CHROM. 23 746

Rapid, two-step purification process for the preparation of pyrogen-free murine immunoglobulin G_1 monoclonal antibodies

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ABSTRACT

A cost-efficient process was specifically designed for the preparation of gram amounts of highly pure murine immunoglobulin (Ig) G_1 monoclonal antibodies (mAbs). This rapid, simple and scalable purification process employs a unique binding and elution protocol for IgG_1 mAbs on a silica-based, mixed-mode ion-exchange resin followed by conventional anion-exchange chromatography. mAbs are bound to BakerBond ABx medium at pH 5.6 directly from serum-supplemented hybridoma culture supernatants. Contaminating proteins and nucleic acids are removed by an intermediate wash at pH 6.5, followed by the specific elution of IgG_1 mAbs with 100 mM Tris–HCl (pH 8.5). The mAb eluate is then loaded directly on to QAE-Sepharose Fast Flow medium and eluted with 10 mM sodium phosphate buffer (pH 7.4), containing 150 mM sodium chloride. The resulting IgG_1 mAbs are greater than 98% pure, free from measurable endotoxin, formulated in a physiological buffer and suitable for *in vivo* applications.

INTRODUCTION

Exciting developments in monoclonal antibody (mAb) technology over the past several years have increased the demand for large amounts of highly pure, non-pyrogenic mAbs suitable for *in vivo* diagnostic and therapeutic usage [1–5]. This demand has led to the design of a variety of chromatographic matrices for the isolation of mAbs from both hybridoma cell culture supernatants and ascites fluids. Typically, mAbs are present as minor protein components in serum-supplemented hybridoma culture media and require extensive purification in order to remove serum proteins, nucleic acids, pyrogens and other non-proteinaceous contaminants prior to use as *in vivo* reagents. Classic methodologies for mAb purification often employ the use of ion-exchange, hydrophobic interaction (HIC) and hydroxyapatite resins [4–8]. These matrices require both labor-intensive method development and purification processes that utilize multiple chromatographic steps to assure a suitably pure, pyrogen-free product.

Affinity-based matrices employing both protein A and protein G have gained widespread acceptance for their ability to specifically purify mAbs from many different species and subclasses [9]. Although the use of these resins can result in preparations of >90% pure mAb in a single step, disadvantages such as their high cost, relatively harsh elution conditions and the potential for leaching of the affinity ligand make these resins less desirable than conventional matrices for designing an economical process for the preparation of gram amounts of highly pure mAbs. In particular, when the desired mAb is a murine immunoglobulin (Ig) G_1 subtype, protein A affinity resins are less useful owing to the lack of binding specificity for this murine mAb isotype at neutral pH [9].

Recently, the silica-based mixed-mode ion exchange medium BakerBond ABx has been successfully used to isolate murine and rat IgG_1 mAbs

from ascites and cell culture supernatants with purities similar to those obtained with either protein A or protein G resins [10,11]. This paper describes a novel and exceptionally cost-efficient process employing both ABx and QAE-Sepharose Fast Flow media for the purification of murine IgG$_1$ mAbs from serum-containing cell-culture supernatants. This rapid, two-step procedure utilizes a unique ABx binding and elution protocol designed specifically for the purification of murine IgG$_1$ mAbs, followed by a second chromatographic step on QAE-Sepharose Fast Flow medium to further purify, concentrate and depyrogenate the mAb preparation for potential *in vivo* applications.

EXPERIMENTAL

Monoclonal antibodies

Anti-LFA3 mAb was purified from cell-culture supernatants harvested from a hybridoma cell line (ATTC HB205) grown in CellPharm hollow-fiber bioreactors (CD Medical, Miami Lakes, FL, USA). Tissue culture medium was composed of α-MEM supplemented with L-glutamine (4 mM), penicillin (50 units/ml), streptomycin (50 mg/l) and 5% fetal bovine serum (FBS). The anti-Lewis mAb was purchased from Helix Biocore (Minneapolis, MN, USA) as a 5% FBS-supplemented RPMI harvest from the hybridoma cell line grown in suspension culture.

Liquid chromatography

All chromatographic procedures were performed on a Pharmacia FPLC system that had been depyrogenated with 0.1 M NaOH as described by the manufacturer. All buffers were made with USP-grade reagents, when available, prepared with Sterile Water For Injection (SWFI) (Baxter Healthcare, Miami, FL, USA), and sterile filtered using Millipore HV 0.22-μm membranes. BakerBond ABx medium (J.T. Baker, Phillipsburg, NJ, USA) and Pharmacia QAE-Sepharose Fast Flow medium (Pharmacia–LKB, Piscataway, NJ, USA) were depyrogenated according to the protocols described by the respective manufacturers prior to use.

mAb characterization

mAb purity was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [12] under reducing and non-reducing conditions using either 12% precast or 4–20% gradient precast minigels (Bio-Rad Labs., Richmond, CA, USA). Total protein was determined using the absorption coefficient of 1.4 ml mg^{-1} cm^{-1} at 280 nm [13]. The amount of IgG$_1$ mAb was determined by employing a sandwich enzyme-limited immunoassay (ELISA) as follows: NUNC maxiSorb 96-well microtiter plates (Cat. No. 468667) were coated with 300 ng of goat anti-mouse whole IgG (Sigma, St. Louis, MO, USA; M8642) in phosphate-buffered saline (PBS), pH 7.2. Plates were blocked with 2% bovine serum albumin (BSA) in PBS for 2 h at 25°C. Aliquots of 100 μl containing IgG$_1$ cell-culture supernatants, purified mAbs or IgG$_1$ standard (murine IgG$_1$ monoclonal anti-human IgM, clone SA-DA4; Fisher Scientific, Pittsburgh, PA, USA) in the range 0.5–20 ng/ml were added and incubated for 2 h at 37°C. Plates were washed with PBS–0.05% Tween 20 and incubated with 100 μl of goat anti-mouse IgG$_1$ conjugated to alkaline phosphatase (Southern Biotech Assoc., Birmingham, AL, USA; Cat. No. 0101-04; diluted 1:500 in 2% BSA–PBS) for 2 h at 37°C. After washing, the plates were developed for 10 min with 100 μl of DNP substrate [1 mg/ml of *p*-nitrophenyl phosphate in diethanolamine, pH 8.9] and read at 405 nm.

Isoelectric focusing (IEF)

IEF was performed using Serva ultra-thin gels having a pI range from 3 to 10. The cathode buffer consisted of 23 mM L-arginine free base, 4 mM L-lysine, 2 M ethylenediamine and 0.1 M NaOH and the anode buffer was 25 mM L-aspartic acid, 25 mM L-glutamic free acid and 0.1 M phosphoric acid. Gels were prefocused (1700 V, 5 mA, 4 W) for 25 min. Protein samples were focused at 200 V, 5 mA, 4 W for 90 V h followed by 1700 V, 5 mA, 4 W for 5500 V h. Gels were fixed in 20% trichloroacetic acid (TCA) for 10 min and protein samples were rendered visible by staining with Serva Blue GW for 15 min. Gels were cleared with water.

Fluorescence-activated cell sorting (FACS) analysis

The functionality of the mAbs was examined by FACS analysis of cells bearing the respective antigens.

Limulus amebocyte lysate (LAL) assay

Endotoxin units were measured using a chromogenic LAL assay purchased from Whittaker Bioproducts (Walkersville, MD, USA). All buffers were routinely tested for acceptable endotoxin levels [< 0.25 endotoxin units (EU)/ml] prior to chromatographic experiments.

RESULTS AND DISCUSSION

The purpose of this study was to develop a rapid, simple, scalable and economical process for the preparation of gram amounts of highly pure, pyrogen-free murine monoclonal IgG$_1$ mAbs which can be used for *in vivo* applications. Ideally, this process would consist of a minimum number of chromatographic steps that could be performed in series without subjecting the mAbs to either dialysis or diafiltration. The presence of high concentrations of albumin and other contaminating proteins in serum-supplemented cell culture supernatants requires that the initial step of the process employ a chromatographic resin having a high capacity, selectivity and specificity for mAbs. The final step of the process should depyrogenate, concentrate and formulate the mAbs in a physiological buffer, thereby making them suitable for potential *in vivo* applications. In addition, the entire chromatographic process should be performed in an environment that maintains sterility and prevents contamination by exogenous endotoxins.

A number of mAbs from different species have been successfully purified using BakerBond ABx medium, a mixed-mode ion-exchange chromatographic resin [2–5,10,11]. ABx medium was developed for process-scale chromatography with properties such as a high capacity for protein binding, good flow characteristics and excellent chemical and physical stability. This resin binds a wide variety of antibodies, while exhibiting weak affinity for albumin, transferrin and other serum proteins at selected pHs and ionic strengths [2–5]. The unique combination of the silica-based, hydrophilic polymer backbone paired with zwitterionic and weakly hydrophobic side-groups capitalizes on the principles outlined by the multi-point interaction hypothesis [14,15], which suggests that the summation of weak forces such as electrostatic, hydrophobic and hydrogen-bonding forces can significantly enhance the affinity for the ligand of interest.

Most protocols for the purification of murine IgG$_1$ mAbs from serum-based cell culture supernatants and ascites using ABx resin require that a significant dilution of the starting medium be made in order to effectively bind the antibody of interest [2–5,10]. Elution of the mAb is usually accomplished by applying linear or stepwise salt gradients to the column at or near neutral pH. For example, Ross *et al.* [10] have reported the isolation of murine IgG$_1$ mAbs from ascites using ABx chromatography where the ascites fluid is diluted with four volumes of 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 5.6) and eluted with a linear gradient from 0 to 1 M sodium acetate (pH 7.0). Although the resulting mAb preparation is >95% pure, the high ionic strength of the ABx eluate is incompatible with subsequent anion-exchange chromatographic steps. Therefore, we sought to develop unique binding and elution conditions for murine IgG$_1$ mAbs which minimize initial sample manipulation and avoid the use of salt gradients. Thus, one could eliminate the need for a dialysis, diafiltration or a significant dilution step prior to anion-exchange chromatography.

The binding efficiency of IgG$_1$ mAbs present in both diluted and undiluted conditioned cell culture supernatants from two different hybridoma cell lines was compared on ABx. The diluted supernatants were prepared by diluting fivefold with 25 mM MES (pH 5.6), while the undiluted supernatants were adjusted to 50 mM MES (pH 5.6) using a stock solution of 1.0 M MES (pH 4.0). No difference in IgG$_1$ binding efficiency was detected when the diluted or undiluted supernatants from either of the two different hybridoma cell lines were compared, based on determination of the mouse IgG$_1$ present in the flow-through fraction by ELISA. This was surprising, considering the significant difference in conductivity between the diluted (2 mS/cm) and undiluted (10 mS/cm) hybridoma cell culture supernatants.

Once the initial parameters for binding of IgG$_1$ mAbs to ABx had been established, we investigated conditions for specifically eluting the mAbs, whereby the ionic strength of the elution buffer would be compatible with a subsequent anion-exchange chromatographic step. A linear gradient from 0 to 100% buffer B (15 mM sodium acetate–35 mM sodium
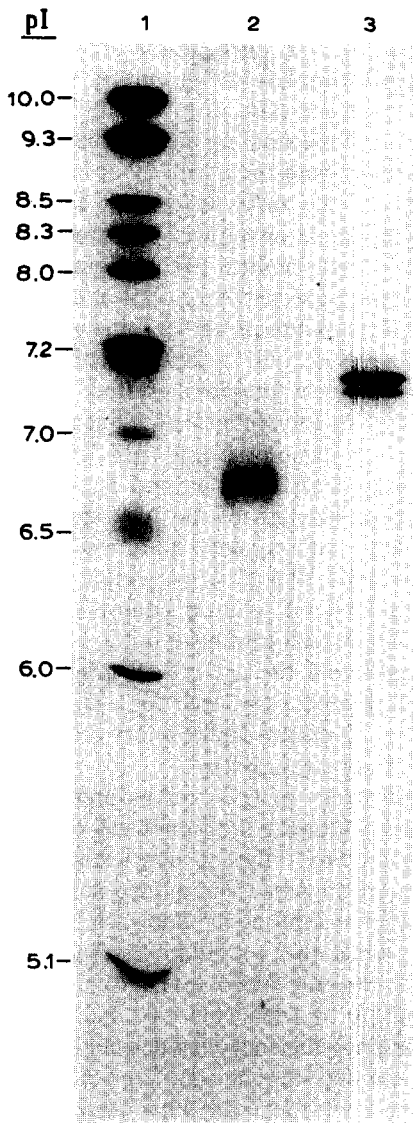
Fig. 1. IEF analysis of purified IgG_1 mAbs. Lanes: 1 = pI standards; 2 and 3 = anti-LFA3 and anti-Lewis mAbs, respectively. A 20-μg amount of purified mAb was loaded for each corresponding lane.

strength of the eluate was not compatible with a subsequent anion-exchange chromatographic step.

The characteristics of the ABx resin, such as a predominance of anionic or cationic side-chains and a relatively narrow pH range within which mAbs bind to the matrix [2–4] suggested to us that a strong buffer with pH > 7.5 could also be effective in eluting IgG_1 mAbs from ABx. We observed that both 100 mM HEPES buffer (pH 7.5) and 100 mM sodium phosphate buffer (pH 7.8) were capable of eluting each of these two mAbs from ABx. However, precipitation of the mAbs was detected in the leading fractions of the mAb eluates using either of these two buffers. Precipitation of the mAbs appeared to be pH dependent, as the fractions from the leading edge of the mAb eluate had a pH between 6.0 and 7.0, while the trailing fractions (which exhibited no precipitation) had a pH of 7.5. The appearance of a precipitate was not entirely unexpected, as the pH of these eluates was near the isoelectric point of both mAbs (Fig. 1). In contrast, no precipitation of either mAb was observed when the ABx column was eluted with 100 mM Tris–HCl buffer (pH 8.5). By stepwise eluting the ABx resin with a strong buffer at a pH significantly above the isoelectric point of the mAb (⩾ 1.5 pH units), precipitation of the mAb was avoided and the mAb eluate is in a buffer compatible for direct application to a sterile, pyrogen-free anion-exchange column.

Based on these results, cell-culture supernatants containing $ca.$ 1.0 g of mAb (3 mg IgG_1/ml) from two different hybridoma cell lines were adjusted to 50 mM MES (pH 5.6) and loaded directly on to separate columns packed with 100 ml of BakerBond Prepscale 40-μm ABx. Based on the quantitative murine IgG_1 ELISA, ABx exhibited a binding capacity of $ca.$ 15 mg mAb/ml resin for undiluted cell culture supernatants containing 5% FBS at pH 5.6. Contaminating proteins were removed by washing the column by stepwise adjustment with 10% buffer B (100 mM Tris–HCl, pH 8.5), where buffer A is 50 mM MES (pH 5.6). This intermediate washing step (resulting pH of 6.5) was very important as it also facilitated the removal of significant amounts of DNA and/or RNA non-specifically bound to the resin (Table I). Both mAbs were specifically eluted with 100 mM Tris–HCl (pH 8.5) (100% buffer B and collected aseptically in sterile, pyrogen-free

phosphate–700 mM ammonium sulfate, pH 7.5), where buffer A is 50 mM MES (pH 5.6), showed that both IgG_1 mAbs eluted between 35% and 50% buffer B. Eluting stepwise with 25% buffer B was sufficient to recover >90% of the applied mAbs with >90% purity, as determined by SDS-PAGE. However, under these conditions, the high ionic

TABLE I

MAB PURIFICATION SUMMARY

Condition/step	Total protein (mg)	Total mAb (mg)	Purity (%)	Recovery (%)	Absorbance ratio (260/280 nm)	Endotoxin (units/mg)
Hybridoma supernatant (ABx load)	9980	1050	10	100	1.2	20
ABx flow-through	8670	40	N.D.	3	N.D.	N.D.
ABx wash	1310	10	N.D.	1	N.D.	N.D.
ABx eluate/QAE load	850	850	>90	85	1.5	3.8
QAE-Sepharose wash	80	0	N.D.	N.D.	1.5	N.D.
QAE-Sepharose eluate	800	800	>98	80	1.8	<0.25

containers. Average mAb recoveries using this protocol were *ca.* 85% based on a murine IgG$_1$ ELISA, with protein purities $\geq$90% (as judged by SDS-PAGE) (Fig. 2). Pyrogen levels were also reduced significantly by over five fold for both mAb samples (Table I).

In order to assure a purity of >98% and a preparation which was free from detectable pyrogens, a second process step was developed to meet the above criteria and to concentrate and formulate the purified mAbs for *in vivo* studies. QAE-Sepharose Fast Flow and BakerBond MAb media are anion-exchange resins which have a high binding capacity for antibodies (8–10 mg/ml resin) and bacterial endotoxins, and display good flow characteristics [3,4,16]. For anion-exchange chromatography,
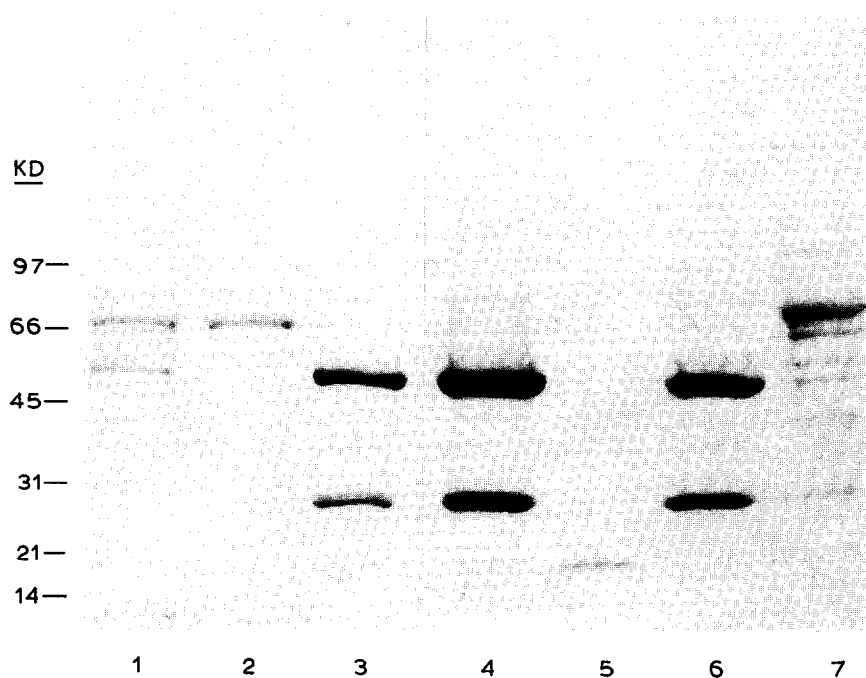

Fig. 2. SDS-PAGE of mAb purification by ABx and QAE-Sepharose Fast Flow. Lanes: 1 = initial ABx load from hybridoma culture supernatants; 2 = ABx flow-through; 3 = 100 m*M* Tris (pH 8.5) eluate from ABx column; 4 = QAE-Sepharose Fast Flow load (ABx eluate); 5 = QAE-Sepharose Fast Flow intermediate wash step; 6 = PBS eluate of QAE-Sepharose Fast Flow; 7 = 300 m*M* NaCl eluate of QAE-Sepharose Fast Flow. kD = kilodalton.

QAE-Sepharose Fast Flow was chosen based on the ability to elute the purified mAbs with an isotonic phosphate buffer. Each mAb eluate was diluted twofold with SWFI to reduce the conductivity to less than 2 mS/cm and applied to 150 ml of sterile, pyrogen-free QAE-Sepharose Fast Flow medium equilibrated in 10 mM sodium phosphate buffer (pH 7.4). The resin was washed extensively with equilibration buffer (> 10 column volumes) to remove loosely bound contaminants, and the mAbs were recovered by stepwise elution with 10 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl. Recoveries for each mAb were between 85 and 90% based on the murine IgG$_1$ ELISA, with purities > 98% as judged by SDS-PAGE. Endotoxin levels were reduced further by ten fold (in comparison with the levels after ABx chromatography) to less than 0.25 EU/ml. The 280/260 nm absorbance ratio increased from 1.5 following ABx chromatography to 1.8 after chromatography on QAE-Sepharose Fast Flow, indicating the removal of additional nucleic acids contaminants (Table I). Most of the remaining protein contaminants remain bound to QAE-Sepharose Fast Flow and can be eluted by a 300 mM NaCl wash (Fig. 2).

Protein A medium has also been utilized to achieve > 90% purity for murine IgG$_1$ mAbs from cell culture supernatants in a single chromatographic step [9,17]. However, murine IgG$_1$ mAbs do not have a high affinity for protein A under physiological conditions [18]. Therefore, the ionic strength must be raised above 1.0 M NaCl, and preferably to 3 M NaCl, and the pH adjusted to 9.0 in order to achieve quantitative binding. For large-scale processes, this pH and ionic strength adjustment is cumbersome and may lead to mAb aggregation or precipitation as the medium is titrated through the isoelectric point of the mAb. The mAb can then be eluted from protein A medium by decreasing both the pH and ionic strength, but after this step the ionic strength of the eluate is too high (owing to residual NaCl in the binding buffer) for subsequent chromatography on anion-exchange resins. Therefore, an intermediate manipulation such as dialysis, diafiltration or a significant dilution is required to reduce the conductivity of the eluate sufficiently in order to achieve effective binding on anion-exchange resins. An alternative to diafiltration of protein A purified mAbs would be to utilize hydrophobic interaction chromatography (HIC) as a second step, whereby the mAb sample is bound to an HIC matrix in high salt at neutral pH and eluted from the HIC resin by lowering the ionic strength of the buffer [3,7]. However, when this method was employed with the both anti-LFA3 and anti-Lewis IgG$_1$ mAbs, the samples were dilute (< 200 μg/ml) and the recovery was poor (< 50%), thus requiring an extra concentration step to be performed under sterile, pyrogen-free conditions prior to use. These observations, combined with the relatively low capacity of protein A for murine IgG$_1$ mAbs (ca. 3 mg mAb/ml protein A Fast Flow Sepharose), and the much higher cost, make protein A-based media less attractive than ABx for the initial step in large-scale mAb purification processes.

The economics of this large-scale chromatographic process for the purification of murine IgG$_1$ mAbs are worth noting. Using the protocol described here, the cost of purifying ca. 1 g of mAb in a single experimental run using 75 ml of ABx medium (assuming a capacity of 15 mg mAb/ml resin) is ca. 30 times less than using 350 ml of protein A Fast Flow Sepharose from the most economical source available. Although one could certainly use less protein A medium and employ multiple binding and elution passages, the labor cost required to conduct numerous runs must be included in the overall production cost, aside from the practical constraints of pH and ionic strength adjustments which must be done and the inability to link the steps of a purification process together in a practical and convenient manner. In summary, the process described here takes advantage of both the excellent chromatographic properties and the cost-efficient nature of both ABx and QAE-Sepharose Fast Flow media for the routine preparation of gram amounts of highly pure, pyrogen-free murine IgG$_1$ mAbs.

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