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Study of an anti-human transthyretin immunoadsorbent Influence of coupling chemistry on binding capacity and ligand leakage

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Abstract

A variant of transthyretin (TTR Val30Met) has been identified as the main protein precursor of the amyloid fibrils deposited in familial amyloidotic polyneuropathy (FAP). Specific removal of TTR in an extracorporeal immunoadsorption procedure is currently under investigation as a possible treatment of FAP. Immunoadsorbents were constructed by immobilizing murine anti-TTR monoclonal antibody 88.6.BA9 onto agarose gel supports via several different coupling chemistries. The influence of coupling conditions such as pH and antibody density, and of perfusion variables, such as antigen concentration and applied flow-rate, on the TTR capture efficiency, was determined. Cyanogen bromide-, carbonyldiimidazole- and aldehyde-activated (ALD) supports conjugated with antibody at optimal pH, provided immunoadsorbents with comparable TTR binding capacities. Regarding stability, leakage was lowest for the ALD based immunoadsorbents, particularly at high pH. © 1998 Elsevier Science B.V.

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1. Introduction

Familial amyloidotic polyneuropathy (FAP) type I is an autosomal dominant hereditary disease with high prevalence in the northern part of Portugal, that was first described by Andrade in 1952 [1]. This disease is characterized by the systemic deposition of amyloid, with particular involvement of the peripheral nerves. A variant of the plasma transport protein transthyretin (TTR) has been found to be the main component of the fibril deposits [2]. Although over 40 different amyloidogenic mutations in TTR have been described [3], TTR Val30Met remains the most common variant related to FAP [4]. This disease follows a progressively incapacitating and fatal course with death occurring, on average, about 12 years after onset. Therapies that are currently under clinical investigation, including hepatic transplant [5], and immunoadsorbent apheresis [6,7], are all aimed at the prevention of further amyloid deposition. In the case of immunoadsorbent apheresis, TTR is specifically removed from plasma through the extracorporeal circulation of plasma over a filter with immobilized anti-TTR antibodies. Immunoadsorption is a well-established technique for the treatment of familial hypercholesterolemia [8], and other studies

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have demonstrated the effectiveness of similar procedures for the removal from plasma of β2-microglobulin [9] and lipoprotein (a) [10]. Preliminary results, regarding safety and TTR removal in FAP patients, are promising. For this trial, murine anti-TTR monoclonal antibodies (mAb) have been immobilized onto Sepharose CL-4B via the cyanogen bromide (CNBr) activation method according to Cuatrecasas et al. [11]. This is the most commonly used method of immobilization; however, it is known that the resulting N-substituted isourea bond is relatively unstable [12,13] causing significant antibody leakage. This leakage may cause an humoral antibody response, that in our patients can be demonstrated by rising human antimouse antibody (HAMA) titers (unpublished results). A similar phenomenon has been described in patients submitted to immunoadsorbent LDL-apheresis, and in one case was related to hypersensitivity reactions that dictated the discontinuation of the treatment [14]. We also discontinued treatment in one of our patients due to hypersensitivity reactions. These problems prompted us to investigate other immobilization methods, in order to optimize TTR binding capacity, ligand leakage, and reproducibility.

2. Experimental

2.1. Immunoglobulins

Murine hybridoma cell lines producing anti-human transthyretin mAbs were developed as described previously [7]. Large-scale production in serum-free cell culture medium of 88.6.BA9, the mAb (IgGl) used in this study, was carried out by Bio-Intermediair (Groningen, The Netherlands), in the setting of a process development study. Rabbit anti-human prealbumin (TTR) was purchased from DAKO-Immunoglobulins (Glostrup, Denmark).

2.2. Resins

Sepharose 4FF, Sepharose 6FF, and CNBr-activated Sepharose 4B were all from Pharmacia Biotech (Uppsala, Sweden). Actigel ALD and Actigel ALD Superflow were purchased from Sterogene Biosepa-rations (St. Arcadia, CA, USA), and Affi-Gel 102 was obtained from Bio-Rad (Richmond, CA, USA).

2.3. Chemicals

CDI (1,1′-carbonyldiimidazole), EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide), DVS (divinyl sulphone), and MOPS (3-(N-morpholino)-propanesulfonic acid) were all from Sigma (St. Louis, MO, USA). Veronal was a product from Gelman (Ann Arbor, MI, USA), and sodium cyanoborohydride was obtained from Sterogene. IODOBEADS and NHS (N-hydroxysulfosuccinimide) were purchased from Pierce (Rockford, IL, USA). CNBr, N,N′-dimethylbarbituric acid, TEA (triethylamine), and pyridine were all from Fluka (Buchs, Switzerland). ^{125}Iodine (17.4 Ci mg^{-1}) was a product of Amersham (Buckinghamshire, UK). All other reagents, of analytical grade, were from Merck (Darmstadt, Germany). Plasma from healthy donors was provided by the Serviço de Hematologia Clínica, Hospital Geral de Santo António (Oporto, Portugal).

2.4. Preparation of immunoadsorbents

The murine anti-TTR mAb, 88.6.BA9, was covalently coupled to agarose via different activation methods, and at different pH, during the immobilization reaction. Coupling reactions, at pH 8 and higher, were carried out in 0.1 *M* carbonate buffer; at pH 5 to 7 in 0.1 *M* phosphate buffer; at pH 4 to 5 in 0.1 *M* acetate buffer, and at pH 3.0 and lower in 0.1 *M* tartarate buffer. All buffer solutions contained 0.5 *M* NaCl.

2.4.1. CNBr activation

Sepharose 4FF was activated with CNBr at neutral pH according to Kohn and Wilchek [15]. Briefly, 10 g of suction-dried Sepharose 4FF were washed with 30% acetone, followed by 60% acetone, and resuspended in 10 ml of 60% acetone in a temperature controlled reaction vessel. The mixture was cooled to −15°C, and 4 ml of 1 *M* CNBr in anhydrous acetone were added. With cooling and vigorous stirring, 4 ml of 1.5 *M* TEA in 60% acetone were added dropwise over a 2 min period. Then the reaction was stopped by quickly pouring the entire mixture into 100 ml ice-cold washing medium (acetone–0.1 *M* HCl, 1:1,

v/v). The amount of cyanate esters on the gel was determined according to Kohn and Wilchek [16]. The resin was used for coupling either immediately, or after storage in 60% acetone at −20°C for up 1 month.

2.4.2. Coupling procedure

CNBr-activated Sepharose 4FF was washed sequentially with cold 60% acetone, 30% acetone, water, and coupling buffer. Commercial CNBr-preactivated Sepharose 4B was used following the manufacturer's protocol. A ratio of 1 ml of mAb solution (5–10 mg 88.6.BA9 ml^{-1} coupling buffer) to 1 g of suction-dried gel was used. Coupling reactions were allowed to proceed overnight at +4°C under gentle agitation. Unbound protein was removed by successively washing in a sintered glass funnel with 5 gel volumes of each of the following buffers: (a) 0.3 *M* glycine–0.5 *M* NaCl, pH 2.8; (b) 0.1 *M* glycine–0.1 *M* NaCl, pH 10.5; and (c) phosphate-buffered saline (PBS), pH 7.4. The calculation of the coupling yield was based on the concentration of 88.6.BA9 in the coupling and washing solutions, determined by measuring the absorbance at 280 nm using $A_{1\%}=14.3$ [17], or with the bicinchoninic acid protein assay. The excess activated groups were blocked by incubating with 1 *M* ethanolamine, pH 8.5, at room temperature for 2 h. After washing and equilibration with PBS, immunoadsorbents were stored in 25% ethanol, at 4°C.

2.4.3. CDI activation

This mode of activation was carried out according to Bethell et al. [18]. Suction-dried Sepharose 4FF or Sepharose 6FF (5 g) were washed with 100 ml each, of ice cold water, water–acetone (7:3, 5:5, 3:7 v/v), and anhydrous acetone on a sintered glass funnel. The gel was suspended in anhydrous acetone (0.7 g ml^{-1}) and 40 mg of CDI per g of suction-dried Sepharose were added. After 2–3 h at room temperature, the gels were washed with acetone, cold water, and coupling buffer and used immediately. Immobilization procedures were the same as described above.

2.4.4. Reductive amination

88.6.BA9 was covalently coupled to Actigel ALD and Actigel ALD Superflow according to the manufacturer's protocol. Immobilization procedures were the same as described above. However, coupling and blocking steps were carried out in the presence of 0.2 ml of 1 *M* $NaCNBH_3$ per ml of resin.

2.4.5. Carbodiimide activation

88.6.BA9 was immobilized onto Affi-Gel 102 gel (aminoalkyl agarose) via EDC activation with NHS as enhancer, at pH 7.0 [19]. A ratio of 1 ml mAB solution (~10 mg 88.6.BA9 ml^{-1} of 25 m*M* MOPS, 0.5 *M* NaCl, pH 7.0) per g of suction-dried gel was used. After mixing the gel and the mAb solution for 1 h at room temperature, EDC and NHS were added from freshly prepared solutions in water, to final concentrations of 25 m*M* and 4 m*M*, respectively. Reaction pH was immediately adjusted to and kept for the next 15 min at 6.95–7.05. Coupling was allowed to proceed overnight at room temperature. Washing of the beads and the blocking step were as described above.

2.4.6. DVS activation

Sepharose 4FF was activated with DVS according to the procedure described by Porath et al. [20]. Sepharose 4FF was washed with five volumes each of 0.3 *M* glycine–HCl, pH 2.8; 0.1 *M* glycine–NaOH, pH 10.5; water; and 0.5 *M* Na_2CO_3–NaOH, pH 11. The gel was suspended in one volume of this last buffer, then DVS was added (0.15 ml per ml of gel), and the activation reaction was allowed to proceed for 3 h at room temperature, with gentle agitation. The activated beads were washed with an excess of cold water and used immediately.

2.5. Typical immunoadsorption procedure

Each immunoadsorbent (~5 ml) was packed in a column (Pharmacia Biotech C 10/10; I.D.=1 cm), and washed with PBS pH 7.4, 0.1 *M* glycine–NaOH–0.1 *M* NaCl pH 10.5, and again PBS. An excess of citrated normal blood plasma (100 ml) was pumped through the columns (Minipuls 3, Gilson), at a flow-rate of 0.5 ml min^{-1}, resulting in an average residence time of 10 min. Nonspecifically bound proteins were washed from the columns with PBS at a similar flow-rate, until absorbance (*A*280 nm) reached baseline (Gilson 112 UV/Vis detector). It has been demonstrated [7] that elution of TTR at

acid pH results in relatively poor desorption yields and protein precipitation. Therefore, bound TTR was desorbed with 0.1 *M* glycine–NaOH–0.1 *M* NaCl pH 10.5. Immunoadsorbents were regenerated by extensive washing with PBS, and stored between chromatographic runs in 25% ethanol, at 4°C. The amount of TTR adsorbed on the gel was calculated by determination of the TTR levels in the eluates, as well as in the influent and effluent plasma. Specificity of adsorption was ascertained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

2.6. Determination of TTR levels

TTR concentrations were determined by rocket immunoelectrophoresis [21]. Electrophoresis plates were prepared with 1% agarose M (Pharmacia Biotech) in veronal buffer containing 6 $\mu l\,ml^{-1}$ rabbit anti-human prealbumin (TTR). The electrophoreses were run at 10 V. cm^{-1} for 12 h. After electrophoresis, gels were dried and subsequently stained with Coomassie Brilliant Blue R-250. Standard plasma from Behringwerke (Marburg, Germany) was used for the calibration.

2.7. Radioiodination

88.6.BA9 was radiolabeled by the IODOBEADS method of Markwell [22]. Briefly, 2 mg of 88.6.BA9 in 2.5 ml of 0.1 *M* PBS pH 7.4 were incubated with ^{125}Na (2.5 mCi) in the presence of 10 IODOBEADS. After 20 min, the reaction was stopped and the mixture was gel-filtered with a PD-10 column (Pharmacia Biotech). The protein fractions with the highest radioactivity were pooled and ultrafiltered twice in a Centricon-10 concentrator (Amicon, Beverly, MA, USA), for a more complete separation of free iodine. Final antibody preparations had a specific activity of 200–300 $\mu Ci\,mg^{-1}$ protein. Counting was done on a LKB-Wallac CliniGamma 1272 (Pharmacia Biotech).

2.8. Leakage experiments

Radiolabeled immunoadsorbents were allowed to stand under different conditions, as described in Table 2 (below), and shaken regularly. At specified times, aliquots of the supernatants were taken, and subjected to ultrafiltration (Ultrafree-4, Millipore, Bedford, MA, USA) for the removal of free iodine. Ligand leakage was determined by measuring the radioactivity of the retentate.

In order to evaluate antibody leakage during apheresis, model studies using radiolabeled immunoadsorbents were carried out, as described in Section 2.5. All effluents with the exception of the ethanol fraction were pooled and assayed for radioactivity.

3. Results and discussion

3.1. Optimization of the immunoadsorption procedure

Previous studies demonstrated that many experimental parameters affect affinity interactions, such as ligand concentration [23,24], column dimensions, flow-rate, incubation time [25], pH, ionic strength and temperature [25,26]. In order to optimize the TTR binding capacity of the different immunoadsorbents, the following parameters were examined.

3.1.1. The influence of immobilized mAb density on TTR binding

In order to determine the effect of immobilized mAb density on the maximum TTR binding capacity of the different immunoadsorbents, 100 ml of citrated normal blood plasma ([TTR]=18–27 $mg\,dl^{-1}$) were passed through immunoadsorbent columns of approximately 5 ml (d=1 cm), at a flow-rate of 0.5 $ml\,min^{-1}$, to provide a residence time of about 10 min. Fig. 1 shows the dependency of the TTR binding capacity on the antibody density of CDI-activated Sepharose 4FF, immobilized at pH 4.0. Immunoadsorbents constructed via other immobilization chemistries showed similar behavior: an increase of the absorption capacity with the concentration of ligand on the matrix, up to about 7–8 mg 88.6.BA9 ml^{-1} of support. The efficiency of the TTR binding (mol TTR bound per mol of immobilized mAb) decreases rapidly from 30% of the theoretical maximum at an antibody concentration of <2 mg ml^{-1} to less than 10% at a mAb density >10 mg ml^{-1}. Thus, from this point of view, a low mAb

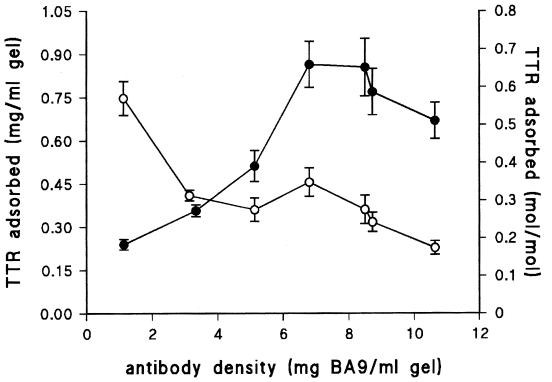

Fig. 1. TTR binding vs. antibody density. In this case, 88.6.BA9 was coupled to CDI-activated Sepharose 4FF at pH 4.0, as described in Section 2. The coupling efficiencies were around 90% for all immunoadsorbents, with the exception of the one with the lowest antibody density (1.1 mg ml^{-1}) which was 60%. Closed circles represent the amount of TTR bound in mg ml^{-1}, whereas open circles refer to antigen binding efficiency (mol TTR mol^{-1} BA9). Data are the means and S.D. of 5–10 chromatographic runs.

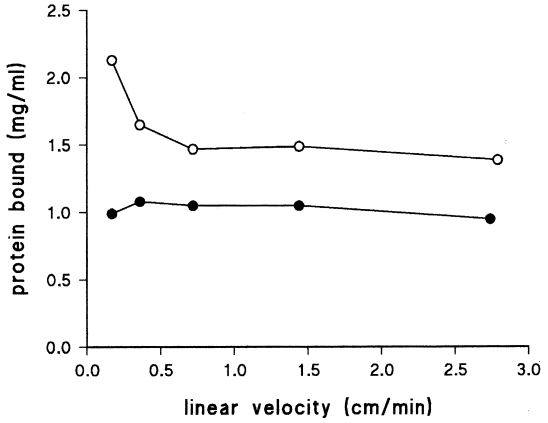

Fig. 2. Effect of the linear velocity on the antigen binding capacity. In this case, 101.3±3.0 ml of plasma ([TTR]=23.9±0.7 mg dl^{-1}) was pumped through a 9.2 ml immunoadsorbent at various flow-rates. A linear velocity of 1 cm min^{-1} corresponds to a flow-rate of 0.79 ml min^{-1}, resulting in a residence time of 11.7 min. Closed circles refer to the amount of TTR bound in mg ml^{-1} of gel, whereas open circles are an estimation of the amount of total protein in the eluents, per ml of gel, as determined by measuring the absorbance at 280 nm ($A_{1\%}$=14.3).

density on the gel is favorable. However, it has to be taken into consideration that the patient's extracorporeal blood volume is limited. So, in order to remove sufficient amounts of TTR, with reasonable column dimensions, it is necessary to use immunoadsorbents with a mAb density of 7–8 mg ml^{-1}. Comparisons among different immobilization chemistries were done for immunoadsorbents with mAb densities near this value.

3.1.2. Linear velocity

The effect of the residence time on the TTR binding capacity was evaluated by passing plasma through the columns at various flow-rates. Fig. 2 shows the dependency of the total adsorbed protein, and of bound TTR, on the applied linear velocity. Over the range tested, there is practically no influence of the residence time on the TTR binding capacity of the immunoadsorbent. However, at low flow-rates more protein binds nonspecifically to the column. Therefore, in order to reduce the extension of the treatments as well as to minimize the loss of plasma proteins other than TTR, the applied linear velocity should be higher than 0. 75 cm min^{-1}.

3.1.3. Antigen concentration

FAP patients are known to have plasma levels of TTR significantly lower than normal subjects [27]. To determine the influence of the TTR concentration in plasma on the binding capacity of the immunoadsorbents, the TTR concentration in the effluent was monitored. We found little or no influence of the TTR concentration of the influent ($[TTR]_{inf}$=18–30 mg dl^{-1}), neither in the amount of TTR bound, nor in the saturation time.

Fig. 3 shows a typical profile of the TTR concentration in the effluent, of plasma passing through an immunoadsorbent ($[TTR]_{inf}$=28.4 mg dl^{-1}); of the total amount of TTR adsorbed, more than 80% is bound within the first hour.

In the case of extracorporeal removal of TTR from plasma during a treatment session, in which the plasma is recirculated, TTR levels of the influent decrease drastically. To simulate this situation, approximately 250 ml of normal citrated plasma ([TTR]=22.3 mg dl^{-1}) were recirculated through an immunoadsorbent (7.5 ml) at a flow-rate of 1.1 ml min^{-1} for a period of 4 h. After washing the gel, desorption of the bound TTR, and regeneration, a

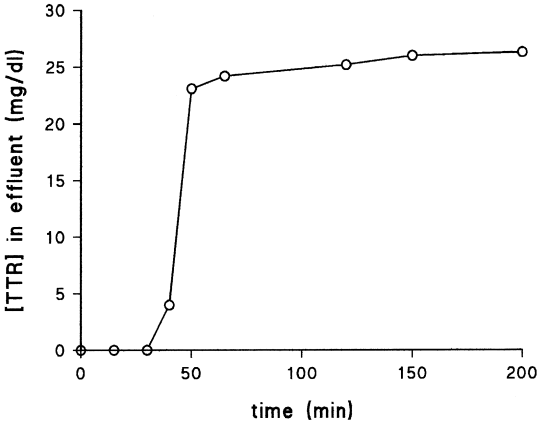

Fig. 3. TTR concentration in the effluent when normal plasma was passed through a 4.8 ml immunoadsorbent column (9.6 mg ml^{-1} 88.6.BA9 covalently coupled to CDI-activated Sepharose 4FF at pH 3.0). The TTR concentration of the influent was 28.4 mg dl^{-1} and the applied linear velocity was 0.63 cm min^{-1}.

second experiment was performed by recirculating the plasma used in the first experiment ([TTR]=15.8 mg dl^{-1}) through the same immunoadsorbent under similar conditions. This procedure was repeated once more ([TTR]=9.3 mg dl^{-1}). The amount of TTR recovered in the desorption solutions, 9.1 mg, 8.6 mg, and 9.3 mg, respectively, were in close agreement with the decrease of TTR concentration in the influent. Since initial TTR levels do not influence the TTR binding capacity of the immunoadsorbents, performance can be compared even when different batches of plasma are used.

3.1.4. Multiple use

It was observed that for a particular immunoadsorbent, the amount of TTR bound in the first run often differed markedly from the average amount of TTR bound in the subsequent runs (data not shown). Plausibly, the antibodies change their conformation during the first perfusion as a result of interactions with the different plasma components. Therefore, in order to be able to compare the performances of different immunoadsorbents, all experiments were done at least in quadruplet, and the data of the first run of each immunoadsorbent were disregarded.

3.2. Influence of pH on coupling

Mouse anti-TTR mAb 88.6.BA9 can be covalently coupled to activated matrices over a broad pH range (Fig. 4). Couplings at pH<3 have not been carried out due to precipitation of the antibody. Although the pH of the medium has an influence on the coupling yield of weakly activated gels, 88.6.BA9 can be immobilized onto solid supports with mAb densities of up to 10 mg ml^{-1} by all of the methods tested.

The pH during the coupling reaction has, nevertheless, a dramatic influence on the ability of the immunoadsorbent to bind TTR (Fig. 5). This effect is most pronounced with CDI activation. As shown, optimal antigen binding was observed for the immunoadsorbents prepared at pH 3–4, whereas those prepared at pH 5–7 virtually did not bind TTR. Immunoadsorbents prepared at pH 8.5 were able to bind TTR, but with only about 30% of the capacity of those constructed at pH 4. Similar results were seen for Actigel ALD Superflow derived immunoadsorbents: the TTR binding capacity is constant over a wide pH range (5–8.5), but more than doubles for the immunoadsorbents obtained at pH 4. The pH effect for CNBr-activated immunoadsorbents

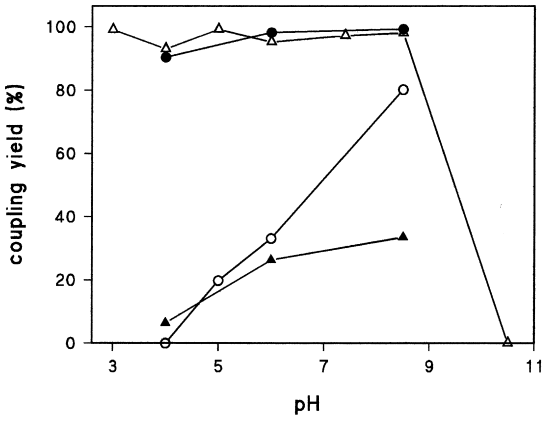

Fig. 4. Effect of the reaction pH on the coupling efficiency. (●)=CNBr preactivated Sepharose 4B (commercial, 16 μmol cyanate esters); (○)=CNBr-activated Sepharose 4FF (TEA, 4 μmol cyanate esters); (△)=CDI-activated Sepharose 4FF (200 mg CDI ml^{-1}); and (▲)=CDI-activated Sepharose 4FF (20 mg CDI ml^{-1}). In all cases, a ratio of 1 ml of 10 mg ml^{-1} 88.6.BA9 in coupling buffer to 1 g of suction-dried activated resin was used.

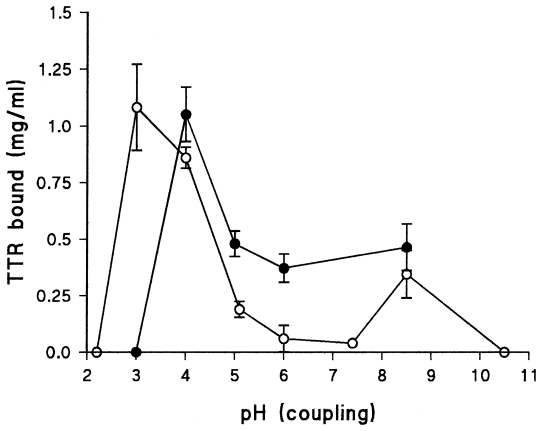

Fig. 5. Influence of the pH during immobilization on the performance of the immunoadsorbent. Closed circles refer to immunoadsorbents constructed via coupling of 88.6.BA9 onto Actigel ALD Superflow via reductive amination, whereas immunoadsorbents obtained via immobilization of the antibody onto CDI-activated Sepharose 4FF are represented by open circles. Data are the means and S.D. of several chromatographic runs with at least two independently constructed immunoadsorbents. Antibody densities on the gels were all in the range of 6–9 mg ml^{-1}.

is less pronounced (data not shown). It has to be taken into account that data shown in Fig. 5 were obtained using immunoadsorbents with different antibody densities. However, if these data are represented as molar ratios, the overall picture is very similar. Table 1 gives an overview of the TTR binding capacity of the immunoadsorbents constructed with the different immobilization chemistries at their optimum pH. Both CDI activation and reductive amination at low pH proved to be good alternatives to the CNBr activation method of Cuatrecasas et al. [11], currently used in the clinical trials, regarding TTR binding capacity. Therefore, they have been selected for further studies.

3.3. Other immunoadsorbents tested

The 88.6.BA9 antibody was also immobilized onto Sepharose 6FF via CDI activation. However, the TTR binding capacities of these immunoadsorbents were considerably lower than with Sepharose 4FF. Sepharose 6FF is a 6% agarose gel, whereas Sepharose 4FF contains 4% agarose. Since both the antibody and TTR are rather large molecules, the reduced binding capacity may reflect a steric hindrance effect of the denser gel. Interestingly, when we compared the performances of immunoadsorbents obtained via coupling to Actigel ALD (4% agarose gel) and Actigel ALD Superflow (6% agarose gel), we found exactly the opposite: the TTR binding capacity of the 6% resin was significantly higher than that of the 4% gel (data not shown). The aldehyde groups on Actigel ALD Superflow are

Table 1
TTR binding capacities of immunoadsorbents

Matrix	Activation	Coupling pH	Coupling yield (%)	mAb density (mg ml^{-1})	TTR binding (mg ml^{-1})	Binding efficiency (%)
Sepharose CL-4B	CNBr–NaOH[a]	8.5	82	6.4	0.99	21
Sepharose 4B	CNBr[b]	8.5	99	9.2	1.04	15
Sepharose 4FF	CNBr–TEA[c]	8.5	80	6.9	1.01	20
Actigel ALD Superflow	ALD	4.0	97	7.3	1.05	20
Sepharose 4FF	CDI	3.0	98	9.6	1.04	15
Affi-Gel 102 gel	EDC–NHS	7.0	94	7.0	0.16	3
Sepharose 4FF	DVS	8.5	97	7.3	0.18	3

Molar ratios were determined using a molecular mass of 150 000 for murine IgG and 55 000 for TTR. The TTR binding efficiency is the ratio of the experimentally determined capacity to the theoretical maximum capacity (2 mol of TTR per mol of immobilized antibody). Data are averages of multiple runs with each immunoadsorbent.

[a] Following the method of Cuatrecasas et al. [11].

[b] Commercial, preactivated (Pharmacia Biotech).

[c] Following the method of Kohn and Wilchek [15].

located on a 5-atom spacer arm, whereas in all other resins, the activated groups are located directly on the matrix. That spacer arm may make the antibody more accessible for antigen binding, resulting in higher TTR binding capacities.

Proteins can also be coupled to activated supports through their carboxylic groups. 88.6.BA9 was immobilized onto Affi-Gel 102 gel via carbodiimide activation, with and without NHS as enhancer [19]. Although coupling yields were high, TTR binding capacities were very low. This is most likely due to crosslinking by the carbodiimide, either within or among protein molecules.

Other researchers have found that DVS activation results in good antigen binding capacities [28], but for our mAb the measured TTR binding capacities were very low.

3.4. Leakage studies

Radiolabeled immunoadsorbents were constructed with the three most promising immobilization methods, in order to study their stability. Final antibody densities on the gels were 10.2 mg ml^{-1} for the CNBr-activated Sepharose 4FF (coupled at pH 8.5); 9.5 mg ml^{-1} for the CDI-activated Sepharose 4FF (coupled at pH 4.0); and 8.4 mg ml^{-1} for the Actigel ALD Superflow (coupled at pH 4.0). Coupling yields were 95%, 88%, and 82%, respectively.

Immunoadsorbents were taken in four equal volumes, three of which were kept in different storage solutions, and the remaining one was packed in a column for evaluating leakage under dynamic conditions. The leakage of ligand from the gel during storage was highest for the CDI-activated gel and lowest for the ALD-activated gel. Table 2 summarizes the results of the storage experiment in detail. This experiment demonstrated clearly that storage at high pH results in a significantly higher release of ligand than at low pH, and therefore, it might be favorable to elute the antigen with a low pH buffer. Unfortunately this is precluded by antigen precipitation, as mentioned before. Also included in Table 2 is the accumulated IgG leakage between runs, during storage in 25% ethanol. Although leakage is low and differences are small, ALD based immunoadsorbents proved to be the most stable ones. Data regarding IgG leakage during chromatography are summarized in Table 3. Again, it shows that immunoadsorbents based on Actigel ALD Superflow are the most stable, followed by the CNBr based gels. In the course of chromatography, leakage from CNBr-activated immunoadsorbents is about five times higher, and from CDI-activated immunoadsorbents more than 20 times

Table 2
Antibody leakage during storage

Immunoadsorbent	Storage solution	Ligand leakage after 3 months		Ligand leakage after 7 months	
		(μg ml^{-1})	(%)	(μg ml^{-1})	(%)
CNBr	A	167	1.64	514	5.06
	B	14	0.14	56	0.55
	C	59	0.58	67	0.66
	D	–	–	27	0.27
CDI	A	284	3.00	477	5.03
	B	33	0.35	72	0.76
	C	36	0.38	73	0.77
	D	–	–	34	0.47
ALD	A	62	0.75	93	1.11
	B	33	0.40	46	0.55
	C	20	0.24	57	0.68
	D	–	–	17	0.20

IgG leakage was determined by assaying the supernatants of the immunoadsorbents for radioactivity, after a storage period of 3 and 7 months in: (A) 0.1 *M* glycine–NaOH pH 10.5; (B) 0.3 *M* glycine–HCl pH 2.8; (C) 3 *M* NaSCN pH 6.0; (D) 25% EtOH at 4°C. Data is presented as the amount of IgG released per ml of gel, and as a percentage of the initially immobilized antibody.

Table 3
Antibody leakage during chromatography

Immunoadsorbent	Run	Time after coupling (days)	IgG leakage ($\mu g\ ml^{-1}$ gel)
CNBr	1	10	6.1
	2	17	5.1
	3	34	3.1
	4	224	2.8
CDI	1	10	40.8
	2	17	19.0
	3	34	9.5
	4	224	11.4
ALD	1	10	0.8
	2	17	0.8
	3	34	0.8
	4	224	0.4

Columns were run as described in Section 2. All eluents, with the exception of the ethanol fraction, were pooled and assayed for radioactivity.

higher than for ALD-activated immunoadsorbents. Most of the leakage, however, occurs during the elution of the antigen (regeneration of column), and therefore the majority of the released antibodies (60–80%), will not end up in the plasma. The leakage during plasma passage is roughly proportional to that of the whole chromatographic run, as determined for run 2 (data not shown). Extrapolating to true size therapeutical immunofilters (300 ml), this corresponds to an IgG leakage into the patient's plasma of 1.0, 2.0, and 0.2 mg per run, for CNBr-, CDI-, and ALD-activated immunoadsorbents, respectively. Such amounts can easily account for the observed patients' HAMA response.

4. Conclusions

Our results support the notion that antigen–antibody based methods have to be optimized case by case, especially when mAbs are involved. With our mAb, DVS- and EDC-activated immunoadsorbents have very low TTR binding efficiencies, and are consequently of no use for selective apheresis. CDI- and ALD-activated immunoadsorbents have similar TTR binding capacities as the currently used CNBr-activated immunoadsorbents, and can be considered as alternatives. Of these, Actigel ALD Superflow proved to be the most suitable regarding stability, particularly under alkaline conditions. This is important in our system, because regeneration of the TTR immunoadsorbents currently used for therapeutic apheresis is done at pH 10.5 [7]. Our results also show a bimodal dependence of antigen binding efficiency of CDI- and ALD-activated immunoadsorbents on the pH of coupling reaction, with optima at high and low pH, something that to our knowledge has not been documented previously.

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