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COMPARISON OF MONO Q, SUPEROSE-6, AND ABx FAST PROTEIN LIQUID CHROMATOGRAPHY FOR THE PURIFICATION OF IgM MONOCLONAL ANTIBODIES

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SUMMARY

Nine immunoglobulin M (IgM) monoclonal antibodies (MAbs) produced in ascites fluids or in cell culture supernatants, have been purified on a fast protein liquid chromatography (FPLC) system using anion-exchange, size-exclusion, or mixed-mode chromatography matrices. The use of a mixed-mode ABx column provided an IgM that had a purity of greater than 99% after a single purification step. Anion-exchange chromatography using a Mono Q column, provided a partial purification of the IgM which could subsequently be purified to a product of *ca.* 90% purity (determined from sodium dodecyl sulfate polyacrylamide gel electrophoresis) by size-exclusion chromatography on a Superose-6 column. Alternatively, the ascites containing the IgM was ammonium sulfate precipitated and chromatographed on the Superose-6 column under normal- as well as high-ionic strength conditions, which also yielded a product of *ca.* 90% purity. The purification of IgM from concentrated cell culture supernatants was evaluated using the Superose-6 or the ABx column. IgM purified from this source was greater than 99% pure when chromatographed on the mixed-mode column and *ca.* 60% pure on the size-exclusion column. MAbs from each of the procedures retained their immunoreactivity, as shown by indirect immunofluorescence staining of fixed cell preparations. A comparison of these methods revealed that mixed mode chromatography was simple, efficient, and yielded a product of high purity. The optimization of these methods facilitates the large-scale purification of mouse IgM MAbs and provides practical procedures for generating IgMs for use as diagnostic and therapeutic reagents.

INTRODUCTION

Immunoglobulin M (IgM) is a common immunoglobulin, secreted by hybridoma cell lines, which can be prepared from cell culture supernatants or mouse ascites fluids. Although the applications of IgM monoclonal antibodies (MAbs) have been restricted due to the large size of the molecule, enzymatic cleavage to smaller Fab and $F(ab')_2$ fragments[1,2] can improve the efficacy of IgM. The use of IgM $F(ab')_2$ fragments in tumor visualization has been documented by Maillet *et al.*[3] and also in

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our laboratory[4]. In addition, these low-molecular-weight molecules are shown to be more efficient in immunocytochemistry by localizing antigens that could not be detected with an intact IgM[5]. To exploit the applications of IgM MAbs, especially for radioimmunoimaging and immunotherapy, rapid and efficient purification methods are required to obtain large quantities of pure and reactive immunoglobulins.

IgM has traditionally been purified by size-exclusion chromatography[6] by taking advantage of its high molecular weight relative to other protein components found in serum or ascites fluid. These purification methods, however, are time consuming, inefficient, and only modestly effective[7,8]. With advancements in liquid chromatographic techniques and the availability of new chromatographic matrices, IgM may now be purified more efficiently. These procedures include anion- and cation-exchange chromatography[9–14], mixed-mode chromatography[13,15], size-exclusion chromatography[16], and combinations of several techniques[17,18]. Additionally, methods based on polyethylene glycol (PEG) precipitation and affinity chromatography for the purification of monoclonal IgMs from mouse ascites fluids and hybridoma supernatants have recently been described[19–21]. Despite these advancements in the purification of IgM, none of the methods are simple one step procedures that yield a high recovery and purity of IgM. In addition, many methods neglect the importance of recovering an IgM that has retained its immunoreactivity. For instance, the strong elution conditions involved with the affinity method, using a low pH eluent, should be avoided to prevent the denaturation of the immunoglobulin.

Recently, a mixed-mode chromatographic matrix, ABx (antibody exchanger) has been shown to purify many IgG MAbs and some polyclonal IgMs in a single step from ascites fluids[15]. Its usefulness, however, in the purification of IgM MAbs has not been adequately defined. We have selectively examined fast protein liquid chromatography (FPLC) methods for the purification of nine IgM MAbs from ascites fluids and cell culture supernatants using a mixed-mode matrix (ABx), an anion exchanger (Mono Q), and a size-exclusion chromatographic column (Superose-6). In this report, a comparison of the chromatographic efficiency for each of the methods is presented and logical purification schemes for IgM MAbs are described.

EXPERIMENTAL

Materials and instruments

All chemicals used in this study were of reagent grade. Buffers and IgM-containing samples were filtered through a 0.22-μm Millex-GV filter unit (Millipore, Bedford, MA, U.S.A.), or Nalgene disposable filterware (Nalge, Rochester, NY, U.S.A.) before injection into columns. Ultrafiltration membrane cones, Centriflo, were obtained from Amicon (Danvers, MA, U.S.A.). For the concentration of cell culture supernatants, 300 000 and 100 000 molecular-weight cut-off (MWCO) tangential flow membranes were used in a Minitan concentrator (Millipore). The columns used include the Mono Q HR 5/5 (50 × 5 mm), Superose-6 HR 10/30 (300 × 10 mm) (Pharmacia, Uppsala, Sweden), and ABx (5 μm) (100 × 7.75 mm) (J. T. Baker, Phillipsburg, NJ, U.S.A.). Chromatography was performed on the Pharmacia FPLC system, equipped with two P-500 dual piston pumps, a V-7 injection valve, solvent mixer, prefilter, UV-1 monitor with an HR 10 flow cell, FRAC-100 fraction collector, and REC-482 dual channel recorder.

Monoclonal antibodies

Hybridomas producing IgM monoclonal antibodies were developed in our laboratory, as described previously[22], and were grown as cell culture supernatants or ascites fluids.

Ascites. Six hybridomas, secreting antinuclear MAbs 244-7, 898-9, 877-8, 899-4, 891-5 and 780-3, were grown in six week old Balb/c mice (Simonsen Labs., Gilroy, CA, U.S.A.) that had been pristane primed for 7–10 days prior to intraperitoneal injection of $5 \cdot 10^6$ hybridoma cells, as described[23]. Ascites growth was monitored and harvested by aspiration 10–18 days later. Cells and debris were removed by centrifugation at 2000 *g* for 10 min at 4°C, and the ascites was then filtered through a 0.45-μm Nalgene filter unit and frozen at −70°C.

Supernatants. Cell culture supernatant of two IgM MAbs (LN-1 and LN-6) were prepared by growing the cells in RPMI-1640 medium (Gibco, Grand Island, NY, U.S.A.) supplemented with 1% fetal calf serum (Hyclone, Logan, UT, U.S.A.), Nutriclone-M (40 μg of protein per ml) (Techniclone, Santa Ana, CA, U.S.A.), 1% L-glutamine, and 1% antibiotics. After three or four days of growth, the supernatants were harvested by centrifugation for 10 min at 1000 *g*. Supernatants were continuously pooled and stored at −20°C. For this study, a maximum of 2.5 l was used at any one time for the purification of the MAb. Monoclonal antibody 7D4, a rat/mouse hybridoma developed by Malek *et al.*[24], was prepared by growing the cells in Dulbecco's Minimum Essential Media supplemented (Gibco) with 1% fetal calf serum (Gibco), Nutriclone-M, and 1% L-glutamine. The supernatant was collected as described above.

FPLC evaluation

Anion exchange. For analytical evaluation, 100 μl of ascites were injected into the Mono Q column, and eluted with a 20 m*M* Tris buffer and a sodium chloride gradient, as described in Fig. 1. Fractions were collected and their IgM reactivity was assayed by indirect immunofluorescence (IF). In the rechromatographic procedure, a 2-ml sample was injected into the Mono Q column and eluted by the same buffers and gradient. Fractions positive for IgM were then concentrated by ultrafiltration using 50 000-MWCO membrane cones, and subjected to rechromatography on Superose-6.

Size exclusion. Ascites fluid (50 ml) was added to an equal volume of a saturated ammonium sulfate solution and immediately clarified by centrifugation at 12 000 *g*. The supernatant was decanted, and the precipitate was dissolved in 5 ml of deionized water (dH_2O) and filtered through a 0.22-μm Millex-GV filter unit. The Superose-6 column was preequilibrated with normal ionic strength (0.1 *M*) phosphate buffered saline (PBS) or with low ionic strength buffer (1:20 dilution of PBS). A maximum of 500 μl of sample was loaded onto the column and eluted with either 0.1 *M* PBS or 5 m*M* phosphate buffer containing 1.7 *M* sodium chloride. Peak fractions were collected and tested for IgM activity by IF. These same elution procedures were used for the rechromatography of IgM-containing fractions collected from Mono Q, as described above.

Mixed mode. Crude ascites (500 μl), or Superose-6 fractions to be rechromatographed, were injected into the ABx column with a 10 m*M* 2-[N-morpholino]-ethanesulfonic acid (MES) binding buffer, pH 5.6, and the IgM was eluted with a

gradient of 1 *M* sodium acetate (pH 7.0) buffer, as described in Fig. 3. Peak fractions were collected and examined for IgM activity by IF.

IgM purification from cell culture supernatants

Concentration of supernatant. Supernatant was filtered through a 0.22-μm Nalgene filter unit and concentrated via the Minitan tangential flow concentrator. The bulk of the supernatant was first concentrated through a 300 000-MWCO membrane to eliminate lower-molecular-weight contaminants. The 300 000-MWCO concentrate (30–50 ml) was diluted in 1 l of the initial buffer used in the purification system chosen and concentrated through the Minitan 100 000-MWCO membrane to a final volume of 50 ml. This step functioned to dialyze the sample into the necessary buffer while further purifying the sample.

Chromatography. Purification of 500 μl of MAb LN-6 concentrated supernatant was carried out on the Superose-6 column under normal ionic strength conditions, as described. A 50-ml volume of MAb LN-1 and 7D4 concentrated supernatant was purified by binding the antibody to the ABx matrix with a 10 m*M* MES (pH 5.6) buffer and eluted by a gradient of 1 *M* sodium acetate (pH 7.0), as shown in Fig. 5.

Indirect IF and sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

IgM activity was assayed in collected fractions, using indirect IF described by Epstein[25]. Briefly, Raji cells were fixed onto Teflon-coated microscope slides containing ten 5-mm wells per slide (Cel-Line Assoc., Newfield, NJ, U.S.A.) using 2% paraformaldehyde in PBS. After fixation the slides were placed in acetone at −20°C to permabilize the cells. Rinsed slides were stored at 4°C in PBS with 0.02% sodium azide. IgM activity was assayed by incubating the collected fractions on the fixed cells followed by a secondary incubation with a fluorescein-conjugated goat anti-mouse IgM specific antibody (Cappel Labs., Malvern, PA, U.S.A.). Slides were examined by epifluorescence microscopy with a Leitz-Orthoplan microscope with Ploemopak 2.1 fluorescence illuminator. IgM positive fractions were analyzed for purity by running both 5 and 7.5% polyacrylamide gels in the presence of sodium dodecyl sulfate under non-reduced conditions as described by Laemmli[26].

RESULTS

When mouse ascites containing MAb 780-3 was separated on a Mono Q column, the IgM peak was eluted with 0.34 *M* sodium chloride (Fig. 1). Similar chromatograms were produced when ascites of MAbs 899-4 and 877-8 were eluted under identical conditions. The IgM via this procedure was estimated to be *ca.* 50% pure by Superose-12 chromatography and SDS-PAGE analysis (data not shown). All percent purities were estimated from both 7.5% and 5% polyacrylamide gels. The 7.5% gels revealed the lower-molecular-weight contaminants while the 5% gels allowed the IgM to migrate from the origin, demonstrating its purification from larger molecules. These same analyses revealed that the major contaminant from the Mono Q purification was albumin. The IgM-rich fractions from the Mono Q column were concentrated by ultrafiltration and then injected into the Superose-6 column for further

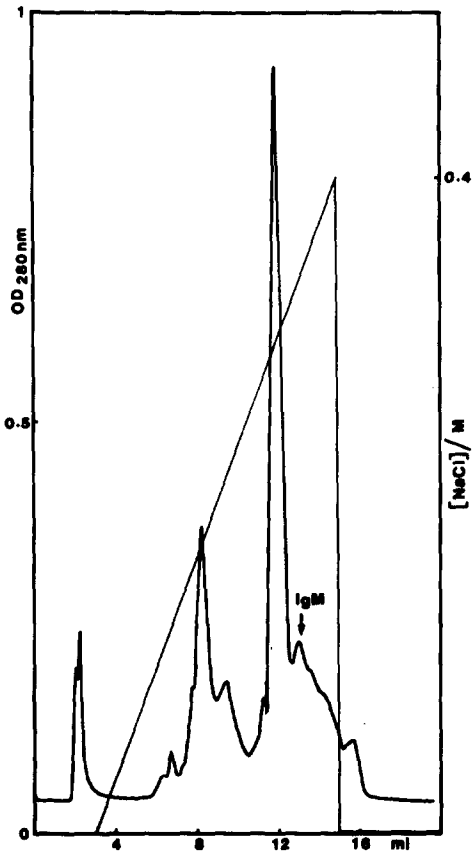

Fig. 1. Elution of IgM MAb 780-3 from a Mono Q HR 5/5 column; flow-rate, 1.5 ml/min; temperature, 25°C; buffer A, 20 m*M* Tris (pH 8.0); buffer B, 20 m*M* Tris + 1 *M* sodium chloride (pH 8.0); linear gradient, 0 to 40% B from 3 to 15 ml, 100 μl ascites fluid; 0.2 a.u.f.s.

purification. The final product from this tandem procedure was found to have retained its immunoreactivity (IF) and to be more than 95% pure (Fig. 5, lane A2).

Ascites containing IgM that was to be purified on Superose-6 was first precipitated with ammonium sulfate and the resulting pellet was resuspended in dH_2O. Indirect IF studies revealed that IgM was completely precipitated by this procedure. A maximum of 500-μl was injected into the Superose-6 column and eluted with either a normal- or high-ionic strength phosphate buffer (Fig. 2). In the first method, the sample was eluted using the normal-ionic strength buffer (0.1 *M* PBS, pH 8.0), with the IgM eluting as the first peak and α-2-macroglobulin (α-2M) as the major contaminant (Fig. 5, lane A1). By sacrificing a portion of the IgM present, it was possible to obtain a relatively pure product of *ca.* 90% purity. Further analysis by SDS-PAGE and IF showed that IgM was present throughout a broad range in the lower-molecular-weight fractions (Fig. 2a). To enhance the recovery of IgM, the size-exclusion chromatography was performed by a modified technique previously described by Bouvet *et al.*[16]. In this procedure, the Superose-6 column is preequilibrated with 5

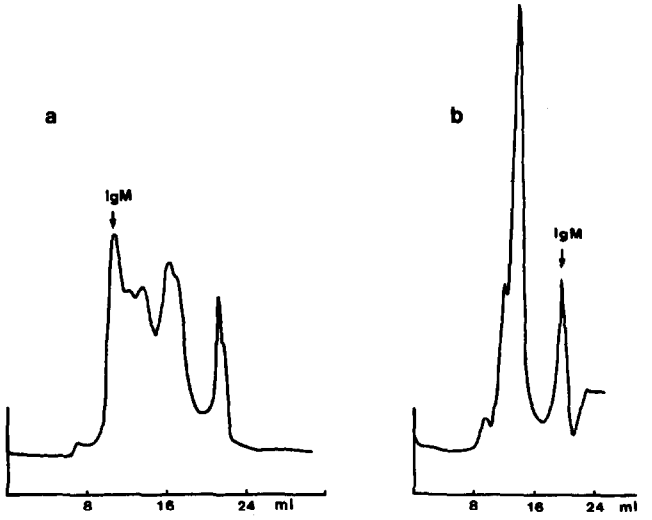

Fig. 2. Comparison of chromatograms for IgM MAb 244-7 obtained with a Superose-6 HR 10/30 column. Volume injected, 500 μl; 2.0 a.u.f.s. (a) Column equilibrated and eluted with 0.1 *M* phosphate buffer (pH 8.0). (b) Column equilibrated with 5 m*M* phosphate buffer (pH 8.0) and eluted with 5 m*M* phosphate buffer (pH 8.0), containing 1.7 *M* sodium chloride.

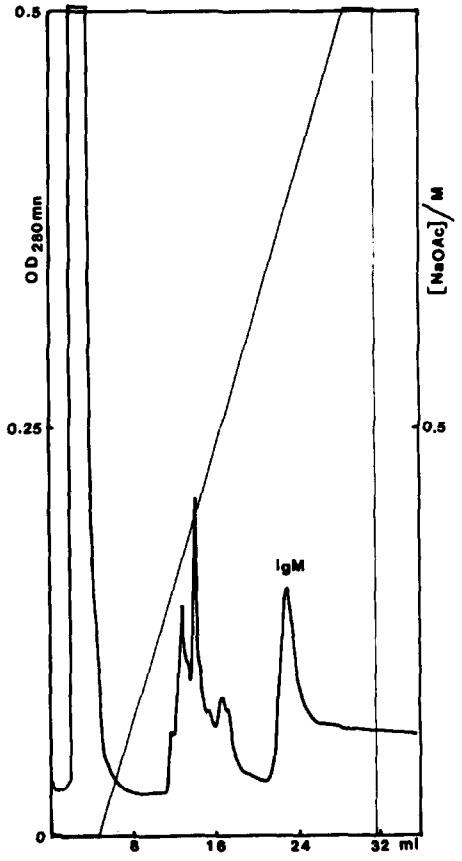

Fig. 3. Elution of IgM MAb 877-8 from a 100 × 7.75 mm ABx column. Flow-rate, 0.8 ml/min; temperature, 25°C; buffer A, 10 m*M* MES (pH 5.6); buffer B, 1 *M* sodium acetate (NaOAc) (pH 7.0); linear gradient, 0 to 100% B from 5 to 30 ml, 100% B from 30 to 32 ml; 500 μl ascites fluid; 0.5 a.u.f.s.

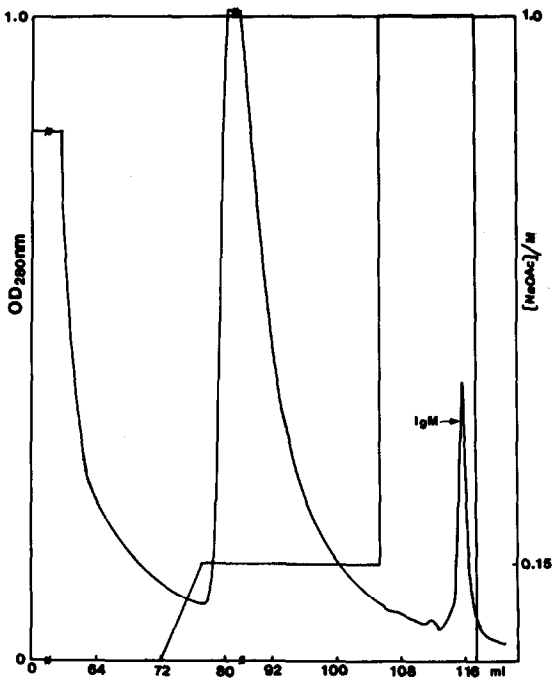

Fig. 4. Elution of IgM MAb LN-1 from a 100 × 7.75 mm ABx column. Flow-rate, 1.0 ml/min; temperature, 25°C; buffer A, 10 m*M* MES (pH 5.6); buffer B, 1 *M* sodium acetate (NaOAc) (pH 7.0); linear gradient, 0 to 15% B from 72 to 77 ml, 15% B from 77 to 105 ml, 100% B from 105 to 118 ml; 50 ml ascites fluid; 1.0 a.u.f.s.

m*M* phosphate buffer and the sample was eluted with 5 m*M* phosphate buffer containing 1.7 *M* sodium chloride (Fig. 2b). Under these conditions the IgM was found to elute as the last peak, resulting in a product that had retained its immunoreactivity (IF) and was *ca.* 95% pure, with small peptides or other low-molecular-weight molecules as contaminants (Fig. 5, lane A3). Furthermore, only a trace amount of IgM activity was detected in the early fractions, demonstrating a higher recovery yield under these conditions. In Fig. 2b, the IgM was purified in a tandem procedure on the Superose-6. First, the IgM was eluted using the high salt method, and then the IgM fraction was injected and eluted under normal-ionic strength conditions. Combining procedures alleviates the problems encountered with the individual methods by increasing the recovery (high-salt conditions) and eliminating the low-molecular-weight contaminants (low-salt conditions).

The highest degree of purity in a single step purification with ascites was achieved when it was chromatographed on the ABx column. Fig. 3 shows the profile for MAb 877-8 ascites, which is representative of the other IgM antinuclear antibodies that were purified on the ABx. Profiles indicated that a large percentage of the contaminating proteins passed through the column under the initial buffer conditions. Although α-2M was the major contaminant bound to the matrix, ascites concentrations of this protein were minimal and its affinity was low enough to allow

good separation from the stronger-binding IgM. The purified IgM retained its activity, and SDS-PAGE indicated that the product approached 100% purity.

Because cell culture supernatant is the predominant raw material for large-scale antibody production, we extended our studies to include the purification of IgM from this source. Using the Minitan apparatus, MAbs were concentrated up to 100-fold from cell culture supernatants. Analysis by SDS-PAGE (not shown) of the concentrate revealed that IgM and α-2M constitute *ca.* 80% of the total protein, with α-2M being the major component. Of the three MAb supernatants studied (LN-1, LN-6 and 7D4), MAb LN-6 was found to pass through the column with the other major contaminants under the initial buffer conditions described. Consequently, this antibody was purified on Superose-6 under normal ionic strength conditions, producing an IgM product that contained significant amounts of α-2M contamination (Fig. 5, lane B). By contrast, MAbs LN-1 and 7D4 bound to the ABx column under the initial buffer conditions. The α-2M contaminant was washed from the column using 0.15 *M* sodium acetate and the IgM was then eluted with 1.0 *M* sodium acetate (Fig. 4). When purified by this method, IgMs retained their activity, and SDS-PAGE indicated that the purity was *ca.* 100% (Fig. 5, lane C).

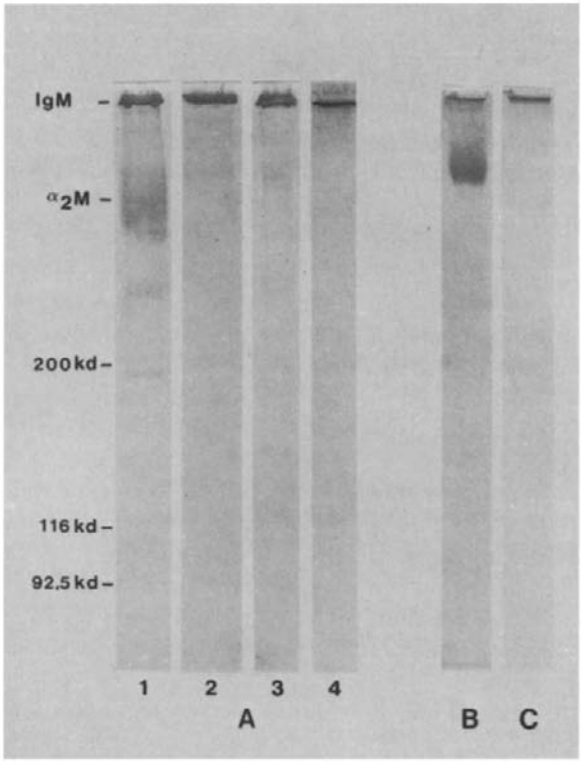

Fig. 5. SDS-PAGE (7.5%) of IgM fractions from different purification procedures. Lanes A1-A4: IgM MAb 244-7, isolated from ascites by (1) ammonium sulfate precipitation and Superose-6 chromatography with normal-ionic strength buffer (Fig. 2A), (2) Mono Q and Superose-6 chromatography with normal-ionic strength buffer, (3) ammonium sulfate precipitation and Superose-6 chromatography with high-salt buffer (Fig. 2B), (4) ABx chromatography (Fig. 3). Lane B: IgM MAb LN-6 isolated from concentrated cell supernatant by Superose-6 chromatography with normal-ionic strength buffer. Lane C: IgM MAb LN-1 isolated from concentrated cell supernatant by ABx chromatography (Fig. 5).

DISCUSSION

This study was performed to identify an effective method of IgM purification using different chromatographic matrices and FPLC techniques. Because of the large molecular weight of IgM (*ca.* 900 000), size-exclusion chromatography was examined for comparative purposes. Affinity chromatography and hydrophobic interaction chromatography were not studied because these methods require harsh elution conditions, such as organic solvents and highly acidic buffers, which may denature the antibodies. In addition to the parameters associated with affinity and size-exclusion chromatography (antigenic binding site, size and shape), separation parameters used in the other IgM purification procedures[6–21] include solubility, charge, hydrophobicity, and isoelectric point. Because of the complexity of the ascites fluid, it is presently not possible to purify IgM from this source using methods based merely on a single separation parameter. Methods using multiple steps on separate chromatographic matrices or the use of mixed-bed separation are therefore required for a successful purification of IgM.

The results obtained in this study show that ammonium sulfate precipitation is a suitable initial step when size-exclusion chromatography is used for IgM purification from ascites fluid. IgM is completely recovered by precipitation, and more importantly, it serves as a concentration step so that the subsequent chromatographic procedures can be performed with greater efficiency. Purity of the separated IgM by Superose-6 methods was dependent upon whether the normal- or high-ionic-strength elution technique was used (see Results). As expected, size exclusion chromatography on the Superose-6 was only modestly effective because of long run times, small sample loads, poor resolution of proteins, and low recovery of IgM. Despite these inherent characteristics, size-exclusion chromatography is useful if the purity of the IgM is not a critical factor, or if there are no other purification methods available (*i.e.* MAb LN-6 in this study). Using the Superose-6 in tandem, by rechromatographing IgM contained in high salt fractions (Fig. 6b), it is possible to obtain an IgM of high purity, that is free from α-2M and low-molecular-weight peptide contamination.

Purification methods using the Mono Q column (a strong anion-exchanger) separates proteins based on their net charge and charge distribution on the surface. We have shown that IgM MAbs were isolated from ascites fluids to *ca.* 50% purity, with albumin as the major contaminant, when Mono Q was used in a single step under the described chromatographic conditions. A greater than 95% purity of IgM MAb could be achieved when the IgM-rich fractions from the Mono Q column were additionally purified by the Superose-6 column. Similar results were obtained by Clezardin *et al.*[17]. Further studies have demonstrated that this method had several drawbacks for the purification of IgM. When using cell culture supernatants, the pH indicator dyes in the media were found to bind strongly to the Mono Q column, which complicated column regeneration methods. Furthermore, the binding capacity of the matrix and the resolution of the antibody fraction were significantly reduced in large scale methods (*i.e.* more than 10 ml of sample injected into a HR 5/5 Mono Q column) using ascites (data not shown).

In this study, single step purification of IgM from ascites was achieved by ABx mixed-mode chromatography. Of the nine IgM MAbs studied to date, only one MAb (LN-6), from cell culture supernatant, did not bind to the ABx matrix under the

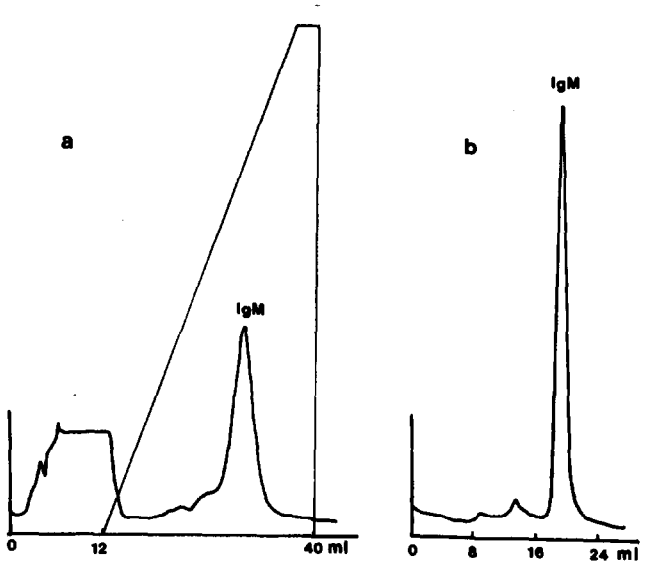

Fig. 6. Rechromatography of 244-7 IgM fractions contaminated with serum proteins (see Fig. 2a, fractions 9–11 ml). (a) Sample (10 ml) was injected into the ABx column via 10-ml Superloop. (b) Sample (500 μl) was injected into a Superose-6 column and eluted with a high-salt buffer.

initial conditions used to purify the other eight MAbs. Using identical binding conditions, the other eight MAbs bound strongly to the mixed-mode sorbent and allowed clean separation of the IgM from the protein contaminants. The final IgM product was free from contaminating proteins and had retained its immunoreactivity. In comparison with the size-exclusion and anion-exchange chromatography described above, the mixed-mode separation is a fast, simple, and efficient method for the purification of IgM MAbs.

Although mixed chromatographic beds containing hydrophobic interaction and ion-exchange media have been successfully used for the purification of a variety of proteins[13,27–29], its use for IgM MAb purification has not been reported. Previously published information describes ABx as a mixed-mode chromatographic matrix which utilizes weakly anionic, cationic, and hydrophobic interactions on a silica gel support[15]. This chromatographic matrix was reported to purify numerous IgG MAbs and several polyclonal IgMs from ascites fluids and tissue culture supernatants[13], and was able to resolve host from MAbs[13], as well as different immunoglobulin components[30]. Although a clear and quantitatively valid explanation is not available for the mixed-mode chromatographic behavior, Hearn[7] and Regnier[31] have recently described the 'multi-site interaction' (or 'multiple groups interaction') hypothesis, which states that the summation of several weak forces such as hydrogen bonding, electrostatic attraction, and hydrophobic interactions, may become a very strong interaction between the proteins and the sorbents because of the involvement of multiple groups. With this concept in mind, and the fact that the ABx was developed to exhibit little or no affinity for albumins and transferrins, the separation of immunoglobulins with high efficiency from ascites on the ABx matrix becomes more practical and permissible because the majority of non-immunoglobulin proteins have

been eliminated. To further understand the nature of the contact region of IgM to the ABx matrix, affinities of several IgG and IgM MAbs, and their $F(ab')_2$ fragments, were examined in the ABx column (unpublished data). It was found that $F(ab')_2$ fragments bound as strongly to the ABx column as their intact parent immunoglobulins. These data suggest that the Fc regions of the immunoglobulins may not be involved in the protein-sorbent interactions. A second conclusion that may be drawn from these observations is that, in molecules with any degree of three-dimensional structure, only a portion of the functional groups are acting simultaneously to control chromatographic behavior.

IgM MAb can be isolated from ascites fluids and culture supernatants by multistep chromatographic methods using anion-exchange and/or size-exclusion chromatography, or by a single step method using mixed-mode chromatography. The latter method has been shown to be simple and efficient, and the technique of choice for most of our studies. The purification results described in this study should facilitate the use of IgM monoclonal antibodies for basic research and in clinical applications.

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