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Comparison of the performance of immunosorbents prepared by site-directed or random coupling of monoclonal antibodies

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ABSTRACT

The majority of methods used to prepare immunosorbents immobilize antibodies through their reactive amino acid residues. The bound antibody activity of these immunosorbents is low. Hydrazide-based matrices couple antibodies through carbohydrate chains frequently located in the Fc region. This paper reports a comparative study of the performance of immunosorbents prepared by cyanogen bromide or hydrazide immobilization methods. The experiments utilized murine monoclonal antibodies to the human plasma proteins Factor IX or Protein C. The antibodies were immobilized at low densities to beaded agarose matrices which had similar properties. The hydrazide immunosorbents had binding efficiencies which were lower (anti-Factor IX) or up to 1.6-fold higher (anti-Protein C) than comparable cyanogen bromide coupled gels. However, there was no improvement in performance due to lower recoveries of bound protein from the hydrazide gels. Control experiments demonstrated that oxidation of antibody which is required for its coupling to hydrazide gels had no effect on antibody binding to antigen. Our results indicate that, as with cyanogen bromide coupling methods, site-directed immobilization through carbohydrate residues results in a restricted ability to bind to antigen. Both monoclonals were found to contain carbohydrate in their Fab′ regions through which coupling may have occurred. The frequency of carbohydrate in the Fab region and the ability to control glycosylation at these sites are factors which may impact the utility of carbohydrate-directed immobilization of antibodies.

INTRODUCTION

Covalent attachment of monoclonal or polyclonal antibodies (Ab) to matrices has long been employed to create highly specific sorbents for haptens or proteins. However once immobilized, a significant reduction in antigen binding capacity occurs. For example the antigen binding capacities (efficiencies) based on divalent Ab activity have been reported to be 30% or less for immunosorbents containing 1 mg or

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more bound Ab per ml of gel [1–5]. This is true for monoclonal or polyclonal antibodies. The application of immunosorbents to large-scale purification is to a large extent constrained by the high cost of Ab. Increasing the acitivty of an immunosorbent would reduce the Ab requirement and significantly lower the cost of the immunosorbent, thus increasing the feasibility of its use in large-scale purification processes.

Changes in immunoaffinity interactions which occur because of Ab immobilization are not well understood. However, it has been suggested that steric hindrance, improper orientation, and alterations of the antigen-binding site contribute to the low bound Ab activity observed for most immunosorbents [6]. Both direct chemical modification of a critical amino acid residue(s) in the antigen-binding site during coupling or indirect distortion of its conformation resulting from multipoint attachment in a nearby region of the molecule, for example, may adversely affect antigen binding.

Several approaches have been used to maximize the efficiency of immunosorbents. Coupling parameters such as the concentration of reactive groups on the gel, pH and reaction time have been varied to minimize multipoint attachment of Ab [7]. It is likely that a uniform and appropriately spaced distribution of reactive sites on the gel is important in optimizing the performance of an immunosorbent [4]. Lower surface densities of Ab have been found to result in greater antigen binding capacity relative to bound Ab, which is thought to be due to decreased steric hindrance between Ab molecules [3,8,9]. In addition, steric hindrance of Ab due to unfavorable interaction with the matrix is thought to be minimized if an adequately long hydrophilic spacer arm is employed [10–12]. An extension of approximately 1–2 nm has been shown to increase the bound activity of immobilized enzymes and co-factors using alkylamines [11] and polyglycine [12] as spacer arms. However, the orientation of antibodies and alteration of their antigen-binding sites have been more difficult to control due to the random nature of the coupling chemistries most frequently utilized. For example, the reactive groups formed by cyanogen bromide (CNBr), N-hydroxysuccinimide, carbonyldiimidazole and toluene sulphonylchloride activation methods immobilize the protein through accessible primary amino groups [7]. Because many such groups are present on the surface of a protein, its site(s) of covalent attachment is thought to be random [6]. This would result in a distribution ranging from coupled antibodies having lowered activity to those which are completely inactive.

Immunoglobulin G (IgG) is an approximately 160 000 molecular weight glycoprotein consisting of two identical heavy and two identical light chains. Each chain consists of several domains which are highly conserved among species (see ref. 13 for review). The CH_2 domain of the heavy chain contains an asparagine residue in the recognition sequence –Asn–X–Thr(Ser) which is glycosylated in human, rabbit, and murine IgG [14–20]. Hence, coupling chemistry specific for carbohydrate has been utilized for site-directed coupling of Ab through the oligosaccharide chain in the Fc region (consisting of the CH_2 and CH_3 domains of the heavy chains) of the Ab. This has been accomplished by oxidation of the vicinal hydroxyl groups of the carbohydrate residues to form aldehydes which are then reacted with the hydrazine groups of a solid support to obtain a covalent hydrazone linkage (Fig. 1). Affinity sorbents prepared using hydrazide-activated solid supports have been reported to have increased binding efficiency compared to random coupling methods [21–25]. In a variation of this approach, the carbohydrate moieties of Ab were selectively biotinylated with hydrazine-biotin [22] and then adsorbed on avidin- or streptavidin-coupled supports [26].

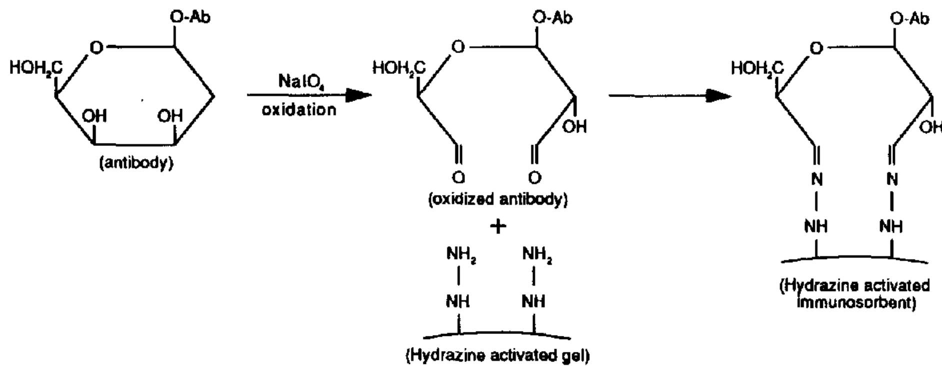


Fig. 1. Schematic representation of antibody attachment to hydrazide-activated gels (adapted from ref. 23). Carbohydrate residues in the oligosaccharide chains of the antibody are oxidized at the vicinal hydroxyls to form aldehydes. The aldehyde groups are coupled with the hydrazine groups of the gel to form the hydrazone-linked immunosorbent.

However, the performance of carbohydrate-linked immunosorbents has not been well documented. Previous studies have examined the effect of hydrazide *vs.* conventional coupling on the binding efficiency of immunosorbents, while little data has been presented on the ability to recover the bound antigen. Furthermore, most published data relates to polyclonal antibodies with little information available on monoclonal antibodies (Mabs), which are more relevant for large scale immunosorbent processes.

In the present study, we compared the performance of immunosorbents prepared by either hydrazide- or CNBr-coupling methods. The matrices for both types of immunosorbents were beaded, cross-linked agarose gels of similar bead size and porosity [27–29]. Two different murine Mabs directed against the human plasma proteins Factor IX (FIX) or Protein C (PC) were used. These Mabs bind to the corresponding antigens in the presence or absence of divalent cations, respectively [30–32]. The resulting antigen–antibody complexes are easily disrupted by chelation or addition of low concentrations of divalent cations. This unique property makes it possible to elute the immunosorbents prepared using these Mabs under mild elution conditions.

METHODS

Materials and reagents

Assera Protein C, a specific rabbit anti-human Protein C antiserum and horseradish peroxidase (HRP)-conjugated anti-human Protein C rabbit antiserum were purchased from American Bio-Products, Parsippany, NJ, USA. Affinity-purified, HRP-conjugated, goat anti-mouse immunoglobulins (IgA + IgG + IgM) antibody was from Cappel, West Chester, PA, USA. *o*-Phenylenediamine (OPD) was from Dakopatts, Denmark. Immulon II microtiter plates were from Dynatech Labs., Chantilly, VA, USA. Protac was from American Diagnostica, New York, NY, USA. S-2366 was from Helena Labs., Beaumont, TX, USA. Aquasil, a water-soluble siliconizing fluid, and Protein A-agarose were from Pierce, Rockford, IL, USA. Papain

(25 mg protein/ml, 27 units/mg) was from Sigma, St. Louis, MO, USA. $NaIO_4$ (certified A.C.S. grade) and glutaraldehyde were from Fisher Scientific, Fair Lawn, NJ, USA. Amicon columns were from Amicon, Danvers, MA, USA. Polypropylene Econo-Columns and Econo-Pac 10DG desalting columns were from Bio-Rad Labs., Richmond, CA, USA and Sepharose CL-2B was from Pharmacia, Piscataway, NJ, USA. Hydrazide derivatized agarose gels were purchased from two manufacturers, BioProbe International, Tustin, CA, USA (Manufacturer A) and Bio-Rad Labs. (Manufacturer B). All other chemicals were reagent grade or better.

Protein purification

Mabs. The murine metal-dependent anti-FIX Mab 1H5B7 was purified from cultured cell supernatant as described by Wang *et al.* [30]. The murine "ethylenediaminetetraacetic acid (EDTA)-dependent" anti-PC Mab 7D7B10 [31] was purified from cultured cell supernatant as follows. The cell supernatant was filtered and concentrated by precipitation with saturated ammonium sulphate at room temperature. The precipitate was dissolved in 0.05 *M* Tris–HCl, 0.1 *M* sodium chloride, pH 7.5 and reprecipitated with 45% ammonium sulphate at 4°C. This precipitate was dissolved in 0.05 *M* Tris–HCl, 0.1 *M* sodium chloride, pH 7.5 and dialyzed extensively against 0.15 *M* sodium chloride. The purity of this material by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) was greater than 90%. Both Mabs were of the IgG_1 subtype with κ light chains. Mab concentrations were determined by absorbance at 280 nm (A_{280}) using an absorption coefficient ($A_{280}^{1\%}$) of 15.5 and assuming a molecular weight of 160 000 [7].

Fab′ fragments were prepared by treatment of the Mab with papain followed by affinity chromatography of the digest on Protein A–agarose using methods as described in ref. 7. The Fab′ fragment preparations contained 10% or less contamination by Fc fragment and no detectable whole IgG, as analyzed by SDS-PAGE. The concentration of Fab′ was calculated from the A_{280} using an absorption coefficient of 10.0 for a 1% solution and a molecular weight of 50 000 [7].

Antigens. Human FIX was purified from cryo (cryoprecipitated antihemophilic factor)-poor plasma by immunoaffinity chromatography using Mab 1H5B7 as described by Tharakan and co-workers [33,34]. The final product was 95% pure by SDS-PAGE and had a specific activity of 202 units/mg. FIX concentrations were determined by A_{280} using an $A_{280}^{1\%}$ of 13.2 and assuming a molecular weight of 57 000 [35]. Human PC was purified from cryo-poor plasma using an immunosorbent based upon the murine anti-human PC Mab 8861. Bound PC was eluted with a pH 10.0 buffer [36]. The final product was 92% pure by SDS-PAGE and had a specific activity of 200 units/mg. PC concentration was determined by A_{280} using an $A_{280}^{1\%}$ of 14.5 and a molecular weight of 62 000 [37]. Purified PC and FIX were used in the experiments described herein to evaluate the performance of the various immunosorbents.

Immunosorbent preparation

Anti-FIX hydrazide immunosorbent A. The coupling was performed by Manufacturer A according to their standard protocol, briefly described as follows. A 20–30 mg/ml solution of anti-FIX Mab in 0.05 *M* sodium acetate, pH 5.0 was oxidized by gently agitating with 0.1 *M* $NaIO_4$ in a 10:1 volumetric ratio (64-fold molar excess $NaIO_4$) for 1 h at room temperature in the dark. $NaIO_4$ was removed by desalting.

An 8–10 mg/ml solution of oxidized Mab was mixed with the hydrazide gel A in a 1:1 volumetric ratio at 4°C overnight. The gel was sequentially washed with 0.05 *M* sodium acetate, pH 5.0, distilled water, and 1 *M* sodium chloride. The coupling efficiency and the immunosorbent density were reported to be 98% and 1.87 mg/ml, respectively. The amount of Mab coupled to the gel was calculated as the difference between total Mab added to the gel and uncoupled Mab recovered in the coupling supernatant and wash pools, as measured by A_{280}. The coupling efficiency was calculated as the ratio of (coupled Mab/total Mab) · 100%.

Anti-PC hydrazide immunosorbents. The anti-PC immunosorbent A(i) was prepared in our laboratory using hydrazide gel from Manufacturer A by a modification of the procedure described above because preliminary experiments resulted in a low coupling efficiency. A 17 mg/ml solution of anti-PC Mab was gently agitated with 0.05 *M* $NaIO_4$ in a 3:1 volumetric ratio (157-fold molar excess $NaIO_4$) for 1 h at room temperature in the dark. $NaIO_4$ was removed by desalting. A 2.5 mg/ml solution of oxidized Mab was gently agitated with hydrazide gel A in a 1:2 volumetric ratio for 48 h at 4°C. The gel was washed with 0.05 *M* sodium acetate, pH 5.0. The coupling efficiency was 90% and the immunosorbent density was 1.0 mg/ml. Anti-PC hydrazide immunosorbent A(ii) was prepared by Manufacturer A according to their standard protocol outlined above for anti-FIX. A 100-fold molar excess of $NaIO_4$ was used in the Mab oxidation step. The coupling efficiency and the immunosorbent density were reported to be 98% and 2.3 mg/ml, respectively. Anti-PC hydrazide immunosorbent B was prepared in our laboratory from hydrazide gel from Manufacturer B using the protocol recommended by the manufacturer. A 2.7 mg/ml solution of anti-PC Mab in 0.05 *M* sodium acetate, pH 5.5 was mixed with 0.1 *M* $NaIO_4$ in a 12:1 volumetric ratio (494-fold molar excess $NaIO_4$) for 1 h at room temperature in the dark. $NaIO_4$ was removed by desalting. A 1.8 mg/ml solution of oxidized Mab was mixed with hydrazide gel B in a 1:1 volumetric ratio for 48 h at room temperature. The gel was washed with 0.05 *M* sodium acetate, pH 5.5. The coupling eficiency was 71% and the immunosorbent density was 1.5 mg/ml. For control experiments to determine the amount of nonspecific binding in the absence of Mab, hydrazide gel from manufacturer A was incubated for 20 h at 4°C with a 3.5-fold molar excess of glutaraldehyde relative to the total hydrazine groups on the gel under conditions as described above.

CNBr-activated immunosorbents. Anti-FIX Mab was coupled to CNBr-activated Sepharose CL-2B according to the method of March *et al.* [38]. The activated gel was coupled to Mab in 0.1 *M* $NaHCO_3$, 0.5 *M* sodium chloride, pH 8.5 and blocked with 1 *M* glycine ethylester pH 8.5. The coupling efficiency was 94% and the immunosorbent density was 1.0 mg/ml. Anti-PC Mab was coupled to CNBr-activated Sepharose CL-2B as described above. The coupling efficiency was 88% and the immunosorbent density was 1.7 mg/ml.

Chromatography

Anti-FIX immunosorbents. The immunosorbents were packed in Aquasil-treated, Amicon G 150 × 10 mm I.D. columns. The columns were equilibrated in 10 m*M* $MgCl_2$, 110 m*M* sodium chloride, 20 m*M* imidazole, pH 7.5. Purified FIX at a concentration of 0.44 mg/ml in 20 m*M* sodium citrate, 110 m*M* sodium chloride, pH 6.8 containing 40 m*M* $MgCl_2$ was loaded onto the columns, rinsed with 10 m*M* $MgCl_2$, 1

M sodium chloride, 20 m*M* imidazole, pH 7.2 and eluted with 20 m*M* sodium citrate, 110 m*M* sodium chloride, pH 6.8 [33,34]. The columns were then washed with 2 *M* sodium chloride, 0.1 *M* sodium citrate, pH 7.2. All column operations were performed at a flow-rate of 1.0 ml/min at 4°C. The corrected absorbance of the effluent fractions was measured as $A_{280} - A_{320}$ and used to calculate the protein concentration. The amount of protein in the load, fall-through and rinse, eluate, and wash pools was determined by summation of the protein in the fractions consituting each pool.

Anti-Protein C immunosorbents. All immunosorbents were packed in 12 ml polypropylene Econo-column with the exception of the anti-PC hydrazide immunosorbent A(i), which was packed in an Aquasil-treated Amicon G 250 × 10 mm I.D. column. The columns were equilibrated in 0.025 *M* Tris–HCl, 0.05 *M* sodium chloride, pH 7.5 [Tris-buffered saline (TBS)]. Purified PC, at a mean concentration of 0.42 ± 0.03 mg/ml (range 0.40–0.47 mg/ml) equilibrated in TBS, was loaded, rinsed with TBS and eluted with 20 m*M* $CaCl_2$ in TBS. The columns were then successively washed with 100 m*M* $CaCl_2$, 2 *M* sodium chloride and 2 *M* NaSCN, each in TBS. Column fractions of 3.0 ml were collected and the A_{280} was measured. All column operations were performed at a flow-rate of 0.5 ml/min at 4°C.

Assays

FIX. FIX activity was measured by a clotting assay according to the method of Biggs [39] as modified by Miekka [40]. A unit is defined as 4 μg of FIX, the amount present in 1 ml of normal pooled human plasma.

PC. The assay was performed according to the method of Odegaard *et al.* [41]. PC was activated by Protac and the chromogenic substrate S-2366 was used to measure the activity of the resulting activitated PC, determined from the rate of change of absorbance at 410 nm using a Vmax kinetic microtiter plate reader (Molecular Devices). A unit of PC is defined as 4 μg, the amount present in 1 ml of normal pooled human plasma.

Enzyme-linked immunosorbent assay (ELISA) of oxidized and native anti-PC Mab. Immulon II microtiter plates were coated with a 1:200 dilution of rabbit anti-PC antisera in 0.1 *M* $NaHCO_3$, pH 9.6 for 1 h at 37°C. Wells were blocked with 1% BSA, 0.1 *M* $NaHCO_3$, pH 9.6 for 1 h at 37°C ad washed with TBS, 0.1% Tween-20. Serially diluted PC samples in TBS, 10 m*M* EDTA, 0.1% BSA were incubated with 32 n*M* oxidized ($NaIO_4$-treated) or native (untreated) anti-PC Mab for 1 h at room temperature. The PC-Mab mixtures were added to the coated wells and incubated for 1 h at room temperature. The wells were washed and incubated with 1:1000 dilution of HRP-conjugated goat anti-mouse IgG in TBS, 10 m*M* EDTA, 0.1% BSA for 1 h at room temperature. The wells were washed and HRP activity was detected with OPD substrate by absorbance at 490 nm using a Vmax plate reader. The results were calculated as a percentage of the signal obtained at saturation.

ELISA of PC purified by different methods. Serially diluted PC samples from a conventional purification [31], and from immunoaffinity purifications using the anti-PC Mabs 8861 or 7D7B10 [32] were preincubated with 32 n*M* native 7D7B10 Mab and assayed by the ELISA procedure outlined above.

SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli [42] using a 12.5% polyacrylamide gel, and stained with 2% Coomassie Brilliant Blue R-250.

Carbohydrate analysis

Sialic acid. Anti-PC and anti-FIX Mabs were heated with 0.05 *M* H_2SO_4 for 1 h at 80°C to liberate sialic acid residues which were quantitated by colorimetric analysis using thiobarbituric acid [43].

Reducing sugars. The reducing sugar content of the parent IgGs and purified Fab′ fragments were analyzed by the *o*-toluidine high-performance thin-layer chromatographic (HPTLC) method [44]. Briefly described, proteins or reference sugars in carbohydrate-free bovine serum albumin (BSA) were hydrolyzed in an identical manner in 6 *M* hydrochloric acid (HCl) at 110°C for 14 h or in 2 *M* trifluoroacetic acid (TFA) at 110°C for 2 and 6 h. The hydrolysates were applied to HPTLC plates. The sugars were resolved by the sequential migration of two solvent systems: *n*-butanol–pyridine–water (16:5:4) followed by ethylacetate–methanol–acetic acid–water (4:1:1:1). The plates were sprayed with *o*-toluidine in acetic acid and allowed to react at 100°C for 25 min and then scanned at 295 nm using a Shimadzu CS 9000 diffuse reflectance densitometer. A linear signal extending from 0.050–1.0 μg was obtained for each reducing sugar standard (data not shown). A 2–4% coefficient of variation was obtained for quadruplicate applications of each sample and reference sugar.

RESULTS

Experiments were performed to directly compare the performance of immunosorbents prepared by coupling purified Mab to hydrazide *vs.* CNBr-activated agarose supports. For each experiment the immunosorbent efficiency, defined as the percent of the theoretical maximum binding capacity assuming a 2:1 molar ratio of protein antigen to Mab, was calculated in two ways: (1) based upon the amount of protein (PC or FIX) that bound to the immunosorbent and (2) based upon the amount of protein (PC or FIX) that was specifically eluted under the mild elution conditions.

Table I summarizes the results of experiments with immunosorbents prepared by immobilizing the anti-PC Mab 7D7B10 to hydrazide or CNBr-activated gels. The hydrazide-coupled immunosorbents utilized were from two manufacturers. Five experimental runs were performed with the anti-PC immunosorbent prepared from the hydrazide gel of Manufacturer A in our laboratory [A(i)]. The efficiency of this immunosorbent remained essentially unchanged through three consecutive uses by dynamic loading (runs 1–3). Despite the fact that these three runs were column-loaded with less than saturating amounts of PC, almost half of the loaded material was recovered in the fall-through fraction. This immunosorbent was also batch loaded with saturating amounts of PC for 1 h with gentle, end-over-end agitation (runs 4 and 5). Similar results were obtained with batch loading under saturating conditions suggesting that the efficiencies observed with dynamic loading were not limited by kinetic phenomena. Table I also contains the results of experiments using the following anti-PC immunosorbents: A(ii), hydrazide immunosorbent prepared by Manufacturer A; B, hydrazide immunosorbent prepared in our laboratory using hydrazide gel from Manufacturer B; CNBr, Sepharose CL-2B immunosorbent prepared in our

TABLE I

PERFORMANCE OF ANTI-PC IMMUNOSORBENTS

Immunosorbents were prepared and operated as described in the Methods section. Hydrazide gels from two manufacturers (A and B) were evaluated. Immunosorbent A(i), prepared from hydrazide gel of Manufacturer A, had a Mab density of 1.0 mg Mab/ml gel and a bed volume of 6.0 ml. Immunosorbent A(ii), prepared by Manufacturer A, had a Mab density of 2.3 mg Mab/ml and a bed volume of 3.2 ml. Immunosorbent B, prepared using hydrazide gel from Manufacturer B had a Mab density of 1.5 mg Mab/ml of gel and a bed volume of 5.0 ml. Immunosorbent CNBr was prepared from Sepharose CL-2B following CNBr activation [38]. It had a Mab density of 1.7 mg Mab/ml gel and a bed volume of 2.0 ml. The immunosorbents were prepared in our laboratory, with the exception of A(ii). All runs utilized dynamic loading, except for runs 4 and 5 with immunosorbent A(i), in which batch loading was performed. Efficiency is defined as the percent of theoretical maximum binding capacity, assuming a 2:1 molar ratio of PC to Mab.

Immunosorbent	Run No.	Load (mg PC)	Fallthrough and rinse (mg PC)	Efficiency[a] (%)	Efficiency[b] (%)
A(i)	1	2.00	0.99	21	15
	2	1.88	0.73	24	13
	3	2.00	0.79	25	13
	4	4.90	4.14	16	10
	5	5.64	4.07	33	15
A(ii)	1	4.51	1.59	51	27
	2	5.29	2.59	47	26
B	1	6.59	3.67	49	26
	2	6.18	3.01	53	23
	3	6.67	4.03	44	28
CNBr	1	2.80	1.78	37	26
	2	2.00	1.45	20	17
	3	2.00	1.14	32	24

[a] Efficiency based upon PC bound to the immunosorbent.
[b] Efficiency based upon PC recovered in the eluate.

laboratory by the CNBr activation method of March [38]. Approximately saturating amounts of PC were column-loaded in these experiments. The immunosorbent efficiencies based upon bound PC were higher for hydrazide immunsorbents A(ii) and B as compared to hydrazide immunsorbents A(i) and the CNBr immunosorbent. However, the higher PC binding efficiencies of these gels did not result in improved performance. This is seen by examining the efficiencies based upon PC recovered in the eluates, which were similar for the CNBr and hydrazide immunosorbents and in all cases lower than efficiencies based upon bound PC.

Table II presents the results of experiments with immunosorbents prepared by immobilizing the anti-FIX Mab 1H5B7 to hydrazide or CNBr-activated gels. In these experiments, 59% of a saturating amount of FIX was column-loaded under identical conditions.The immunosorbent efficiency based upon bound FIX was higher for the CNBr gel (48–51%) compared to the hydrazide gel (29–33%). This was also true for the efficiency based upon recovered FIX, which ranged from 36–40% for the CNBr gel and 17–20% for the hydrazide gel. As was the case with the anti-PC immunosor-

TABLE II

PERFORMANCE OF ANTI-FIX IMMUNOSORBENTS

Immunosorbent A was prepared from hydrazide gel by Manufacturer A and operated as described in the text. The Mab density was 1.87 mg Mab/ml gel and the bed volume was 4.9 ml. Immunosorbent CNBr, prepared from CNBr-activated Sepharose CL-2B, had a Mab density of 1.0 mg Mab/ml gel and a bed volume of 5.0 ml. Efficiency is as in the legend to Table I.

Immunosorbent	Run No.	Load (mg FIX)	Fallthrough and rinse (mg FIX)	Efficiency[a] (%)	Efficiency[b] (%)
A	1	3.84	1.86	29	17
	2	3.69	1.44	33	20
	3	3.81	1.83	29	20
CNBr	1	2.12	0.31	49	37
	2	2.10	0.35	48	40
	3	2.01	0.23	48	38
	4	2.08	0.21	51	36

[a] Efficiency based upon FIX bound to the immunosorbent.
[b] Efficiency based upon FIX recovered in the eluate.

bents, the immunosorbent efficiencies were lower based upon recovered as compared to bound FIX.

Table III summarizes the mean efficiency data for the experiments listed in Table I and II. For the anti-PC immunosorbents, the efficiencies based upon bound PC for hydrazide immunosorbents A(ii) and B were 49 ± 3% and 49 ± 5%, significantly higher than the efficiency of the CNBr gel of 30 ± 9%. However, this 1.6-fold increase in PC binding efficiency of the hydrazide gels did not result in a comparable increase in efficiency based upon PC that could be eluted from these immunosorbents. The efficiency based upon PC recovered in the eluates was 27 ± 1% and 26 ± 2% for

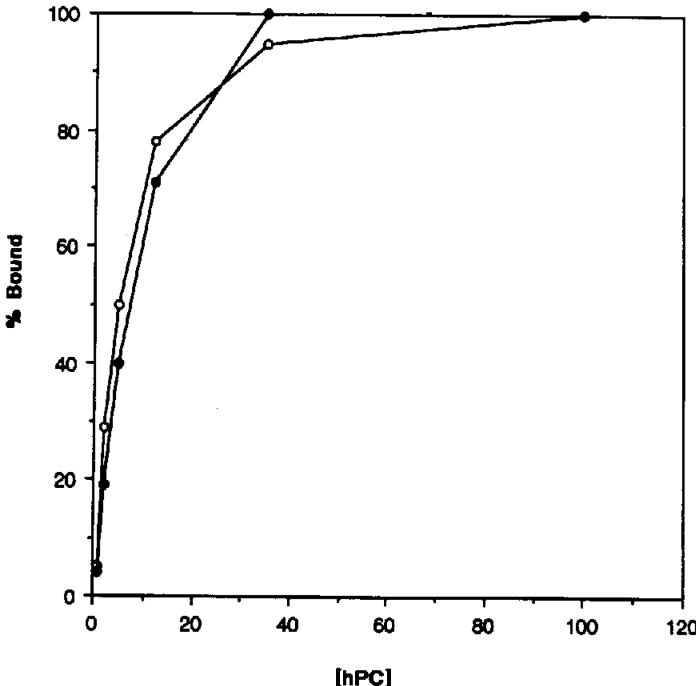
TABLE III

SUMMARY OF MEAN EFFICIENCIES OF IMMUNOSORBENTS

The mean efficiencies for the experiments listed in Table I and II were calculated. Efficiency is as defined in the legend to Table I.

Immunosorbent[a]	Efficiency (%)			
	(Based on protein bound)		(Based on protein recovered)	
	PC	FIX	PC	FIX
A(i)	24 ± 6 (5)[b]	N.A.[c]	13 ± 2 (5)	N.A.
A(ii)	49 ± 3 (2)	30 ± 2 (3)	27 ± 1 (2)	19 ± 2 (3)
B	49 ± 5 (3)	N.A.	26 ± 2 (3)	N.A.
CNBr	30 ± 9 (3)	49 ± 1 (4)	22 ± 4 (3)	38 ± 2 (4)

[a] Immunosorbents are as given in the legends to Tables I and II.
[b] Mean ± S.D. with the number of experiments in parentheses.
[c] Not applicable.



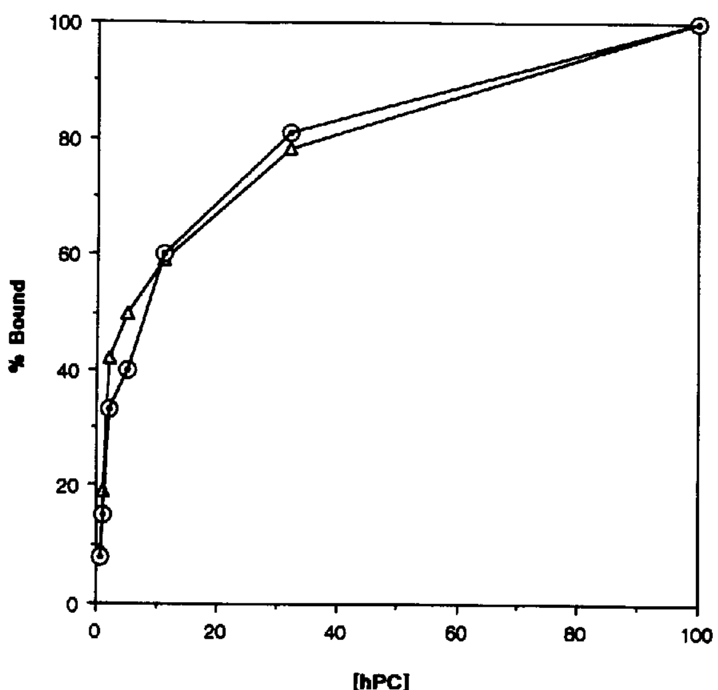


Fig. 2. Comparison of PC binding by native and oxidized anti-PC Mab. Native (untreated) and oxidized (periodate-treated) Mab was incubated with varying concentrations of PC, as indicated. The resulting PC–Mab complex was quantitated by ELISA as described in the Experimental section. ● = native Mab; ○ = oxidized Mab. The concentration of Protein C ([hPC]) is given in n*M*.

Fig. 3. Comparison of anti-PC Mab binding to PC purified by different methods. Anti-PC Mab (7D7B10) was incubated with increasing concentrations of PC, purified by conventional methods (△), by immunoaffinity chromatography using anti-PC Mab 7D7B10 (○), or anti-PC Mab 8861 (●). The resulting PC–Mab complex was quantitated by ELISA.

TABLE IV

SUMMARY OF MEAN RECOVERIES OF IMMUNOSORBENTS

The mean recoveries for the experiments listed in Tables I and II are tabulated. Recovery was calculated based upon the amount of bound protein recovered in the eluate (no brackets) or recovered in the eluate plus subsequent washes (in brackets).

Immunosorbent[a]	Recovery (%)	
	PC	FIX
A(i)	55 ± 12 (5)[b]	N.A.[c]
	[55 ± 12]	
A(ii)	52 ± 1 (2)	61 ± 5 (3)
	[52 ± 1]	[77 ± 6]
B	60 ± 9 (3)	N.A.
	[60 ± 9]	
CNBr	75 ± 8 (3)	75 ± 5 (4)
	[85 ± 5]	[85 ± 9]

[a] Immunosorbents are as given in the legends to Tables I and II.
[b] Mean ± S.D. with the number of experiments indicated in parentheses.
[c] Not applicable.

hydrazide gels A(ii) and B, respectively, compared to 22 ± 4% for the CNBr gel representing approximately a 20% increase in immunosorbent efficiency. The efficiency of hydrazide immunosorbent A(i) was less than that of the CNBr gel based upon both bound and eluted PC. For the anti-FIX immunosorbents, the efficiency of the CNBr gel was 49 ± 1% compared to 30 ± 2% for the hydrazide gel prepared by Manufacturer A based upon bound FIX and 38 ± 2% compared to 19 ± 2% for CNBr and hydrazide gels, respectively, based upon eluted FIX.

Table IV summarizes the mean recovery data for the immunosorbents. For each experiment, both protein specifically recovered in the eluate (specific recovery) as well as total protein recovered in the eluate plus subsequent wash pools (total recovery) was calculated as a percentage of the bound protein. For the anti-PC immunosorbents, the mean specific recovery of PC for the CNBr-coupled gel was 75% which was higher than found for the hydrazide immunosorbents which ranged from 52 to 60%. Likewise for the anti-FIX immunosorbents, the mean specific recovery of FIX was 75% for the CNBr gel as compared to 61% for the hydrazide gel. For both CNBr immunosorbents as well as for the anti-FIX hydrazide immunosorbent, additional protein was recovered in the buffered 2 *M* sodium chloride wash such that the total protein recovery was 77–85%. The anti-PC hydrazide immunosorbents were the exception, however, in that no additional PC was recovered following successive washes with buffered 2 *M* sodium chloride and buffered 2 *M* NaSCN.

In order to determine whether $NaIO_4$ treatment of the anti-PC Mab affected its ability to bind to PC, oxidized anti-PC Mab ($NaIO_4$-treated) and native anti-PC Mab (untreated) were compared by an ELISA (Fig. 2). The dose-response curves of PC binding to both oxidized and native Mab were very similar indicating that the periodate oxidation step did not impair the activity of the Mab.

In further experiments, the binding of 7D7B10 Mab to varying concentrations

TABLE V

CARBOHYDRATE COMPOSITION OF MURINE Mab AND Fab′ FRAGMENTS

Data from 14 h hydrolysis with 6 *M* hydrochloric acid, except for mannose data which is from 2 and 6 h hydrolysis with 2 *M* trifluoroacetic acid.

Sample	NAG (mol/mol)[a]	Galactose (mol/mol)	Mannose (mol/mol)	Sialic acid (mol/mol)
7D7B10 Mab	23.3	4.2	7.0	1-2
7D7B10 Fab′ (nc)[b]	4.1	0.5	nd[b]	na[b]
7D7B10 Fab′ (c)[b]	3.1	nd	nd	na
1H5B7 Mab	16.7	11.7	3.6	1–2
1H5B7 Fab′ (nc)	4.1	1.8	nd	na
1H5B7 Fab′ (c)	2.4	0.6	nd	na

[a] Molecular weights of 160 000 for Mab and 50 000 for Fab′ were assumed. NAG = N-acetylglucosamine.
[b] nc = Not corrected for Fc contamination; c = corrected for Fc contamination (5% in 7D7B10 Fab′ and 10% in 1H5B7 Fab′ as judged by desitometry of SDS-PAGE); nd = none detected; na = not analyzed.

of conventionally purified PC, or PC purified using either 7D7B10 or 8861 Mab was measured by ELISA. As shown in Fig. 3, the binding curves for purified PC from all three sources were indistinguishable. Conversely, similar ELISA experiments verified that an excess of PC was able to bind 100% of a limiting amount of 7D7B10 Mab, indicating that the anti-PC Mab preparation did not contain a subpopulation of Mab molecules that lacked the ability to bind PC (data not shown).

Because hydrazide immunosorbents are formed through the coupling of oligosaccharide chains, the carbohydrate content of the anti-PC and anti-FIX Mabs as well as their Fab′ fragments were analyzed. The results are presented in Table V. N-acetylglucosamine (NAG) was the predominant reducing sugar detected in either Mab with 23 residues per 7D7B10 IgG and 17 residues per 1H5B7 IgG using hydrochloric acid hydrolysis. Similar results were obtained using TFA hydrolysis (data not shown). The galactose content was considerably higher in the 1H5B7 Mab with 12 mol/mol compared to 4 mol/mol of 7D7B10 Mab. Mannose was only detected in the hydrochloric acid hydrolysates of the 7D7B10 Mab (5 mol/mol) and not the 1H5B7 Mab. However, when TFA hydrolysis was used which is less destructive to mannose, 7 mol/mol 7D7B10 was found and 4 mol/mol 1H5B7 Mab. No fucose was detected in either Mab when hydrochloric acid or TFA hydrolyzed samples were analyzed. Both Mabs contained 1–2 residues of sialic acid. Importantly, significant amounts of NAG were also found in the purified Fab′ fragments from either Mab. Correction for the small amount of Fc contamination in the Fab′ preparations (5% Fc contamination in the 7D7B10 Fab′ and 10% Fc contamination in the 1H5B7 Fab′) yielded 3.1 and 2.4 residues of NAG per Fab′ fragment from the 7D7B10 and 1H5B7 Mabs, respectively. Little or no galactose, mannose or fucose was detected in the Fab′ preparations.

DISCUSSION

The hydrazide- and CNBr-activated matrices used in the present studies were beaded agaroses which had similar porosities and particle sizes [27–29] and were

found to have minimal nonspecific adsorptive properties under the conditions used in these experiments. Therefore, these two systems should be sufficiently similar for a direct comparison of the effects of hydrazide and CNBr immobilization chemistry upon immunosorbent performance.

The concentration, distribution, and accessibility of reactive groups (such as hydrazides) on the agarose matrix may be of importance in determining the binding efficiency of a given immunosorbent. These reactive sites should have a uniform distribution and accessibility to achieve proper spacing of Mabs on the matrix and thus avoid clustering which could result in steric hindrance and lower efficiencies. Clustering of Mab for either the hydrazide- or CNBr-activated gels was likely to have been minimal because the total reactive groups on the matrix were in approximately a 1000-fold molar excess over that needed to couple Mabs at the low densities used in these experiments. These calculations are based upon the hydrazide linker concentrations given by the manufacturers [28,29] and the minimum concentration of CNBr-activated groups from the data of March *et al.* [38]. The Mab densities used in these experiments varied from 1.0 to 2.3 mg Mab/ml of gel. While the efficiency of immunosorbents on porous supports has been shown to decrease with increasing Mab densities, these effects were found to occur at considerably higher Mab densities than used in the present experiments [3,8]. In fact, varying the Mab density over the 1–2 mg/ml range had little effect on the efficiency of the CNBr-activated anti-FIX immunosorbent used in the present studies [9,45].

Both hydrazide- and CNBr-activated gels immobilize proteins under mild conditions which do not irreversibly denature or are non-denaturing to the antibodies evaluated in this study. While the hydrazide coupling was performed at a lower pH than the CNBr coupling procedure (pH 5.0–5.5 *vs.* pH 8.5), immobilization through the carbohydrate should preserve the conformational flexibility of the peptide backbone of the molecule thus allowing it to resume its normal conformation under antigen binding conditions at neutral pH. The Mabs used to form the hydrazide immunosorbents were oxidized in a 64- to 494-fold molar excess of $NaIO_4$ for 1 h at room temperature. Although these mild conditions are generally insufficient to oxidize amino acids of proteins [22], it seemed possible that the efficiencies of the hydrazide immunosorbents may have been adversely affected by loss of activity of the Mab as a result of $NaIO_4$ treatment. However, this was not the case as seen for the anti-PC Mab, where the oxidized and native Mab had indistinguishable PC binding curves.

The anti-FIX/FIX and the anti-PC/PC systems behaved similarly in these studies in that essentially quantitative recoveries of bound protein were obtained using the CNBr-coupled immunosorbents. For both the anti-FIX/FIX and anti-PC/PC CNBr-coupled immunosorbents, 75% of bound protein was recovered under specific mild elution conditions (millimolar concentrations of EDTA or $CaCl_2$, respectively). These mild conditions are thought to destabilize a conformation which is recognized by the Mab. In both cases, small amounts of additional protein were eluted in the 2 *M* sodium chloride was pool, such that 85% of the bound protein could be accounted for. For both systems, 52–61% of bound protein was recovered from the hydrazide-coupled immunosorbents under mild elution conditions. For the anti-FIX immunosorbent, additional FIX was recovered with the high salt wash such that 77% of the bound protein could be accounted for. In contrast, for the anti-PC hydrazide immunosorbent, no additional protein was recoverd with 2 *M* sodium chloride of 2 *M*

NaSCN. Because nonspecific protein adsorption was not observed with the glutaraldehyde-blocked hydrazide matrix, it is likely that the lower recovery of PC can be attributed to the presence of covalently attached Mab. The possibility that this was due to a population of immobilized antibodies with higher avidity seems unlikely because further treatment with harsh elution conditions (2 *M* NaSCN) did not result in a desorption of PC not previously recovered by $CaCl_2$ elution. The reason for the lower recoveries of PC from the hydrazide gels is unknown.

Hydrazide-activated gels couple Abs through their carbohydrate residues. This offers several *potential* advantages. First, Ab coupling occurs through residues that are not involved in antigen-binding, thus providing a peptide-sparing effect. In contrast, CNBr-activated gels are linked to the matrix through reactive amino acids. As many such groups are present on a macromolecule like an Ab, linkage can occur through many sites on the protein including peptide regions that may be vital to the Ab-antigen interaction, thus compromising the acitivity of the bound Ab. Second, the carbohydrate groups on the Ab have the potential advantage of being able to act as a linker to space the molecule away from the matrix and thus improve its accessibility to the antigen. Conversely, the Abs of CNBr-activated gels can be expected to be located closer to the surface of the matrix giving rise to steric hindrance effects [10]. Third, linkage through the carbohydrate moieties is expected to give a higher degree of Ab orientation compared to coupling through amino acids, as the coupling is thought to involve only the Fc region of the molecule [21–25].Therefore, the hydrazide immunosorbents were expected to have higher bound Ab activities and efficiencies than CNBr-coupled immunosorbents.

Despite the *potential* advantages of site-directed coupling via carbohydrate residues, both the anti-PC and anti-FIX hydrazide immunosorbents failed to perform better than conventional CNBr-activated gels. In the case of the anti-FIX hydrazide immunosorbent, the efficiency was approximately one-half that of the comparable CNBr-coupled gel whether based upon bound FIX or specifically recovered FIX. In the case of the anti-PC immunosorbents, 2 out of 3 hydrazide gels had a 1.6-fold increase in PC binding efficiency compared to the CNBr-coupled gel. However, this increase was not translated into improved performance, because the efficiencies based upon specifically recovered PC were only 1.2-fold higher. It is interesting to note that 3- to 5-fold increases in antigen binding efficiency have been reported for hydrazide immobilized polyclonal antibodies relative to CNBr immunosorbents [22–25], while no increase [23,24] or at most a 2-fold increase [25] was found for hydrazide immobilized monoclonal antibodies. In most cases, the amount of bound antigen that could be eluted from the hydrazide immunosorbents was not reported in these studies.

Our results indicate that monoclonal antibodies immobilized to agarose matrices by site-directed coupling via carbohydrate residues on the Mab still have a restricted ability to bind to antigen. It is apparent that the presentation of carbohydrate is such that it does not result in significant changes in binding capacity relative to that seen with CNBr-coupled Mabs. Apparently, coupling through the well-conserved carbohydrate chains located in the Fc region [14–20] does not benefit antigen binding, albeit that these carbohydrate moieties could potentially impart several nm greater extension than the 1 nm spacer arms used to increase the bound activity of β-galactosidase on a similar agarose matrix [10]. This may indicate that despite this site-directed coupling, there is still a considerable degree of variation in orientation of Ab in

terms of the spatial relationship, for example, between the Fab antigen-binding region and the matrix.

The presence of oligosaccharide in the CH_2 domain of the Fc fragment of immunoglobulins has been in part a reason for the development of carbohydrate-directed immobilization chemistry. However, N-linked glycosylation is dictated by the peptide sequence Asn–X–Ser which has also been shown to occur in the variable regions of the antigen-binding domain of rabbit, murine and human immunoglobulins [46–48]. For this reason, we analyzed the carbohydrate composition of the Mabs used in the present study, as well as the purified Fab′ fragments derived from them. The sugar composition of both Mabs was consistent with the dibranched complex structure of N-linked oligosaccharides of murine IgG reported by others [19]. Importantly, both Mabs employed in this study had significant amounts of NAG in the Fab′ fragment. Thus, some coupling via the Fab antigen-binding domain may have occurred which may have abrogated the potential advantage of site-directed coupling through the carbohydrate in the Fc region. The frequency of carbohydrate in the Fab region of monoclonal antibodies and the ability to control glycosylation at these sites, for example, by cell culture growth conditions [49] are additional factors which may impact the utility of carbohydrate-directed immobilization of antibodies.

CONCLUSION

There was no advantage in terms of improved efficiency in the use hydrazide-activated immunosorbents over the more conventional CNBr-activated immunosorbents. Furthermore, the heavier protein losses observed with the hydrazide gels were an added disadvantage.

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