μg), (C) IgG1(λ)-type paraproteinemia plasma (sample 7) (50 μg), (D) eluted fraction of C (30 μg).

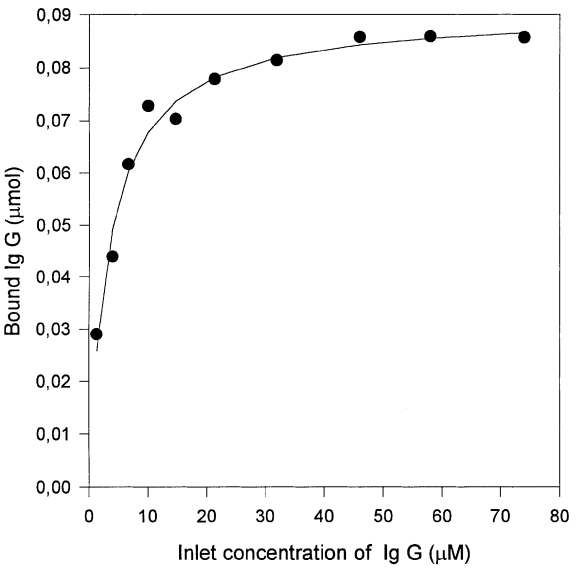

Fig. 3. Adsorption isotherm of IgG1 paraproteins binding to the membrane. Paraprotein IgG1(κ) purified from plasma as described in Section 2.2 was continuously applied to the membrane module I until the effluent concentration was identical to the inlet concentration. After washing the elution was accomplished by 0.5 *M* NaCl to determine the bound IgG. A linear flow-rate of 1.1 mvol/min was used.

adsorption and can be described by the following equation:

$$Q_a = Q_x K_a [C]/(1 + K_a + [C]) \quad (1)$$

where K_a is the association constant, Q_x is the maximum binding capacity of the membrane, and Q_a is the amount of bound protein in equilibrium with the protein concentration, [C], of the solution. A dissociation constant ($K_d = 1/K_a$) of 3.2 μ*M* and a maximum binding capacity of 1.43 mg/cm^2 IgG1 paraprotein were calculated from the curve.

Recently, Langlotz and Kroner [12] used a protein A membrane for adsorption of immunoglobulins and murine monoclonal antibodies. The binding capacity of that affinity membrane was about 0.1 mg/cm^2 which is less compared to the affinity membrane used in this study. Conversely, when filtration membranes were substituted with ion exchanging ligands [17] the capacity for immunoglobulin adsorption approached 1 mg/cm^2 which is equivalent with our findings. The results of the present study demonstrate

that binding and elution of immunoglobulins take place under very mild conditions, which is in contrast to the harsh conditions needed for separation of immunoglobulins from immobilised protein A. This often impairs the immunological properties of purified monoclonal antibodies. It has to be proven whether the affinity membrane used for monoclonal IgG extraction is applicable to purification of monoclonal antibodies derived from human hybridoma cell lines.

It was of interest to know which part of the entire immunoglobulin conferred binding to the affinity membrane. Therefore, purified IgG1 paraprotein was digested by papain to obtain Fc- and Fab-fragments. After fragmentation, 1 mg of the sample was chromatographed on membrane module I under similar conditions as shown in Fig. 1. The distribution of Fc- and Fab-fragments in the unbound and bound fractions, respectively, was analysed by Western blotting using specific enzyme-labelled polyclonal antibodies. As seen in Fig. 4, the Fc-fragments could be detected in the unbound fraction, whereas the main part of the Fab-fragments was adsorbed to the membrane. The results indicate that binding of IgG1 to the membrane is mainly conferred by the Fab-moiety, and more distinctly, by the heavy-chain of the Fab-moiety since the class and subclass specificity is determined by the structure of the heavy chain. This binding mode is different from the binding of immunoglobulins to protein-A or protein-G which occurs via the Fc part of the molecule.

4. Conclusions

The results of the present study clearly demonstrate that human monoclonal immunoglobulins derived from paraproteinemic plasma selectively bind to a commercially available affinity membrane. Since both paraproteins and human monoclonal antibodies are products of human myeloma B cells it can be anticipated that the affinity membrane may be useful in purification of human antibodies from cell culture supernatant.

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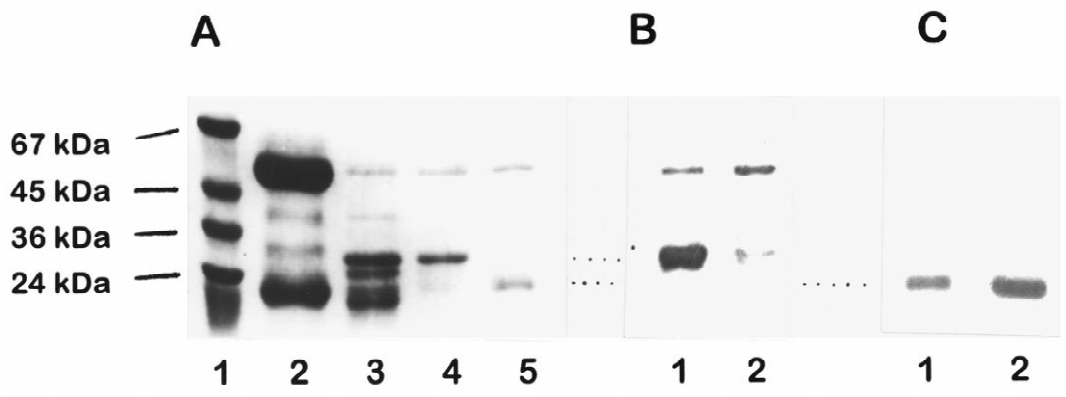

Fig. 4. Analysis of the Fc- and Fab-fragment distribution in papain digests of IgG1 after membrane chromatography on module I. A 50 mg sample of purified IgG1-type paraprotein was digested with papain as described in Section 2.6. An aliquot of 1 mg (1 mg/ml) was subjected to chromatography on the membrane module I under the conditions shown in Fig. 1. The bound and unbound fractions were concentrated to original volume by ultrafiltration and 30 μg were subjected to SDS–PAGE under reducing conditions. The proteins were electroblotted onto membranes and the detection of the fragments was achieved by POD-labelled goat anti-human Ig (Fc) antibody (B) and rabbit anti-human-IgG-(λ)-chain antibody and anti-rabbit-Ig-POD from goat as secondary antibody (C). (A) SDS–gradient PAGE (3–15%); staining with Coomassie R 250: (1) marker proteins; (2) IgG1 paraprotein before papain digest (50 μg); (3) papain digest of IgG1 (20 μg); (4) break-through fraction (10 μg); (5) eluted fraction (10 μg). (B) Western blot for detection of Fc-fragments: (1) break-through fraction (broad band); (2) eluted fraction. (C) Western blot for detection of Fab-fragments: (1) break-through fraction; (2) eluted fraction.

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