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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF AMINO ACIDS, PEPTIDES AND PROTEINS

XCIII[a]. COMPARISON OF METHODS FOR THE PURIFICATION OF MOUSE MONOCLONAL IMMUNOGLOBULIN M AUTOANTIBODIES

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SUMMARY

A comparison of methods for the purification of naturally occurring mouse monoclonal autoantibodies, of the immunoglobulin M (IgM) isotype, has been performed to determine the optimal strategies for the isolation of IgM from ascites fluid and *in vitro* tissue culture hybridoma supernatants. In order to quantify each purification procedure, the concentration of IgM in eluted fractions was determined by using a double-sandwich μ-chain-specific anti-IgM enzyme-linked immunosorbent assay, and the purity of the IgM was determined by a bicinchoninic acid-based protein assay and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The most efficient single-step purification was based on size-exclusion chromatography on high-resolution Superose 6 HR 10/30 fast protein liquid chromatography (FPLC) columns. This procedure resulted in recoveries of monoclonal IgMs of *ca.* 71–86% with purities between 68 and 86%. Single-step chromatography of monoclonal IgM, on Superose 6 FPLC columns resulted in a 21-fold purification of IgM, prepared by the *in vitro* culture of hybridoma cells in dialysis membrane. Size-exclusion chromatography, performed with Sephacryl S-300 columns, resulted in reduced resolution of monoclonal IgM, with yields of *ca.* 57–80% and purity of *ca.* 42–58% compared with the high-resolution Superose 6 FPLC columns. "Non-ideal" size-exclusion chromatography on Superose 6 FPLC columns resulted in selective retention of monoclonal IgMs and elution of IgM with high-ionic-strength buffers in the trailing peak. Recovery of IgM with this strategy was high (*ca.* 82–92%) but the purity was not comparable to the single-step fractionation of IgM on Superose 6 FPLC columns. Single-step anion- and cation-exchange and mixed-mode hydroxyapatite chromatography resulted in only partial purification of monoclonal IgM with the applied procedures. With these latter separation techniques, monoclonal IgM was eluted with a variety of other ascites fluid or supernatant proteins, including those with apparent molecular weights identical to those of mouse IgG and albumin. Sequential purification of monoclonal IgMs by Mono Q anion exchange, followed by Superose 6 FPLC columns, resulted in a 2- to 3-fold purification of IgM but did not

[a] For Part XCII, see ref. 36.

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separate IgM from high-molecular-weight contaminants with apparent molecular weights similar to those of α_2-macroglobulin and IgG.

Enrichment of monoclonal IgM from ascites fluid by ammonium sulphate precipitation revealed increasing IgM recovery with increasing ammonium sulphate final concentrations up to 60%. Isolation of IgM, based on euglobulin properties, following dialysis in either 2% (w/v) boric acid (pH 6.0), 5 m*M* Tris–HCl (pH 7.8), or distilled water, resulted in low IgM yields ($\leqslant$ 10%) and purity (*ca.* 10–20%). Precipitation of monoclonal IgM from ascites fluid with polyethylene glycol 6000 (PEG 6000) resulted in *ca.* 63% recovery and purification of 4- to 5-fold, indicating that this procedure may be advantageous for enrichment or concentration of IgM. Antibody reactivity with intracellular and cell surface murine autoantigens, as determined by indirect immunofluorescence, was maintained following all purification procedures. The results indicate that optimal purification of monoclonal IgMs, on the laboratory scale, was performed in a single step by size-exclusion chromatography on Superose 6 HR 10/30 FPLC columns. This procedure will allow the isolation of naturally occurring mouse monoclonal IgM autoantibodies for the characterization of their autoreactive specificities.

INTRODUCTION

Fusion of B lymphocytes from the spleens of healthy mice with myeloma cells has revealed that a high proportion of the resulting hybridomas produce autoantibodies[1–3]. These autoantibodies, which react with a variety of intracellular, extracellular and cell surface autoantigens, demonstrate highly polyspecific properties and are almost exclusively of the Immunoglobulin M (IgM) class[4]. In order to understand the significance of these autoantibodies in apparently healthy animals and their relationship to immunological self tolerance and autoimmunity, characterization of the autoantigenic specificities of these autoantibodies is required. Such analyses are most easily undertaken by using purified forms of the monoclonal autoantibodies.

Many methods for the purification of monoclonal IgM from ascites fluid and tissue culture supernatant have been described[5]. Purification of IgM, based on its molecular weight (900 000 daltons) has been successfully undertaken with size-exclusion chromatography (SEC)[6–8]. Variations in purity and yield depend on the chromatographic matrices used for size-based separations. In the purification of IgMs advantage can also be taken of "non-ideal" SEC and the propensity of IgM to precipitate in low-ionic-strength buffers[9]. In these latter procedures, IgM is eluted from the size-exclusion matrix as the last peak, being selectively detained and eluted with high-ionic strength buffers.

Alternatively, IgM has been purified by anion- and cation-exchange chromatography[8,10,11], mixed mode chromatography[8,12] and combinations of these chromatographic techniques[8,11,13,14]. Affinity chromatography has also been used to purify IgM from sera, ascites fluids, or tissue culture supernatants. Using anti-IgM antibodies or specific antigen coupled to a solid phase, IgM has been highly purified[15–17]. Affinity chromatography based on protein A[18,19] or concanavalin A[20] has not resulted in isolation of IgM in a highly purified form. Isolation of IgM from hybridoma ascites fluid and supernatant has also been undertaken by utilizing the euglobulin

properties of IgM[21], polyethylene glycol (PEG)[22,23] and ammonium sulphate precipitation[24].

Few of the procedures described above have been quantitatively analysed in relation to the yield and purity of the recovered IgM, often relying on estimates obtained by scanning of Coomassie Blue-stained sodium dodecyl sulphate-polyacrylamide gels. In order to identify the optimal procedures for isolation of naturally occurring autoreactive IgM monoclonal antibodies in the functional state, a quantitative comparison of purification methods was undertaken. Here we describe the results of purification of monoclonal IgM autoantibodies derived from three hybridoma lines by means of ammonium sulphate precipitation, low-ionic-strength precipitation, and PEG enrichment procedures. Separation of monoclonal IgMs, based on size-exclusion, anion- and cation-exchange and hydroxyapatite chromatography, and combinations of anion-exchange and size-exclusion chromatography have also been examined.

MATERIALS AND METHODS

Materials

Chemicals used in this study were of the analytical reagent grade. All buffers were prepared with distilled deionized water (Milli Q purified, Millipore, Bedford, MA, U.S.A.). Buffers were filtered through 0.22 μm filters (Millipore).

All chromatographic procedures were performed at room temperature. Chromatography in open columns was performed by using an ISCO UA-5 absorbance monitor with an ISCO Type 6 optical unit reading at 280 nm, coupled to an ISCO 328 fraction collector (ISCO, Lincoln, NB, U.S.A.). Constant flow-rate was maintained by means of a P-3 peristaltic pump (Pharmacia, Uppsala, Sweden).

All fast protein liquid chromatography (FPLC) experiments were performed on a Pharmacia FPLC system, consisting of an LCC-500 gradient programmer, two P-500 syringe pumps, an MV-7 injector, a UV-1 280 nm fixed-wavelength UV detector, coupled to an REC-482 two-channel pen recorder and a Perkin-Elmer LCI-100 integrator (Perkin-Elmer, Norwalk, CT, U.S.A.). Column eluates were generally collected in 1-ml fractions by using a FRAC-100 fraction collector (Pharmacia).

Hybridomas producing naturally occurring IgM autoantibodies for this study were produced by fusion of myeloma cells and splenocytes from either unimmunized healthy 8 day old neonatal Balb/c mice or germ-free adult Balb/c mice, as previously described[3]. Indirect immunofluorescence characterization revealed that the germ-free mouse-derived autoantibodies GFM-5 1B12 reacted with nuclei of acetone-fixed mouse cells, while GFM-4 1G8 reacted with the Golgi complex of these cells. The hybridoma antibodies derived from neonatal mice (NNS-10 2D5) reacted with membrane antigens of Balb/c mouse thymocytes. IgM-containing ascites fluids or supernatants were produced from the three hybridoma cell lines.

Ascites fluids were generated for two hybridoma cell lines GFM-5 1B12 and NNS-10 2D5. Ascites fluids containing IgM autoantibodies were produced by injection of $5 \cdot 10^6$ hybridoma cells intraperitoneally into 4 week old Balb/c mice primed 10 days earlier with 0.5 ml 2,6,10,14-tetramethylpentadecane (pristane)[25]. Ascites fluids were collected 14–23 days after injection of cells, clarified by centrifugation at

2000 *g* for 10 min, and stored at −20°C with 0.02% sodium azide as preservative.

Tissue culture supernatants containing increased concentrations of IgM were prepared by culturing $5 \cdot 10^7$ hybridoma cells ($5 \cdot 10^6$ cells/ml) in dialysis membrane (Spectropor 4, Spectrum Medical Industries, Los Angeles, CA, U.S.A.) in 200-ml tissue culture flasks on a roller bottle apparatus (Wheaton Instruments, U.S.A.) at 1 rotation per 4.5 min[26].

All supernatants and ascites fluids were ultracentrifuged (100 000 *g*, 1 h at 4°C) after collection, followed by dialysis into the appropriate chromatography buffers prior to loading onto chromatography columns.

Methods for purification of hybridoma IgM

Enrichment methods

Salting-out precipitation procedures with saturated ammonium sulphate. Appropriate volumes of saturated ammonium sulphate (pH 7.4) were added dropwise at 4°C, at a constant rate (200 μl/min, P3 peristaltic pump, Pharmacia) to 2-ml aliquots of ascites fluid to achieve final concentrations of 40, 45, 50, 55 and 60%. The ammonium sulphate–ascites fluid slurry was stirred for 2 h at 4°C, and the precipitate was collected by centrifugation (12 000 *g*, 30 min, 4°C). The precipitate was resuspended in phosphate-buffered saline (PBS, pH 7.2) and dialysed against at least 100 volumes at 4°C with three buffer changes.

Precipitation of IgM with low-ionic-strength buffers. Aliquots of 2 ml of ascites fluids were dialysed against either 5 m*M* Tris–HCl (pH 7.5), 2% (w/v) boric acid (pH 6.0), or doubly distilled water. Dialysis was carried out against 3 × 100 ml of buffer for 16 h at 4°C. The resulting precipitate was collected by centrifugation at 12 000 *g* for 30 min and redissolved in PBS containing 0.5 *M* NaCl. Both the redissolved precipitate and supernatant from dialysis were stored at −20°C prior to analysis.

Precipitation of IgM with PEG 6000. The procedures used for partial purification of mouse IgM from ascites fluid using PEG 6000 precipitation were based on the methods described by Neoh *et al.*[23]. Monoclonal antibody-containing ascites fluids were delipidated either by centrifugation (10 000 *g*, 30 min, 4°C) or by incubation with silicon dioxide[23]. The optimal PEG 6000 concentration for IgM precipitation was determined by adding appropriate volumes of a 50% (w/v) PEG solution to 100 μl aliquots of ascites fluid to give final PEG concentrations of 0–25%. After incubation on ice for 15 min, the precipitated protein was removed by centrifugation (12 000 *g*, 20 min, 4°C), and the supernatants were analysed by agarose electrophoresis (Corning, Palo Alto, CA, U.S.A.). The lowest concentration of PEG 6000 that precipitated the IgM with the least contaminating protein was selected for preparative analysis. Preparative PEG precipitation was performed by addition of appropriate volumes of 50% PEG 6000 in veronal-buffered saline (pH 7.2) to 2-ml samples of clarified, delipidated ascites fluids. Samples were incubated on ice for 30 min with occasional mixing, and precipitated protein was collected by centrifugation (12 000 *g*, 20 min, 4°C). The precipitate containing IgM was redissolved in PBS and stored at −20°C prior to analysis.

Chromatographic procedures

SEC. Open-column SEC was performed by using Sephacryl S-300 columns (100 × 1 cm I.D., Pharmacia). For single-buffer determinations, isocratic elution with 100 m*M* Tris–HCl (pH 8.0), containing 50 m*M* NaCl was employed at a flow-rate of 30 ml/h. Prior to analyses, the S-300 columns were calibrated by chromatography of Blue dextran (M_r $2 \cdot 10^6$, Pharmacia), ferritin (M_r 475 000, Sigma), myosin (M_r 200 000, Sigma), phosphorylase B (M_r 94 000, Sigma), bovine serum albumin (BSA) (M_r 66 200, Sigma) and carbonic anhydrase (M_r 30 000, Sigma). For analyses of IgM purification, 1-ml aliquots of ascites fluids were dialysed into appropriate buffers, ultracentrifuged (100 000 *g*, 1 h, 4°C) and applied to the S-300 columns.

FPLC purifications of mouse monoclonal IgM were performed on Superose 6 HR 10/30 columns with single- and dual-buffer systems. For the single-buffer determinations the Superose 6 columns were equilibrated and eluted with 100 m*M* Tris–HCl (pH 7.8), containing 50 m*M* NaCl, at a flow-rate of 0.5 ml/min. For dual-buffer determinations, performed essentially as described by Bouvet *et al.*[9], the Superose 6 columns were equilibrated with 5 m*M* L-histidine (pH 6.0) and eluted with 50 m*M* L-histidine (pH 6.0), containing 1.7 *M* NaCl at a flow-rate of 0.5 ml/min. Loadings of 200 µl of IgM-containing supernatants or ascites fluids, dialysed into appropriate buffers and ultracentrifuged, were applied to the Superose 6 HR 10/30 columns.

Anion-exchange chromatography. Anion-exchange chromatography was performed with Mono Q HR 5/5 pre-packed columns (5 × 50 mm I.D., Pharmacia). Samples of ascites fluids were dialysed, applied (200 µl) in 20 m*M* L-histidine (pH 6.0), and eluted with a gradient of 20 m*M* L-histidine (pH 6.0), containing 500 m*M* NaCl essentially as described by Clerzardin *et al.*[13] A gradient of 0–100% NaCl was generated in two steps over 25 min at a flow-rate of 1 ml/ml. A 0–50% gradient was generated over 20 min with a 50–100% gradient generated over 5 min.

Cation-exchange chromatography. Cation-exchange chromatography was performed on Mono S HR 5/5 pre-packed columns (5 × 50 mm I.D., Pharmacia) essentially as described by Boonekamp and Pomp[10]. The 200 µl samples of supernatants or ascites fluids, containing monoclonal IgM antibodies, were dialysed and applied in 50 m*M* sodium acetate (pH 5.5), and eluted with 50 m*M* sodium acetate (pH 5.5), containing 1.0 *M* NaCl. The elution conditions for cation-exchange chromatography were as follows. The sample was applied and the column was washed for 10 min with starting buffer. Retained material was eluted with a 0–100% gradient of eluting buffer over 10 min, at a flow-rate of 1.0 ml/min.

Hydroxyapatite chromatography. Hydroxyapatite chromatography was performed on a Bio-Gel high-performance hydroxyapatite (HPHT) column (100 × 7.8 mm I.D., Bio-Rad Labs., Richmond, CA, U.S.A.), essentially as described by Stevens and Brooks[27]. The column was equilibrated with 10 m*M* sodium phosphate butter (pH 6.8), containing 0.3 m*M* calcium chloride, and eluted with 300 m*M* sodium phosphate buffer (pH 6.8), containing 0.01 m*M* calcium chloride. Elution of bound protein was performed with a 0–100% linear gradient generated over 15 min at a flow-rate of 0.5 ml/min.

Combinations of chromatographic procedures. The IgM-rich fractions from Mono Q-purified ascites fluid were pooled and concentrated to 400 µl. Samples of 200 µl were then applied to Superose 6 HR 10/30 size-exclusion columns equilibrated in 100 m*M* Tris–HCl (pH 8.0), containing 50 m*M* NaCl, and chromatographed at 0.5 ml/min as described above.

Analysis of the purity of isolated mouse IgM

The purity of the isolated IgM was analysed by SDS-PAGE, and double-sandwich anti-IgM capture enzyme-linked immunosorbent assay (ELISA).

Polyacrylamide gel electrophoresis

Fractions from all procedures used to purify IgM, were analysed by SDS-PAGE as described by Laemmli[28]. Analysis for purity of IgM was performed on 12.5% polyacrylamide resolving gels under reducing conditions or on 3–12.5% polyacrylamide gradient resolving gels under non-reducing conditions. Electrophoresis was performed at 200 V, 25 mA, per gel until the Bromophenol blue tracking dye had reached the lower edge of the resolving gel. Following silver staining of the electrophoresed proteins according to methods described by Morrissey[29], the purity of the IgM was determined by estimation of detectable contaminants. Highly purified IgM and IgG monoclonal antibodies were used for comparison on all gels (see next section).

Measurement of IgM concentrations by ELISA

To determine the concentrations of IgM in all purified fractions a double-sandwich capture anti-IgM ELISA was performed. ELISA plates (Nunc-Immunoplate, Maxisorp F96, Nunc, Roskilde, Denmark) were coated with 25 ng per well anti-IgM μ-chain-specific antibodies (KPL, Gaithersburg, MD, U.S.A.) in 0.05 *M* bicarbonate buffer (pH 9.6). After 4 h at room temperature, the plates were washed in PBS containing 0.05% Tween 20 (PBS-T) and blocked overnight at 4°C with 100 μl of 1% (w/v) BSA in PBS per well. Duplicate samples of the fractions for which the concentration of IgM was to be determined and highly purified IgM standards were applied to appropriate wells. After 30 min at room temperature, the plates were washed 9 times with PBS-T, and bound IgM was identified by sequential incubations of sheep anti-mouse-biotin, streptavidin horseradish peroxidase (Amersham, Sydney, Australia) and H_2O_2–*o*-phenylenediamine-containing substrate solution. Absorbance values were determined at 492 nm, by using an MCC-340 Multiscan ELISA reader (Flow, Stanmore, Australia). Concentrations of IgM in chromatographic fractions were determined from a standard plot of the concentrations of the IgM standard *versus* absorbance values.

The IgM standard used for all ELISA and SDS-PAGE analyses was derived from ascites fluid of the GFM-5 1B12 hybridoma. Highly purified IgM was produced following 40% ammonium sulphate precipitation, Superose 6 HR 10/30 SEC and absorption on an anti-mouse IgG affinity column. Double-sandwich capture ELISA analysis with anti-IgG γ-chain-specific antibodies (KPL) as well as SDS-PAGE revealed a highly purified IgM preparation containing no contaminating IgG. Monoclonal mouse IgG_1 purified from hybridoma ascites fluid by DEAE-cellulose ion-exchange chromatography was also used as a standard for comparison with IgM in SDS-PAGE.

Estimation of protein concentration of IgM-containing samples

In order to determine the efficiency of the IgM purification procedures compared in this study, protein concentrations of all samples were determined by the use

of the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, U.S.A.). Serial dilutions, in duplicate, of protein standards (BSA) and unknown IgM-containing samples were prepared in PBS. Aliquots of 50 μl of standards or unknown protein solutions were diluted in 1000 μl of BCA diluent buffer. Samples were then incubated at 37°C for 60 min. From the absorbance values, measured at 562 nm, protein concentrations were determined for unknown samples from the standard curve. The assay performed as described could detect protein in the range 5–250 μg/ml.

Determination of the immunoreactivity of isolated IgM autoantibodies

Immunoreactivity of IgM-containing fractions from the various purification procedures was assessed by indirect immunofluorescence. Fractions containing monoclonal IgMs, derived from GFM-5 1B12 or GFM-4 1G8 hybridomas, were incubated for 20 min in a humidified chamber with acetone-fixed Balb/c mouse 3T3 cells, which had been grown on multiwell slides (Flow Labs., Stanmore, Australia) overnight ($2 \cdot 10^4$ cells per well). Following three washes with PBS, the cells were incubated with affinity-purified anti-mouse immunoglobulins, coupled to fluorescein isothiocyanate (FITC) (Silenus, Melbourne, Australia) for 20 min and washed three times. Bound monoclonal IgM was identified by narrow-band blue illumination under a Leitz Dialux epi-illumination fluorescence microscope.

Fractions containing IgM, isolated from NNS-10 2D5 ascites fluid, were allowed to react with $1 \cdot 10^6$ viable intact Balb/c mouse thymocytes for 20 min at 4°C. Following three washes with PBS, containing 10% FCS and 0.1% NaN_3 (PBS–FCS), the cells were incubated with affinity-purified anti-mouse immunoglobulins, coupled to FITC (Silenus) for 20 min at 4°C. Following a further three washes in PBS–FCS, the thymocytes were resuspended in 100 μl PBS–fetal bovine serum (FCS) and examined, after being mounted on slides, by narrow-band blue illumination under a Leitz Dialux epi-illumination fluorescence microscope.

RESULTS

Examination of three procedures routinely used in laboratories for the isolation or enrichment of IgM demonstrated that PEG 6000 precipitation, based on the method of Neoh *et al.*[23], led to the recovery of *ca.* 60% of the IgM from GFM-5 1B12 ascites fluid and a purification factor of between 4- to 5-fold (Table I). SDS-PAGE of PEG 6000-precipitated samples revealed contamination of the IgM-containing fractions with proteins of high and low apparent molecular weights, including those similar to α_2-macroglobulin, IgG, and albumin (Fig. 1). Delipidation of GFM-5 1B12 ascites fluid by using silicon dioxide or centrifugation did not effect levels of monoclonal IgM in the samples prior to PEG 6000 precipitation (data not shown). Analysis of ammonium sulphate enrichment procedures revealed that, with GFM-5 1B12 antibodies, increasing ammonium sulphate concentrations precipitated increasing amounts of IgM. For example, 40% ammonium sulphate precipitated *ca.* 31% of IgM, and 60% ammonium sulphate precipitated *ca.* 83% of the IgM from ascites fluid but with increasing amounts of other protein contaminants (Table I, Fig. 2). For GFM-5 1B12 ascites fluid-derived IgM antibodies, the optimal purification of *ca.* 3-fold was achieved with 45% (v/v) final ammonium sulphate concentration. SDS-

TABLE I

COMPARISON OF METHODS FOR ENRICHMENT OF MONOCLONAL IgM ANTIBODIES FROM ASCITES FLUIDS

Purification procedure	*Antibody*	*Fraction(s) analysed*	*Protein applied (μg)*	*IgM applied (μg)*	*Protein recovered (μg)*	*IgM recovered (μg)*	*Yield of IgM (%)*	*Purification factor*
Ammonium sulphate precipitation	GFM-5 1B12	Ascites fluid	16 850.0	2520.0[a]	–	–	100.0	1.0
		40% (v/v) ammonium sulphate			2620.0	780.0	31.0	2.0
		45% (v/v) ammonium sulphate			3340.0	1350.0	53.5	2.7
		50% (v/v) ammonium sulphate			3720.0	1440.0	57.0	2.5
		55% (v/v) ammonium sulphate			5600.0	1610.0	63.0	1.9
		60% (v/v) ammonium sulphate			6500.0	2080.0	82.5	2.1
Euglobulin precipitation	GFM-5 1B12	Ascites fluid	16 850.0	2520.0[a]	–	–	100.0	1.0
		2% (w/v) boric acid (pH 6.0)			1250.0	237.0	9.4	1.3
		5 m*M* Tris–HCl (pH 7.5)			700.0	136.0	5.3	1.3
		Double distilled H_2O			950.0	100.0	4.0	0.7
Polyethylene glycol precipitation	GFM-5 1B12	Ascites fluid	16 850.0	2520.0[a]	–	–	100.0	1.0
		PEG 6000/SiO_2 clarified			2060.0	1600.0	63.5	5.2
		PEG 6000/centrifuge-clarified			2600.0	1660.0	66.0	4.3

[a] Amount of monoclonal IgM in 1.0 ml of GFM-5 1B12 ascites fluid.

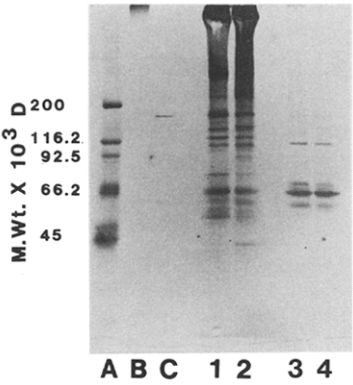


Fig. 1. Fractions from PEG 6000 precipitation of GFM-5 1B12 ascites fluid analysed on a 3–12.5% SDS–polyacrylamide gradient gel under non-reducing conditions. Lanes A, B and C represent molecular weight, IgM, and IgG standards respectively. Lanes 1 and 2 contain redissolved samples from the centrifuge- and SiO_2-clarified PEG 6000 precipitates of GFM-5 1B12 ascites fluid, respectively. Lanes 3 and 4 contain supernatants from the precipitates of centrifuge- and SiO_2-clarified GFM-5 1B12 ascites fluid, respectively.

PAGE revealed considerable contamination of the precipitated IgM with lower-molecular-weight species, particularly IgG and albumin (Fig. 2). The euglobulin properties of IgM have often been used for IgM purification[30]. However, the results with 2% (w/v) boric acid (pH 6.0) and 5 m*M* Tris–HCl (pH 7.5) indicated little purification and poor yields of GFM-5 1B12 ascites fluid IgM (*ca.* 9 and 5% respectively, Table I). Dialysis of GFM-5 1B12 ascites fluid against distilled water was least successful, with 100 μg of IgM present in 950 μg of total protein (Table I). Similarly, analysis of samples derived from euglobulin precipitations by SDS-PAGE also demonstrated poor purity (Fig. 3). Another consideration in the use of the euglobulin properties of hybridoma IgMs for purification or enrichment was the observed in-

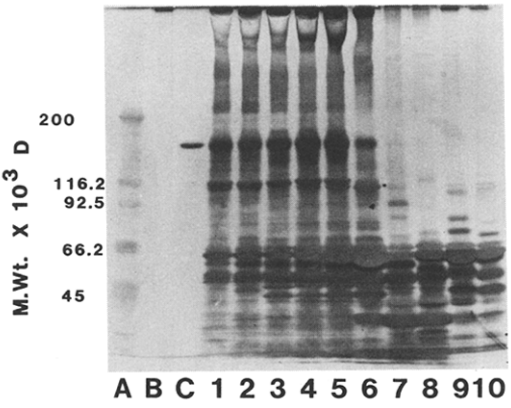


Fig. 2. Analysis of samples of ammonium sulphate precipitates and supernatants from GFM-5 1B12 ascites fluid on a 3–12.5% SDS–polyacrylamide gradient gel under non-reducing conditions. Lanes A–C contain molecular weight, IgM and IgG standards, respectively. Lanes 1–5 contain samples of redissolved ammonium sulphate precipitates in the order of 40, 45, 50, 55 and 60%, respectively. Lanes 6–10 contain samples of supernatants from the ammonium sulphate precipitates in the order of 40, 45, 50, 55 and 60%, respectively.

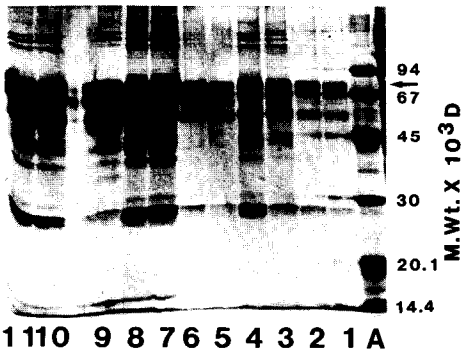


Fig. 3. SDS–PAGE (12.5%), under reducing conditions, of precipitate and supernatant fractions, following dialysis of GFM-5 1B12 ascites fluid in low-ionic-strength buffers. Lane A contains molecular-weight standards. Lanes 1 and 2 contain samples of redissolved precipitate following dialysis into 5 m*M* Tris–HCl (pH 7.8). Lanes 3 and 4 contain samples of redissolved precipitate, following dialysis into 2% (w/v) boric acid. Lanes 5 and 6 contain redissolved samples of precipitates following dialysis against distilled water. Lanes 7–11 contain samples from supernatants of precipitates following dialysis against 5 m*M* Tris–HCl pH 7.8 (lanes 7 and 8) 2% boric acid (lane 9), and distilled water (lanes 10 and 11). The arrow indicates heavy chain of IgM (M_r 80 000; ref. 24).

ability of the IgM-containing precipitates to redissolve completely, indicating denaturation.

Resolution of hybridoma IgMs based on SEC resulted in purification of all the monoclonal antibodies examined. Sephacryl S-300 purification resulted in *ca.* 2- to 4-fold purification of IgM, derived from ascites fluid of GFM-5 1B12 and NNS-10 2D5 hybridomas, with yields of *ca.* 57–80% (Table II, Fig. 4). The fractions initially eluted from S-300 contained IgM in a purity of *ca.* 80–86% but in low yield (Table II). These fractions were contaminated with other high-molecular-weight species, having apparent molecular weights similar to α_2-macroglobulin and its subunits. Subsequent fractions were contaminated with a variety of lower-molecular-weight species (data not shown). Purification of IgM from crude ascites fluids and supernatant on the Superose 6 HR 10/30 columns resulted in separation of IgM from other proteins and recoveries of IgM of *ca.* 71–86% (Table II, Fig. 5). Purity, as assessed by SDS-PAGE (Fig. 6) was very high, with *ca.* 68–86% of the total protein being actually IgM (Table II). Interestingly, analysis of silver-stained SDS–polyacrylamide gels alone, in the absence of actual measurements of amounts of IgM and protein in IgM-containing fractions, would indicate an IgM purity of $\geqslant$90% from Superose 6 chromatography (Fig. 6). This observation demonstrates that caution must be exercised when estimating IgM purity solely from polyacrylamide gels. Both the NNS-10 2D5 and GFM-5 1B12 ascites fluid-derived IgM antibodies were also separated by 'non-ideal' SEC, similar to that described by Bouvet *et al.*[9]. The Superose 6 HR columns were equilibrated with 5 m*M* L-histidine, pH 6.0, and eluted with 50 m*M* L-histidine (pH 6.0), containing 1.7 *M* NaCl. This procedure is based on the euglobulin properties of IgM. Under the equilibration conditions of this chromatographic procedure, IgM is selectively slowed as it approaches the limits of solubility in the low-ionic-strength buffer and is eluted with the high-ionic-strength buffer in the trailing peak (Fig. 7). Recoveries of hybridoma IgM under these conditions was *ca.* 82–92% (Table III). However

TABLE II

PURIFICATION OF MOUSE MONOCLONAL IgM ANTIBODIES FROM HYBRIDOMA CULTURE SUPERNATANT AND ASCITES FLUIDS BY SINGLE-STEP SEC

Purification procedure	*Antibody*	*Fraction(s) analysed*	*Protein applied (μg)*	*IgM applied (μg)*	*Protein recovered (μg)*	*IgM recovered (μg)*	*Yield of IgM (%)*	*Purification factor*
Sephacryl S-300	NNS-10 2D5	Ascites fluid	18 500.0	4693.5[a]	–	–	100.0	1.0
		8			24.0	20.7	0.44	2.3
		9–12			6630.0	3812.5	57.2	
	GFM-5 1B12	Ascites fluid	13 750.0	1501.9[a]	–	–	100.0	1.0
		8			3.2	2.7	0.18	3.9
		9–12			2845.0	1195.0	79.5	
Superose 6 HR 10/30	NNS-10 2D5	Ascites fluid	7510.0	1877.4[b]	–	–	100.0	1.0
		10–13			1880.0	1613.8	86.0	3.4
	GFM-5 1B12	Ascites fluid	5100.0	619.0[b]	–	–	100.0	1.0
		10–12			630.0	503.0	80.0	6.6
	GFM-4 1G8	Dialysis bag supernatant	2850.0	90.0[b]	–	–	100.0	1.0
		10–13			94.3	64.3	71.0	21.5

[a] Amount of IgM present in 1.0 ml of ascites fluid following dialysis into 100 m*M* Tris–HCl (pH 7.8)–50 m*M* NaCl.

[b] Amount of IgM present in 200 μl of ascites fluids or supernatants following dialysis into 100 m*M* Tris–HCl (pH 7.8)–50 m*M* NaCl.

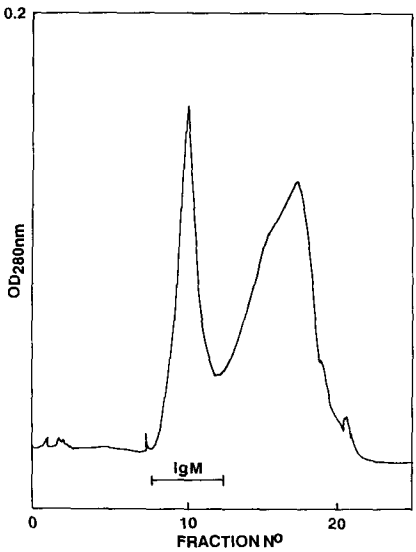


Fig. 4. Chromatography of monoclonal IgM from NNS-10 2D5 hybridoma on a Sephacryl S-300 column (100 × 1 cm I.D.), equilibrated in 100 m*M* Tris–HCl (pH 8.0), containing 50 m*M* NaCl. Sample applied, 1 ml ascites fluid; flow-rate, 0.5 ml/min; 0.2 a.u.f.s.

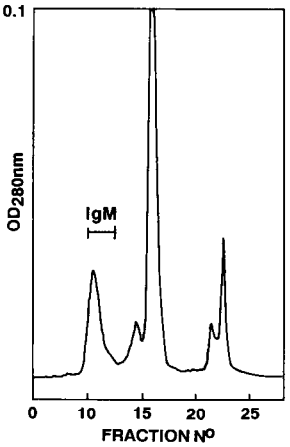


Fig. 5. Purification of IgM from GFM-5 1B12 ascites fluid by SEC on a Superose 6 HR 10/30 FPLC column equilibrated in 100 m*M* Tris–HCl (pH 8.0), containing 50 m*M* NaCl. Volume of ascites fluid applied, 200 μl; flow-rate, 0.5 ml/min; 0.1 a.u.f.s.

SDS-PAGE, IgM, and protein determinations demonstrated that low-molecular-weight protein contaminants, including those with apparent molecular weights similar to mouse IgG and albumin are not separated from IgM. (Table III, Fig. 8). Using this procedure, purification of IgM in the range 3.5- to 4.2-fold was achieved.

Purification of IgM from ascites fluids and supernatants by anion- and cation-exchange chromatography did not result in highly purified IgM. Anion-exchange chromatography on Mono Q FPLC columns produced only *ca.* 1.5-fold purification of GFM-5 1B12 and NNS-10 2D5 IgMs (Table IV). These hybridoma IgMs were eluted with *ca.* 160 m*M* NaCl in the trailing shoulder of the second-eluted peak through Fractions 25–38 and 27–38, for GFM-5 1B12 and NNS-10 2D5 antibodies,

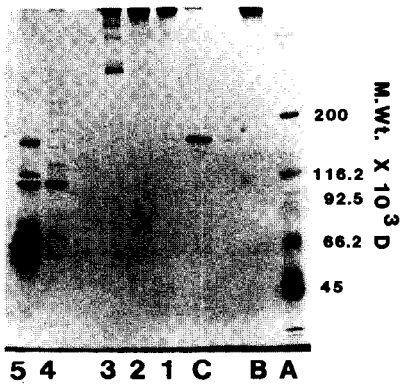


Fig. 6. SDS-PAGE of fractions eluted from a Superose 6 HR FPLC column following application of GFM-5 1B12 ascites fluid (3–12.5% gradient gel, non-reducing conditions). Lanes A, B, and C represent molecular-weight, IgM, and IgG standards, respectively. Lanes 1–5 represent samples from fractions 10, 11, 12, 14, and 15 eluted from the Superose 6 column.

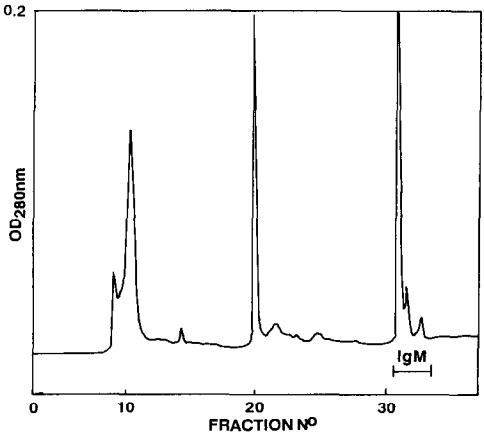


Fig. 7. "Non-ideal" SEC of GFM-5 1B12 ascites fluid on a Superose 6 HR 10/30 FPLC column, equilibrated in 5 m*M* L-histidine (pH 6.0) and eluted with 50 m*M* L-histidine (pH 6.0), containing 1.7 *M* NaCl. Volume applied, 200 ml; flow-rate, 0.5 ml/min; 0.2 a.u.f.s.

respectively (Fig. 9). Recovered IgM consisted of *ca.* 50–80% of the amount loaded and was significantly contaminated with co-eluting species (Table IV, Fig. 10). For NNS-10 2D5 ascites, only *ca.* 2.0% of the protein recovered from the Mono Q column was IgM, whilst for the GFM-5 1B12 antibody this value was *ca.* 5.0% (Table IV). Cation-exchange chromatography on Mono S FPLC columns lead to IgM recoveries of 52–94% (Table IV). The IgMs were eluted, commencing at *ca.* 150 m*M* NaCl, in a broad band through fractions 15–22, 15–27 or 18–28 depending on the specific antibodies (Table IV, Fig. 11). The eluted IgM-containing fractions also contained species with apparent molecular weights similar to α_2-macroglobulin, IgG and albumin (Fig. 12). The IgM represented less than *ca.* 12% of the total protein in the IgM-containing fractions for the three hybridoma antibodies eluted from the Mono S column (Table IV).

Mixed-mode chromatography on hydroxyapatite (HPHT) resulted in lower resolution of IgM than did SEC or mixed-mode chromatography on Abx col-

TABLE III

FRACTIONATION OF MOUSE MONOCLONAL IgM FROM ASCITES FLUIDS BY "NON-IDEAL" SEC ON SUPEROSE 6 HR COLUMNS

Antibody	*Fraction(s) analysed*	*Protein applied (μg)*	*IgM applied (μg)*	*Protein recovered (μg)*	*IgM recovered (μg)*	*Yield of IgM (%)*	*Purification factor*
NNS-10 2D5	Ascites fluid	4600.0	44.7[a]	–	–	100.0	1.0
	31–34			1000.0	41.2	92.3	4.2
GFM-5 1B12	Ascites fluid	3750.0	130.0[a]	–	–	100.0	1.0
	31–34			876.8	106.8	82.2	3.5

[a] Amount of IgM present in 200 μl of ascites fluids following dialysis into 5 m*M* L-histidine (pH 6.0).

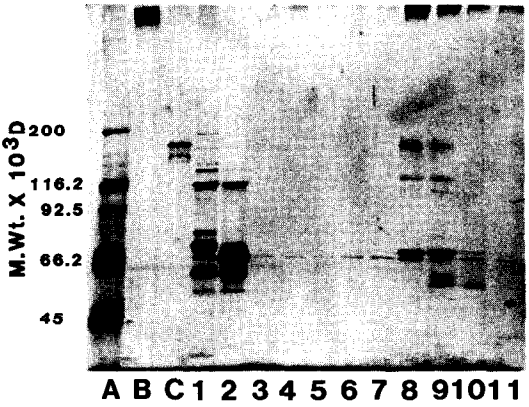


Fig. 8. Analysis of fractions of GFM-5 1B12 ascites fluid with SDS–PAGE (3–12.5% gradient gel, non-reducing conditions), following chromatography on a Superose 6 HR 10/30 FPLC column under "non-ideal" size-exclusion conditions. Lanes A–C represent molecular weight, IgM, and IgG standards, respectively. Lanes 1, 2, 8–11 contain samples from eluted fractions 9, 10, 31–34, respectively.

umns[8,31]. The IgMs derived from NNS-10 2D5 and GFM-5 1B12 ascites fluids were eluted in the second-elution peak from HPHT (Fig. 13). The recovery of IgM was *ca.* 40% with significant contamination from a variety of lower-molecular-weight species, including several of apparent molecular weights similar to α_2-macroglobulin, IgG, and albumin (Table V, Fig. 14).

TABLE IV

ANION- AND CATION-EXCHANGE CHROMATOGRAPHY OF MOUSE MONOCLONAL IgM ANTIBODIES FROM HYBRIDOMA SUPERNATANT AND ASCITES FLUIDS

Purification procedure	*Antibody*	*Fraction(s) analysed*	*Protein applied (μg)*	*IgM applied (μg)*	*Protein recovered (μg)*	*IgM recovered (μg)*	*Yield of IgM (%)*	*Purification factor*
Mono Q anion exchange	NNS-10 2D5	Ascites fluid	4500.0	62.6[a]	–	–	100.0	1.0
		27–38			1532.0	31.6	50.6	1.5
	GFM-5 1B12	Ascites fluid	4480.0	176.0[a]	–	–	100.0	1.0
		25–38			2696.8	142.8	81.1	1.3
Mono S cation exchange	NNS-10 2D5	Ascites fluid	4675.0	75.0[b]	–	–	100.0	1.0
		18–28			1165.6	70.3	93.7	3.8
	GFM-5 1B12	Ascites fluid	4300.0	476.8[b]	–	–	100.0	1.0
		15–28			3662.0	441.3	92.5	1.1
	GFM-4 1G8	Dialysis bag supernatant	504.0	2.0[b]	–	–	100.0	1.0
		15–27			137.0	1.0	50.0	1.9

[a] Amount of IgM present in 200 μl of ascites fluids following dialysis into 20 m*M* L-histidine (pH 6.0).
[b] Amount of IgM present in 200 μl of ascites fluids following dialysis into 50 m*M* sodium acetate (pH 5.5).

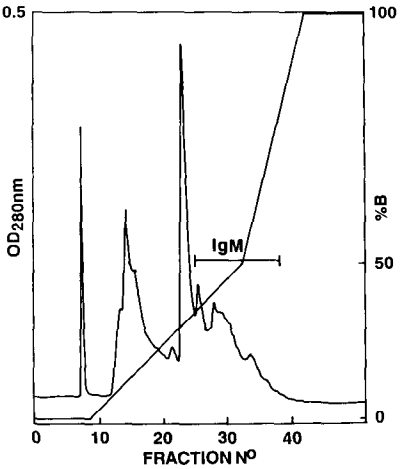


Fig. 9. Mono Q anion-exchange chromatography of GFM-5 1B12 ascites fluid. Volume applied, 200 μl; flow-rate, 1.0 ml/min; 0.5 a.u.f.s. Column equilibrated in 20 m*M* L-histidine (pH 6.0) (buffer A) and eluted with 20 m*M* L-histidine (pH 6.0), containing 500 m*M* NaCl (buffer B). Linear gradient, 0–50% B generated over 20 min and 50–100% B over 5 min.

Combinations of anion-exchange chromatography and SEC have been used to purify hybridoma IgM from supernatants and ascites fluids[8,11,13]. In our study, ascites fluid-derived hybridoma IgMs were initially separated with Mono Q FPLC, the IgM-containing fractions were pooled, concentrated and applied to Superose 6 HR 10/30 columns. Recovery of the IgMs, initially eluted from Mono Q columns following SEC, was *ca.* 60% (Table VI). However, their purity was less than 6%. The proportional changes in the molecular mass of IgM in relation to lower-molecular-

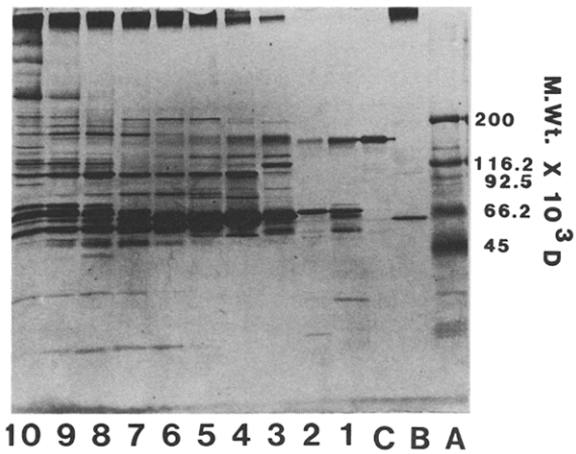


Fig. 10. Analysis with SDS-PAGE of fractions eluted from a Mono Q FPLC column, following the application of 200 μl of GFM-5 1B12 ascites fluid (3–12.5% gradient gel, non-reducing conditions). Lanes A–C represent molecular weight, IgM, and IgG standards, respectively. Lanes 1–10 correspond to aliquots from fractions 8 and 15, and 25–32, respectively.

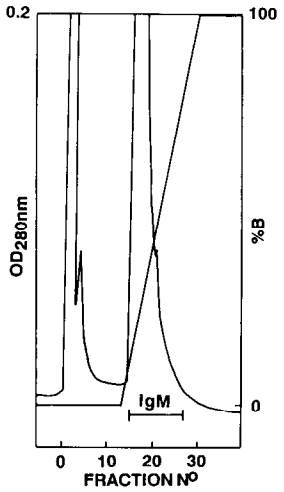


Fig. 11. Cation-exchange chromatography of GFM-5 1B12 ascites fluid. Volume applied, 200 μl; flow-rate, 1.0 ml/min; 0.2 a.u.f.s. Columns equilibrated in 50 m*M* sodium acetate (pH 5.5) (buffer A) and eluted with 50 m*M* sodium acetate (pH 5.5), containing 1.0 *M* NaCl (buffer B). Linear gradient, 0–100% B over 10 min.

weight contaminants following elution from Mono Q columns resulted in poor resolution of IgM by SEC (Fig. 15) in comparison with ascites fluid or supernatant IgM chromatographed in a single step on Superose 6 columns (Fig. 5).

The immunoreactivity of hybridoma IgMs was determined, following fractionations, by indirect immunofluorescence. In all cases, fractions containing IgM from GMF-5 1B12 produced anti-nuclear immunofluorescence and from GFM-4 1G8, anti-Golgi complex reactivity, when tested on acetone-fixed mouse 3T3 cells. Similarly, with the NNS-10 2D5 hybridoma, anti-Balb/c mouse thymocyte cell surface

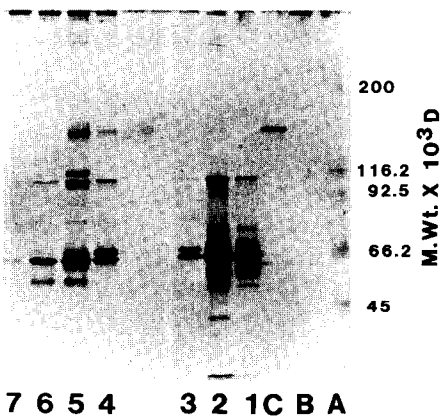
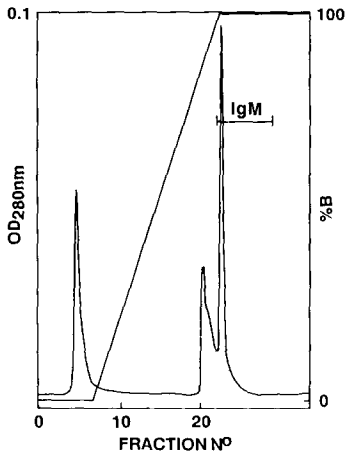


Fig. 12. SDS-PAGE of fractions of GFM-5 1B12 ascites fluid eluted from a Mono S FPLC column (3–12.5% gradient gel, non-reducing conditions). Lanes A–C contain molecular weight, IgM and IgG standards, respectively. Lanes 1–3 contain aliquots from eluted fractions 2–4, respectively. Lanes 4–7 contain aliquots from eluted fractions, 15, 17, 19, and 21, respectively.



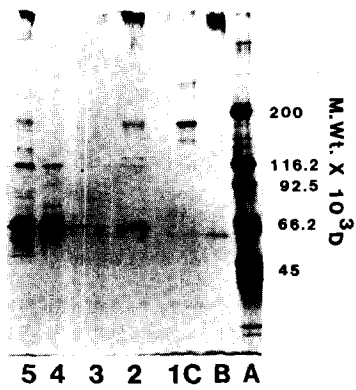


Fig. 13. Mixed-mode chromatography of NNS-10 2D5 ascites fluid on hydroxyapatite. Volume applied, 200 μl; flow-rate, 0.5 ml/min; 0.1 a.u.f.s. Column equilibrated in 10 m*M* sodium phosphate buffer (pH 6.8)–0.3 m*M* calcium chloride (buffer A) and eluted with 300 m*M* sodium phosphate buffer (pH 6.8)–0.01 m*M* calcium chloride (buffer B). Linear gradient 0–100% B generated over 15 min.

Fig. 14. Analysis of fractions with SDS-PAGE (3–12.5% non-reducing gradient gel) of NNS-10 2D5 ascites fluid, following HPHT chromatography. Lanes A–C represent molecular weight, IgM, and IgG standards, respectively. Lanes 4 and 5 contain aliquots from fractions 21 and 23, respectively. Lane 2 contains an aliquot from fraction 23, following HPHT chromatography of a 45% ammonium sulphate-precipitated sample of NNS-10 2D5 ascites fluid.

immunofluorescence could be detected in all isolated fractions containing NNS-10 2D5 hybridoma IgM following the various purification strategies. These results indicate the maintenance of immunoreactivity of each of the hybridoma IgMs following purification by all procedures.

TABLE V

ISOLATION OF MOUSE MONOCLONAL IgMs FROM ASCITES FLUID BY HYDROXYAPATITE CHROMATOGRAPHY

Antibody	*Fraction(s) analysed*	*Protein applied (μg)*	*IgM applied (μg)*	*Protein recovered (μg)*	*IgM recovered (μg)*	*Yield of IgM (%)*	*Purification factor*
NNS-10 2D5	Ascites fluid	4900.0	330.0[a]	–	–	100.0	1.0
	22–28			2362.5	128.8	40.0	0.8
GFM-5 1B12	Ascites fluid	4440.0	342.1[a]	–	–	100.0	1.0
	20–30			3159.0	135.3	39.5	0.6

[a] Amount of IgM present in 200 μl of ascites fluids following dialysis into 10 m*M* sodium phosphate (pH 6.8) – 0.3 m*M* calcium chloride.

TABLE VI

SEQUENTIONAL PURIFICATION OF MOUSE MONOCLONAL IgM ANTIBODIES BY MONO Q ANION-EXCHANGE CHROMATOGRAPHY AND SUPEROSE 6 SEC

Antibody	*Fraction(s) analysed*	*Protein applied (μg)*	*IgM applied (μg)*	*Protein recovered (μg)*	*IgM recovered (μg)*	*Yield of IgM (%)*	*Purification factor*
NNS-10 2D5	Mono Q eluate	610[a]	5.8	–	–	100.0	1.0
	8–12			132.6	3.6	62.0	2.8
GFM-5 1B12	Mono Q eluate	848[b]	41.5	–	–	100.0	1.0
	8–12			260.0	26.8	64.5	2.1

[a] Pooled fractions 27–38 from Mono Q FPLC column.
[b] Pooled fractions 25–38 from Mono Q FPLC column.

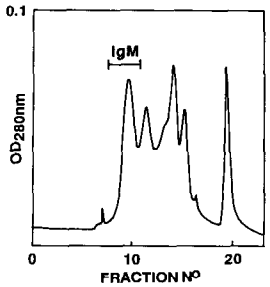


Fig. 15. Separation of IgM-containing fractions eluted from a Mono Q column of NNS-10 2D5 ascites fluid, following chromatography on a Superose 6 HR 10/30 FPLC column. Volume of sample, 200 μl; flow-rate, 0.5 ml/min; 0.1 a.u.f.s.

DISCUSSION

The above comparison of methods for the purification of monoclonal IgM autoantibodies was undertaken in order to ascertain an efficient procedure for the recovery and isolation of IgM. Data in the literature indicate that assessment of purity of isolated IgM has usually been undertaken by SDS-PAGE relying on either densitometric or manual estimation for quantification of IgM purity[8,13,30,31]. We have compared the methods for IgM purification by a double-sandwich anti-IgM ELISA for the measurement of IgM concentrations in ascites fluids, supernatant, and fractions from purification procedures. With ELISA we were able to detect as little as 2.0 ng of IgM per ml. In conjunction with this ELISA, IgM purity was further assessed in two ways. First, IgM-containing fractions were subjected to SDS-PAGE under either reducing (12.5% gels) or non-reducing (3–12.5% gel) conditions, and protein was detected by silver-staining[29], a method claimed to be at least 100-fold more sensitive than Coomassie Blue R250 staining[24]. Secondly, protein concentrations of IgM-containing fractions were determined by a BCA-based protein assay

with a detection sensitivity of 5–250 μg protein per ml[32]. These assay procedures enabled the definition of the relative purity of IgM in any given fraction following chromatography and visualization of the apparent molecular weights of protein species contaminating the IgM.

The IgM separation techniques tested in this study included routinely used procedures, such as ammonium sulphate[24], euglobulin precipitation[24,31,33], PEG-induced precipitation[23], and more advanced techniques such as size-exclusion, anion- and cation-exchange and mixed-mode chromatographic methods. Combinations of separation strategies were also tested.

Ammonium sulphate precipitation of immunoglobulins has been used as an initial enrichment and concentration method[24]. This study demonstrates that complete recovery of murine monoclonal IgMs does not occur at the commonly used 40–45% (v/v) final concentration of ammonium sulphate. The highest recovery of IgM (*ca.* 83%) occurred at 60% (v/v) final concentration of ammonium sulphate, suggesting that care should be taken in selecting ammonium sulphate concentrations if recovery of monoclonal IgM is of importance. In contrast, precipitation of monoclonal IgM based on euglobulin properties in low-ionic-strength solutions resulted in low IgM recovery ($\leqslant$ 10%) under conditions known to precipitate serum IgM[21]. Dialysis of ascites fluid against 2% boric acid (pH 6.0) produced the greatest recovery of monoclonal IgM consistent with reports that euglobulins precipitate more readily in boric acid, and boric acid protein complexes are more readily redissolved than other precipitates[33]. Of significance, however, with the low-ionic-strength buffer precipitation of IgM was the observed inability of precipitated material to redissolve completely indicating some protein denaturation.

PEG 6000 precipitation produced *ca.* 4- to 5-fold purification of IgM with *ca.* 65% recovery. Purity of PEG-precipitated hybridoma IgM was *ca.* 64–70%, as assessed by protein estimations contrasting with >90%, reported by Neoh *et al.*[23] who used Coomassie Blue-stained agarose gels. Consequently, the use of PEG 6000 may provide a useful enrichment and concentration procedure for hybridoma IgM antibodies.

The results demonstrate that in a single step, SEC on Superose 6 HR 10/30 FPLC columns produced a purification of 3- to 22-fold for IgMs derived from ascites fluids and supernatants. With a single-buffer system, IgM was recovered in *ca.* 70–80% purity and *ca.* 68–80% yield from Superose 6 columns. These values are slightly less than the estimates from Coomassie Blue-stained SDS-PAGE reported by Chen *et al.*[8] although the resolution in our system was apparently greater (Fig. 6). The differences in IgM resolution following Superose 6 chromatography demonstrated in this study compared to that reported by Chen *et al.*[8] may be due to differences in buffer systems [100 m*M* Tris–HCl, (pH 7.8)–50 m*M* NaCl *versus* 100 m*M* PBS (pH 8.0)] or subtle differences in the monoclonal IgM, allowing hydrophobic or ionic interactions with the chromatographic matrix. Further studies in our laboratory have revealed that resolution of IgM can be achieved with 50 m*M* L-histidine (pH 6.0) buffers, containing 1.7 *M* NaCl on Superose 6 HR 10/30 FPLC columns.

Separation of IgM based on "non-ideal" SEC resulted in high recoveries of IgM (*ca.* 82–92%) but with comparatively low purity ($\leqslant$ 12%). This is in contrast with the results reported by Bouvet *et al.*[9] and Chen *et al.*[8] for isolation of IgMs from ascites fluid. Both authors reportedly purified IgM from ascites fluids using "non-

ideal" SEC to $\geqslant$ 90% purity, as assessed by Coomassie Blue-stained SDS-PAGE. Differences in purities reported here from those reported by others may again be related to either subtle differences in the monoclonal IgM autoantibodies such that poor resolution from other selectively slowed proteins occurred under the conditions applied. Alternatively, variations may be explained by differences between the buffers used for the analyses, or differences in methods for estimating sample purity. However, the results presented here, demonstrate the retention of monoclonal IgMs on Superose 6 columns under low-ionic-strength conditions, possibly due to euglobulin properties of IgM and/or the potential for hydrophobic behaviour of the chromatographic matrix, and their elution in the final peak with high-ionic-strength buffers. The behaviour of the hybridoma IgMs examined in this study with respect to this latter point is similar to that reported by others[8,9]. The contaminating lower-molecular-weight species, including mouse IgG and albumin, found to be eluted with IgM under "non-ideal" SEC conditions may not have been unexpected, as similar contaminants were found in IgM-containing precipitates from the low-ionic-strength analyses (Fig. 3).

Under the conditions investigated, anion- or cation-exchange chromatography and Mono Q and Mono S FPLC columns, was unable to separate the specific monoclonal IgMs from other proteins present in ascites fluids or concentrated tissue culture supernatants in a single step. In contrast to the purity of *ca.* 50% estimated for single-step Mono Q-purified IgM by Chen *et al.*[8], the purity of IgM eluted from the Mono Q column in this study was $\leqslant$ 5%, albumin constituting a major contaminant. In order to achieve high recovery of IgMs in this study, IgM eluted in the broad, trailing shoulder of the second-eluted peak was pooled. Thus, recovered IgMs were of significantly lower purity, as these fractions also contained many other proteins (Fig. 10). The buffers used in this study were those reported by Clerzardin *et al.*[13] for monoclonal IgM purification on Mono Q columns. Chen *et al.*[8] used 20 m*M* Tris–HCl (pH 8.0) for equilibration of the Mono Q column and this buffer, containing 1.0 *M* NaCl, for elution. Variations in the purity of the monoclonal IgMs eluted from Mono Q columns may be related to the different net-charge characteristics and surface distribution of these charges on the IgM molecules at the two different buffer pH values used in these studies.

Results from this study indicate that cation-exchange chromatography on Mono S columns cannot be used to isolate monoclonal IgM in a highly purified form from ascites fluids or tissue culture supernatants. Similarly, mixed-mode chromatography on hydroxyapatite did not result in the purification of IgM reported by others[27,34].

Combinations of anion-exchange chromatography and SEC have been reported to result in the purification of monoclonal IgMs free from α_2-macroglobulin and other supernatant and ascites fluid proteins[8,11,13]. In this study, separation of IgMs eluted from Mono Q columns from other proteins did not occur following passage through Superose 6 HR 10/30 FPLC columns. In this dual-step procedure, recovery of monoclonal IgM was high (*ca.* 65%) whilst the purification factor was 2- to 3-fold. These results were attributable to proportional increases in the amounts of contaminant proteins in relation to IgM, following Mono Q chromatography. This resulted in poor separation of IgM from high-molecular-weight contaminants (Fig. 15). Thus, the purification factor of monoclonal IgMs for this dual-step procedure was lower

than for single-step SEC on Superose 6 columns. Other authors reported significant purification of IgM by "non-ideal" SEC following anion-exchange chromatography[8,9,11].

A notable observation in this study was that of variable recovery of hybridoma IgMs following dialysis of ascites fluids and supernatants into the appropriate buffers for the different chromatographic procedures (see Tables II–VI).

The results of this study indicate that monoclonal IgMs can be readily purified in a single step from hybridoma ascites fluid or dialysis culture supernatants to reasonable purity and yield by SEC on Superose 6 HR 10/30 FPLC columns. This system, rather than "non-ideal" SEC, produced significantly enhanced purification of monoclonal IgMs. Other single-step chromatographic procedures based on anion- and cation-exchange and hydroxyapatite columns could not separate monoclonal IgMs from contaminant proteins under the conditions applied. Similarly, sequential use of anion-exchange chromatography and SEC did not resolve the monoclonal IgMs. Comparison of the procedures tested in this study demonstrates that monoclonal IgMs can be rapidly purified on laboratory scale by size-exclusion FPLC, based on their molecular weight. This finding will enable the isolation of naturally occurring monoclonal IgM autoantibodies from concentrated supernatants and ascites fluids for the characterization of their autoantigenic specificities and further understanding of their relationship to autoimmune diseases.

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