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PURIFICATION OF MONOCLONAL ANTIBODIES AGAINST THE LOW-DENSITY LIPOPROTEIN RECEPTOR BY PREPARATIVE ISOTACHOPHORESIS

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SUMMARY

A preparative free-flow isotachophoretic method for the purification of monoclonal antibodies from mouse ascites fluid and tissue culture media is described. This high-resolution method allows the direct separation of monoclonal antibodies from antibody-containing tissue culture media or ascites fluid and gives a better separation from the major contaminant protein fractions and a higher recovery of the monoclonal antibodies than anion-exchange chromatography. The purification can run continuously and without any time-consuming regeneration procedures; the monoclonal antibody is obtained under mild conditions in a small electrolyte volume.

INTRODUCTION

The use of monoclonal antibodies is increasing rapidly in several fields of natural and medical sciences [1–3]. The procedures generally used for monoclonal antibody production are those in tissue culture media and mouse ascites fluid [4–7]. Antibody concentrations of several mg/ml can be achieved when monoclonal antibodies are purified from mouse ascites, which contains a protein pattern similar to the serum proteins. Cell culture methods, however, result in much lower antibody concentrations of only a few μg/ml and relatively high levels of various protein contaminants, mainly due to the addition of foetal calf serum (FCS). This can be partially prevented when synthetic media are used [8]. Moreover, some other aspects, e.g., costs, simplicity and rapidity, should be taken into consideration when selecting the most suitable procedure for the purification of monoclonal antibodies.

Several methods are available for the preparative purification of monoclonal antibodies from tissue culture media or mouse ascites fluid, e.g., ammonium sul-

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phate precipitation [9], anion-exchange chromatography [10], affinity chromatography (immobilized Protein A or immobilized antigen) [11,12] and preparative high-performance liquid chromatography (HPLC) [13]. Often these procedures must be used in combination and all of them have their own drawbacks.

Anion-exchange chromatography using DEAE-cellulose or DEAE-Affigel Blue causes problems when samples with highly diluted monoclonal antibodies are applied. Under these conditions the gel matrix cannot separate the immunoglobulins from other proteins efficiently and the antibodies from the samples are even more diluted [10]. Affinity chromatography using Protein A–Sepharose overcomes this problem, but the antibodies must be eluted with buffers of low pH, which may alter the characteristics of the antigen–antibody interaction [11,12]. HPLC procedures allow the separation of immunoglobulins and their subclasses to a high degree, but expensive columns must be used [13].

In addition to column chromatography, electrophoretic techniques such as zone electrophoresis [14], isoelectric focusing (IEF) [15] or isotachophoresis (ITP) [16–20] could be used for the analytical or preparative separation of monoclonal antibodies. Free-flow ITP is an electrophoretic technique that allows the separation of ionic sample components based on differences in their net electric mobilities using a discontinuous electrolyte system. The leading electrolyte must contain an ion species with a mobility higher than that of any of the sample ions of interest and the terminating electrolyte must contain an ion species with a mobility lower than that of any of the sample ions of interest. In addition, a common counter ion is used, which should have a good buffering capacity in the pH range within which the separation takes place. When the system has reached equilibrium, all ions move at the same speed, individually separated into a number of zones in immediate contact with each other. Therefore, to achieve optimal separation, intermediate-mobility compounds (spacer ions) are added to the sample solution. The optimal selection of spacer ions has to be determined experimentally [16–20].

In this paper we present a simple and rapid method for the purification or separation of proteins from liquid media by ITP. It allows the continuous purification of monoclonal antibodies from ascites fluid and tissue culture media under mild conditions. Highly diluted samples need not be previously concentrated, as ITP can be performed using optional volumes.

EXPERIMENTAL

Materials

RPMI 1640 tissue culture medium, Hank's solution and hypoxanthine–thymidine (HT) supplement were obtained from Gibco (Karlsruhe, F.R.G.), FCS from Boehringer (Mannheim, F.R.G.), DEAE-Trisacryl from Pharmacia–LKB (Freiburg, F.R.G.), immobilized Protein A from Pierce (Weiskirchen, F.R.G.), mouse IgG as standard and sheep anti-mouse IgG antibody from Cappel/Cooper (Frankfurt/Main, F.R.G.), antisera against mouse IgG subclasses from Camon (Wiesbaden, F.R.G.) and fluoresceinisothiocyanate (FITC)-conjugated goat anti-mouse IgG from Becton Dickinson (Heidelberg, F.R.G.). 6-Aminocaproic acid (EACA), polyvinylpyrrolidone (PVP) (M_r 360 000) and 2-amino-2-methyl-1,3-

propanediol (ammediol) from Sigma (Deisenhofen, F.R.G.). All other biochemicals were purchased from Sigma or Serva (Heidelberg, F.R.G.).

Monoclonal antibodies in tissue culture medium

C7 hybridoma cells (ATCC No. CRL-1691), secreting monoclonal antibodies against the low-density lipoprotein (LDL) receptor [21], were cultivated in HT medium (pH 6.8–7.0) containing RPMI 1640, streptomycin (100 μg/ml), penicillin (10 000 U/l), glutamine (3.18 m*M*) and 15% (w/v) FCS in a humidified carbon dioxide incubator at 37°C. After 3 days in culture the cells were pelleted by centrifugation (200 *g* for 10 min). The supernatant medium, containing the monoclonal antibodies, was collected and cleared by centrifugation (800 *g* for 10 min). The cells were resuspended in culture medium and split for further incubation.

Recloning of the C7 hybridoma cell line was performed using mouse bone-marrow macrophages. C7 hybridoma cells were co-cultivated with macrophages in microtitre plates (each hole containing one cell) for 7 days at 37°C under a 5% carbon dioxide atmosphere. Intact hybrid clones were identified by microscopic inspection and isolated for further cultivation [22].

Monoclonal antibodies in mouse ascites

About 10^6–10^7 hybridoma cells were injected into 2,6,10,14-tetramethylpentadecane (pristane)-primed 3-month-old BALB/c mice [7]. After development of the ascites tumours for 2–3 weeks, the mice were punctured for collection of ascites fluid in phosphate-buffered saline (PBS) containing 50 U/l Liquemin (La Roche). Centrifugation (800 *g* for 10 min) yielded a clear medium containing the monoclonal antibodies.

Preparative isotachophoresis

Preparative ITP for the separation of monoclonal antibodies was carried out with an Elphor VAP 22 (Bender und Hobein, Munich, F.R.G.). Culture medium (100 ml) dialysed overnight against 40 m*M* ammediol or ascites fluid diluted 1:10 in 40 m*M* ammediol were injected into the electrophoresis chamber intermediate between the laminar streaming zones of the leading buffer [20 m*M* HCl–40 m*M* ammediol (pH 8.9)–1% PVP] and the terminating buffer [40 m*M* EACA–40 m*M* ammediol (pH 10.0)–1% PVP]. To achieve a better separation, valine (10 mg/ml) and β-alanine (10 mg/ml) were added as spacers. The sample was separated with a voltage of 700 V and a current of 48 mA at a sample flow-rate of 40 ml/h. Phenol red was used as a marker dye for process control. The separated zones were collected through 90 channels into 5-ml fractions. Collection was monitored at 280 nm. The protein content was measured according to the method of Lowry et al. [23] and mouse IgG determined by enzyme-linked immunosorbent assay (ELISA) (see below).

Anion-exchange chromatography

A 100-ml volume of culture medium was concentrated to 20 ml with an Amicon ultrafiltration cell (YM-100 membrane). For the separation of monoclonal antibodies from higher volumes of medium, an Amicon hollow-fibre system was

used. The concentrated tissue culture medium was dialysed against 0.025 *M* Tris–HCl (pH 8.8) containing 0.035 *M* sodium chloride and 0.05% sodium azide and applied to a 20-ml column of DEAE-Trisacryl, which had been washed with three volumes of 1 *M* sodium chloride equilibrated with three volumes of the Tris buffer. Immunoglobulins were eluted with the equilibration buffer at a flow-rate of 20 ml/h and collected in 2-ml fractions. The protein concentration was determined by the procedure of Lowry et al. [23] and the mouse IgG content of each fraction was measured by ELISA (see below). The IgG-positive fractions were pooled and concentrated to a volume of 10 ml.

Immobilized Protein A chromatography

Mouse ascites fluid and tissue culture medium purified by ITP or anion-exchange chromatography were dialysed against buffer A [1 *M* Tris–HCl (pH 9.9)–0.05% sodium azide] and applied to a Protein A–Sepharose column (10-ml gel bed volume), which had been washed with three volumes of buffer A and buffer B [0.1 *M* glycine–HCl (pH 3.0)–0.05% sodium azide] and equilibrated with three volumes of buffer A. Elution was performed at a flow-rate of 15 ml/h, applying 15 ml of each buffer, first buffer A, then buffer C [0.1 *M* citrate (pH 6.0)–0.05% sodium azide] and buffer D [0.1 *M* citrate (pH 4.5)–0.05% sodium azide] and last buffer B. IgG-containing fractions were collected in 1 *M* dipotassium hydrogen phosphate to raise immediately the low pH. After IgG determination, the positive fractions were pooled, concentrated and dialysed against PBS (pH 7.3).

SDS gel electrophoresis

Cell membranes of human skin fibroblasts were solubilized according to the method described by Havinga et al. [24] and fractionated, as described by Laemmli [25], by one-dimensional SDS gel electrophoresis on 7.5% (w/v) polyacrylamide vertical slab gels (1.5 mm), containing 0.1% (w/v) SDS, in a Bio-Rad Protean II slab gel electrophoresis apparatus. Samples containing 200 μg of protein per slot were adjusted to a final concentration of 2% SDS, 10% (v/v) glycerol, 10 m*M* Tris (pH 6.8) and 0.1% (w/v) bromophenol blue, but without mercaptoethanol. Electrophoresis was carried out at 10°C with 30 mA per slab gel for 5 h. Gels were calibrated with the following M_r standards: α_2-macroglobulin, 180 000; β-galactosidase, 116 500; fructose-6-phosphate kinase, 84 000; pyruvate kinase, 58 000; fumarase, 48 500; lactate dehydrogenase, 36 500; and triose phosphate isomerase, 26 600. Gels were either stained with Coomassie Blue R 250 or silver stained with a modified Bio-Rad Silver Stain kit [26].

Electrophoretic transfer of proteins to Immobilon® membranes

Proteins were transferred from SDS gels on to Immobilon poly(vinylidene difluoride) (PVDF) membranes (Millipore) according to the technique of Burnette [27] in a Bio-Rad Trans Blot cell. Electrophoretic transfer was carried out for 16 h at 4°C and 40 V with the anode on the membrane side of the blot sandwich using 20 m*M* Tris–150 m*M* glycine–20% (v/v) methanol (pH 8.3) as blotting buffer.

Immunoblotting [28,29]

For the determining of LDL receptor protein binding, the Immobilon membrane strips were blocked for 4 h at 0°C with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) [50 m*M* Tris–HCl (pH 8.0)–90 m*M* sodium chloride] and then incubated for 16 h in fresh buffer containing 2 μg/ml monoclonal antibody against the LDL receptor. The strips were transferred for 1 h into a 1:400 dilution of biotinylated rabbit anti-mouse IgG (Boehringer) and then for 30 min into a 1:400 dilution of biotinylated streptavidin HRPO complex (Amersham). Between each incubation step the strips were washed three times for 10 min in PBS containing 1% BSA and 0.05% Tween 20. Bound antibodies were detected by incubation of the strips in 0.018% 4-chloro-2-naphthol (60 mg in 20 ml of methanol) in 100 ml of 10 m*M* Tris–HCl (pH 7.4)–150 m*M* sodium chloride containing 100 μl of 30% hydrogen peroxide.

Detection of mouse immunoglobulins

Mouse immunoglobulins were identified with a sandwich ELISA using sheep anti-mouse IgG coated wells of microtitre plates. Mouse IgGs in appropriate dilutions were used as standards. The reaction of the mouse IgG-bound peroxidase-labelled antibodies with *o*-phenylenediamine–hydrogen peroxide was measured with a Dynatech ELISA reader [30–32].

IgG subclasses of monoclonal antibodies

IgG subclasses were determined by use of antisera against mouse IgGs. The monoclonal antibody-containing samples were incubated in wells of microtitre plates coated with antibodies against mouse IgG subclasses. Peroxidase-conjugated anti-mouse IgG was applied, then *o*-phenylenediamine–hydrogen peroxide was added and the reaction measured at 490 nm.

Separation of monocytes by density gradient centrifugation

Leukocytes were isolated by a modification of the method described by Boyum [33]; 6 ml of EDTA blood, obtained from healthy donors, were overlayered to 4 ml of Neutrophil isolation kit (Packard), followed by centrifugation at 400 *g* for 30 min to separate the granulocyte and the monocyte cell populations. The upper white cell layer was collected with a Pasteur pipette and washed three times with PBS at 200 *g* for 5 min. Cell counting was performed using a TOA CC-800 and the leukocytes were diluted in PBS to a final concentration of $2 \cdot 10^6$ cells/ml.

Incubation of cells with monoclonal antibodies against the LDL receptor

After 48 h preincubation of the monocytes in RPMI containing either 10% FCS or 10% lipoprotein-deficient serum (LPDS), the cells were washed three times in PBS, then $4 \cdot 10^5$ cells were incubated at 4°C for 1 h with the monoclonal antibodies in a total volume of 500 μl. The cells were washed twice as above and then incubated for 30 min with 4 μg/ml FITC-conjugated goat anti-mouse IgG antibody. The cells were washed a further twice and analysed by flow cytometry.

Flow cytometry [34]

Fluorimetric analysis was performed using a Becton Dickinson FACStar equipped with a 2-W argon ion laser. The excitation wavelength was 488 nm; emission was measured at 535 ± 20 nm by means of a band-pass filter. In addition to the fluorescence right-angle light scatter, the electronic volume signal was gained. Cellular fluorescence was analysed after gating the scatter versus the volume dot plot in order to eliminate the fluorescence of destroyed cells or doublets. Each value was determined from the signals of 10 000 cells, and calculation was based on the mean fluorescence.

RESULTS

ITP of mouse ascites fluid and tissue culture medium

Ascites fluid (ca. 10 ml) was collected by puncture of ascites tumours developed in BALB/c mice producing monoclonal antibodies against the LDL receptor (Table I). After 1:10 dilution with 40 m*M* ammediol the solution was injected directly into the VAP 22 electrophoresis chamber between the leading and terminating electrolytes, as described in detail under Experimental.

When monoclonal antibodies were isolated from tissue culture medium, a much larger volume compared with ascites fluid was required to obtain significant amounts of monoclonal antibodies (Table I). Here the tissue culture medium (100 ml) was not diluted, but dialysed overnight against 40 m*M* ammediol. After dialysis the medium was injected without any concentration step, since preparative ITP as described here, can be performed as a continuous procedure purifying optional volumes of monoclonal antibody-containing solutions. The elution conditions were the same as for the purification of mouse ascites fluid. The absorption profiles of mouse ascites fluid (Fig. 1A) and of tissue culture medium (Fig. 1B) are not directly comparable, since the final protein content in the tissue culture medium (5 mg/ml) used here was significantly higher than that in the 1:10 diluted mouse ascites fluid (0.7 mg/ml). As shown in Fig. 1, most of the proteins eluted between fraction 15 and 40, both for the purification of mouse ascites fluid and tissue culture medium. The fractions marked with arrows (Fig. 1A and B), contained the immunoglobulins, as verified by protein and mouse IgG determination (Fig. 2).

The monoclonal antibodies isolated from mouse ascites fluid were found predominantly in the arrow-marked fractions 32–34 and could be separated clearly from most of the contaminating proteins (Figs. 1A and 2A). The total concentration of mouse IgG in these fractions was about 8 mg, i.e., 94% of mouse IgG was recovered after ITP. The quantification of protein in fractions 32–34 (Fig. 2A) showed that mouse IgG represented 92% of the total protein (Table II). Because of the relatively high concentration of monoclonal antibodies obtained from ITP of ascites fluid, a second purification step is not necessary.

Mouse antibodies separated from tissue culture medium were found in fractions 31–33 (Figs. 1B and 2B). The total concentration of mouse IgG was much lower (110 μg) than in purified mouse ascites fluid (8 mg) and the mouse IgG-positive fractions still contained a large amount of other proteins. The mono-

TABLE I

PURIFICATION STEPS FOR THE ISOLATION OF MONOCLONAL ANTIBODIES AGAINST THE LDL RECEPTOR FROM MOUSE ASCITES FLUID (COMPARISON BETWEEN ITP AND PROTEIN A CHROMATOGRAPHY) (A) AND TISSUE CULTURE MEDIUM (COMPARISON BETWEEN ITP/PROTEIN A CHROMATOGRAPHY AND DEAE-TRISACRYL/PROTEIN A CHROMATOGRAPHY) (B)

Defined volumes (10 ml of mouse ascites fluid and 100 ml of tissue culture medium) were used in order to compare the efficiencies of the different purification procedures (for quantitative results see Table II).

(A) Ascites fluid: purification procedure

ITP		Protein A	
Applied volume (ml)	Purification step	Applied volume (ml)	Purification step
10	1:10 dilution	10	Dialysis
	ITP		Protein A
15	Dialysis	20	Dialysis

(B) Culture medium: purification procedure

ITP/Protein A		DEAE-Trisacryl/Protein A	
Applied volume (ml)	Purification step	Applied volume (ml)	Purification step
100	Dialysis	100	Concentration
	↓	20	Dialysis
	ITP		DEAE-Trisacryl
15	Dialysis	30	Concentration
	↓	10	Dialysis
	Protein A		Protein A
10	Dialysis	10	Dialysis
	Concentration		Concentration

clonal antibodies obtained from ITP of tissue culture medium can be applied directly after dialysis against 1 *M* Tris-HCl to the Protein A column without further concentration (Table I). The IgG/protein ratio after ITP of tissue culture medium (5 μg of IgG per mg of protein) was considerably lower than that after ITP of mouse ascites fluid (860 μg of IgG per mg of protein). From a total mouse IgG content of 110 μg per 100 ml of tissue culture medium, 100 μg were recovered after dialysis against 40 m*M* ammediol and 85 μg were found after ITP, i.e., the yield in tissue culture medium was 77% compared with 94% obtained from ITP of mouse ascites fluid (for details see Table II).

Immobilized Protein A chromatography of mouse ascites fluid

Because of the high concentration of monoclonal antibodies against the LDL receptor and the small volume, mouse ascites fluid could be applied directly after

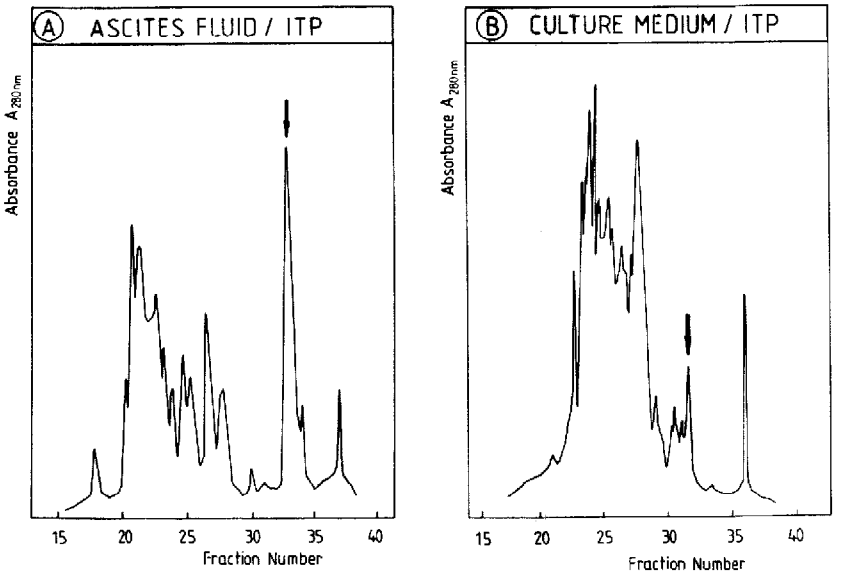

Fig. 1. Absorption profiles at 280 nm obtained from analytical isotachophoresis (ITP) of 10 ml of mouse ascites fluid diluted 1:10 in 40 m*M* ammediol (A), and 100 ml tissue culture medium dialysed overnight against 40 m*M* ammediol (B), both containing the monoclonal antibody against the LDL receptor. Separation conditions: leading buffer, 20 m*M* HCl–40 m*M* ammediol (pH 8.9)–1% PVP; terminating buffer, 40 m*M* EACA–40 m*M* ammediol (pH 10.0)–1% PVP. Spacers: 10 mg/ml valine, 10 mg/ml *β*-alanine. Protein content monitored at 280 nm; IgG peaks are marked with arrows.

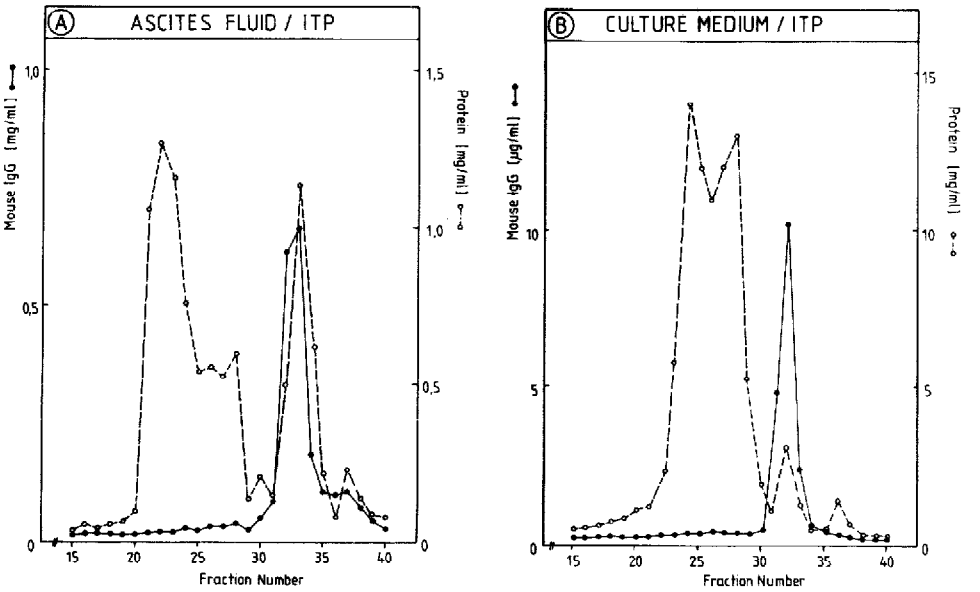

Fig. 2. Determination of protein and mouse IgG of ascites fluid (A) and tissue culture medium (B) after preparative isotachophoresis. Separation conditions as in Fig. 1. Fractions obtained by ITP collected in volumes of 5 ml. Protein content measured according to the procedure of Lowry et al. [23]; determination of mouse IgG performed by sandwich ELISA.

TABLE II

QUANTITATIVE ANALYSIS OF THE ANTIBODY SEPARATION PROCEDURES

The data presented refer to a volume of 10 ml of mouse ascites fluid purified by ITP and Protein A chromatography and 100 ml of tissue culture medium purified by ITP/Protein A chromatography and DEAE-Trisacryl/Protein A chromatography (see Experimental and Table I). After each step of the purification, aliquots of the indicated fractions were removed for quantitation of protein and mouse IgG.

Sample	Method	Protein (mg)		IgG (mg)		mg IgG/ mg protein	Purification factor	Yield (%)
		Applied	Recovered	Applied	Recovered			
Ascites fluid		70		8.5		0.12		100
	ITP	70	9.3	8.5	8.0	0.86	7.2	94
	Protein A	68	8.8	8.3	8.1	0.92	7.7	95
Culture medium		510		0.110		$0.2 \cdot 10^{-3}$		100
	ITP	490	17	0.100	0.085	$5.0 \cdot 10^{-3}$	23	77
	DEAE-Trisacryl	470	35	0.090	0.065	$1.9 \cdot 10^{-3}$	9	59
	ITP/Protein A	16	0.09	0.080	0.073	0.849	3859	66
	DEAE-Trisacryl/ Protein A	32	0.06	0.058	0.051	0.836	3800	46

dialysis to the Protein A column without previous concentration or any other pretreatment (see Table I).

As shown in Fig. 3, almost 90% of the total protein eluted at pH 9 (fractions 4–18) and pH 6 (fractions 19–24), but no mouse IgG appeared. The mouse immunoglobulins were found quantitatively at pH 4.5 (fractions 25–40), the characteristic pH for the mouse IgG 2b subclass [21,35]. Fractions 27–36 contained the mouse antibodies, as determined by ELISA (see below). The purification factor and the yield of mouse IgG after Protein A chromatography of mouse ascites fluid are approximately the same as for ITP of mouse ascites fluid (see Fig. 2 and Table II).

DEAE-Trisacryl chromatography of tissue culture medium

Anion-exchange chromatography using DEAE-Trisacryl gel yielded a better separation of IgG from the major protein fraction and a shorter separation time when a high concentration of IgG in a small volume was applied. Thus, tissue culture medium containing the monoclonal antibody against the LDL receptor was concentrated (Table I) from 100 ml to 20 ml before dialysis against the equilibration buffer and then applied to the DEAE-Trisacryl column. Protein and mouse IgG were determined in every second fraction of the eluate, which was collected in 2-ml aliquots (Fig. 4). When tissue culture medium was purified by DEAE-Trisacryl chromatography, the proteins were separated into two groups; the first group (fractions 4–28) containing the monoclonal antibodies was eluted with the equilibration buffer, whereas the second (fractions 29–52) eluted at a concentration of 1 *M* sodium chloride (Fig. 4). Mouse IgG was found in fractions 10–24, in a total volume of 30 ml (Table I). The IgG-positive fractions were pooled

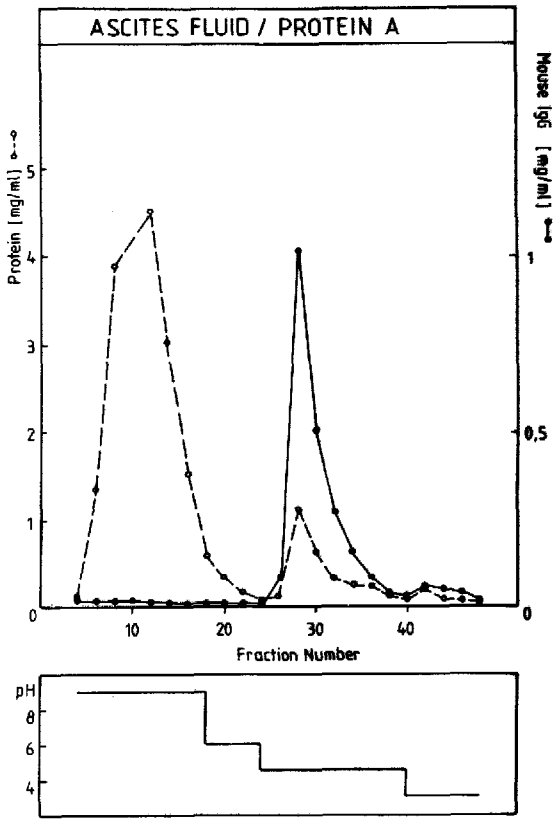

Fig. 3. Determination of protein and mouse IgG content of 10 ml of mouse ascites fluid purified by Protein A chromatography. Elution buffers: 1 *M* Tris–HCl (pH 9.0), 0.1 *M* citrate (pH 6.0), 0.1 *M* citrate (pH 4.5), 0.1 *M* glycine–HCl (pH 3.0). Collection in fractions of 2 ml.

and concentrated to a volume of 10 ml before Protein A chromatography. In comparison with ITP, one additional purification step was necessary (Table I). The amount of recovered IgG (65 μg) and the yield (59%) were not as high as that obtained from ITP (85 μg and 77%; see Table II). The IgG/protein ratio (1.9 μg of IgG per mg of protein) was lower than that determined after ITP (5.0 μg of IgG per mg of protein).

Protein A chromatography of tissue culture medium purified by ITP and DEAE-Trisacryl chromatography

The antibody-containing fractions obtained either from ITP or from DEAE-Trisacryl chromatography of tissue culture medium were applied after dialysis to the Protein A column and eluted using several buffers of different pH (9.0, 6.0, 4.5, 3.0), as described under Experimental. Mouse immunoglobulins were separated effectively from the other proteins (Fig. 5A and B). The major protein fraction was eluted directly at pH 9.0 without binding to the immobilized Protein A (fractions 4–18). At pH 6.0 no protein was found (fractions 19–24). Mouse immunoglobulins were eluted nearly quantitatively at pH 4.5 (fractions 25–40),

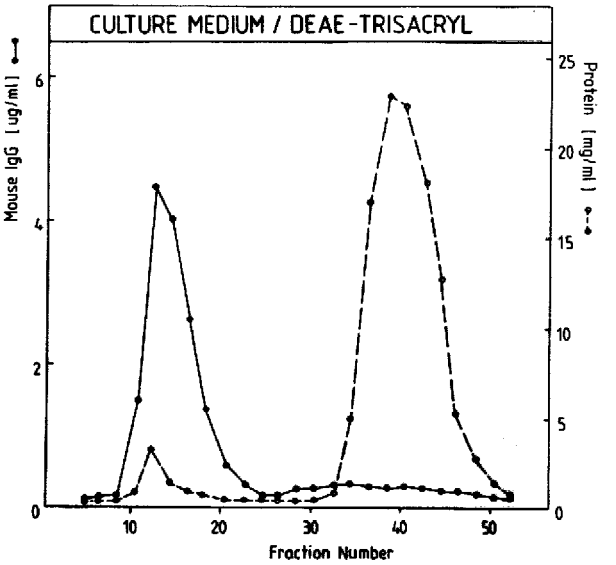

Fig. 4. Determination of protein and mouse IgG after DEAE-Trisacryl chromatography of 100 ml of tissue culture medium concentrated to 20 ml and dialysed against 0.025 *M* Tris-HCl (pH 8.8) containing 0.035 *M* NaCl. Immunoglobulins obtained by elution with the equilibration buffer and collected in 2-ml fractions (fractions 9–28). Elimination of other proteins from the DEAE-Trisacryl gel by 1 *M* NaCl (fractions 29–52). Protein and mouse IgG determined in every second fraction.

the characteristic pH for mouse IgG 2b subclass [21,35]. At a lower pH no further IgGs were found (fractions 42–48). ELISA for the detection of mouse IgG showed that the monoclonal antibodies were present in fractions 28–36 (Fig. 5). These fractions were pooled, dialysed and concentrated to a volume of 2 ml (Table I).

As shown in Fig. 5A and Table II, the amount of recovered mouse IgG (73 μg) obtained from ITP and Protein A chromatography of tissue culture medium was lower than after ITP (85 μg). The determination of the IgG/protein ratio (849 μg of mouse IgG per mg of protein) demonstrated a high degree of purification after Protein A chromatography based on the specific characteristics of Protein A [11]. The purification steps and the elution profile of Protein A chromatography of tissue culture medium after DEAE-Trisacryl chromatography were in principle the same as for Protein A chromatography of ITP-purified medium (Fig. 5A and B). The IgG/protein ratio after DEAE-Trisacryl/Protein A chromatography (836 μg of mouse IgG per mg of protein) was similar to that obtained from ITP/Protein A chromatography (849 μg of mouse IgG per mg of protein). However, the amount of recovered mouse IgG was significantly lower after DEAE-Trisacryl/Protein A chromatography (51 μg of mouse IgG) than after ITP/Protein A chromatography (73 μg of mouse IgG) (Table II).

Determination of the IgG subclass

The purified antibody was determined by ELISA using antisera against mouse IgG subclasses. The monoclonal antibody against the LDL receptor was identified as IgG 2b; this result was verified also from the elution profile of Protein A

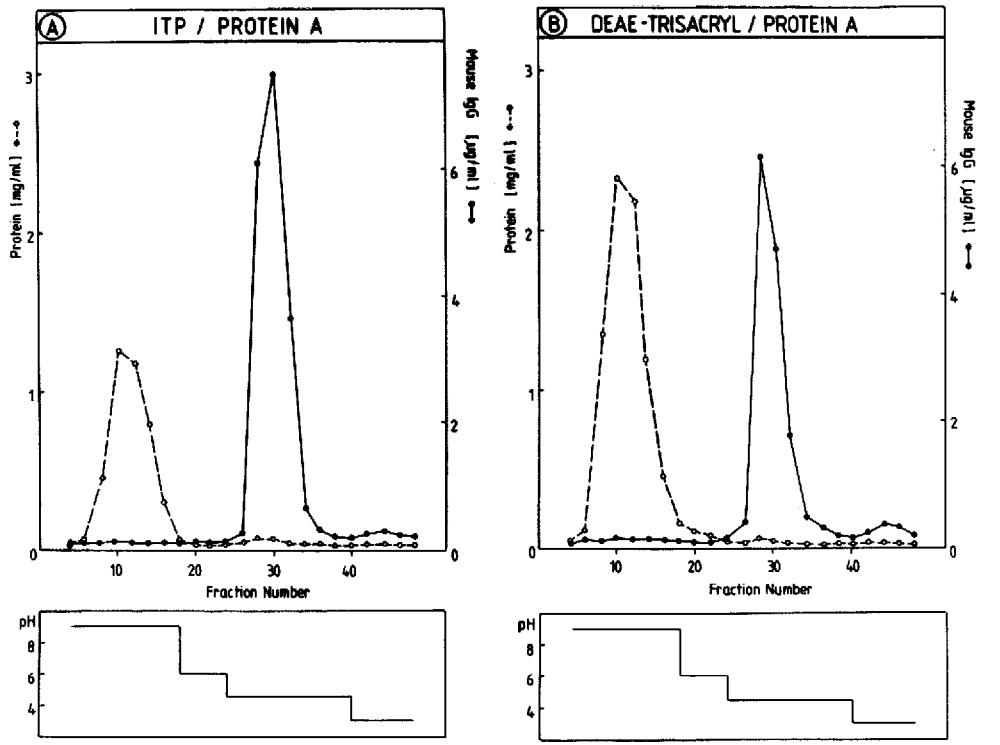

Fig. 5. Determination of protein and mouse IgG of tissue culture medium after ITP/protein A chromatography (A) and DEAE-Trisacryl/Protein A chromatography (B). Pooled IgG-containing fractions obtained from ITP and DEAE-Trisacryl chromatography were treated as described for the purification of mouse ascites fluid by Protein A chromatography (see Fig. 3 and Experimental).

chromatography, where mouse IgG 2b generally elutes at pH 4.5 [21,35]. Other mouse IgG subclasses were found only in extremely small amounts.

Electrophoresis and immunoblotting using the monoclonal antibody against the LDL receptor

In order to verify the nature of the isolated antibody, solubilized membranes of human skin fibroblasts which contain the LDL receptor protein were used for SDS gel electrophoresis and immunoblotting (Fig. 6), as described under Experimental. With the purified monoclonal antibody against the LDL receptor a single band of M_r 130 000 was detected on blot strips from whole membrane protein solubilizates (Fig. 6C). These results are in good agreement with those published by Havinga et al. [24]; they detected the LDL receptor by immunoblotting and ligand blotting on solubilized membranes of fibroblasts, Hep G2 cells and HeLa cells at a similar M_r.

Flow cytometric LDL receptor analysis on human blood monocytes

The saturation curves obtained from flow cytometry using the ITP-purified FITC-conjugated monoclonal antibodies against the LDL receptor for human

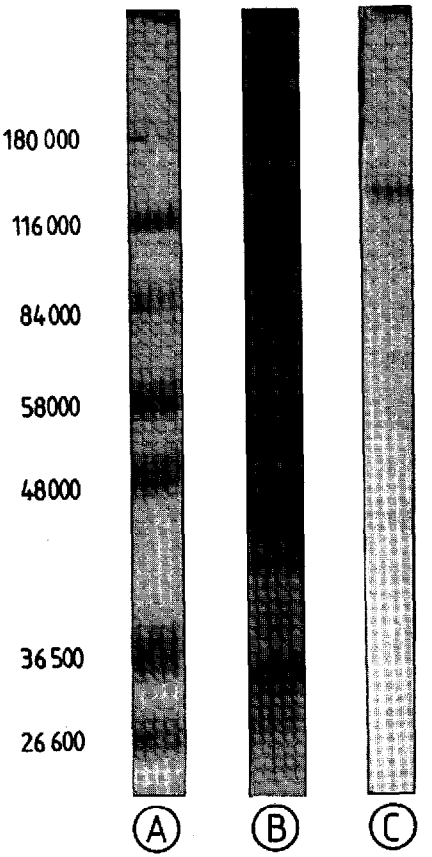

Fig. 6. Immunoblotting of LDL receptors on human skin fibroblasts. Solubilized cell membranes of human skin fibroblasts were fractionated by electrophoresis on 7.5% polyacrylamide gel in the presence of SDS, but without reducing agents. The proteins were transferred to Immobilon (PVDF) membranes. A, M_r standards; B, Coomassie Blue-stained proteins; C, visualization of the receptor after incubation with 2 μg/ml of the LDL receptor antibody using biotinylated anti-mouse IgG and biotinylated streptavidin-peroxidase complex.

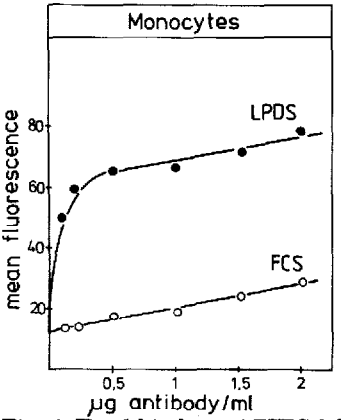

Fig. 7. Total binding of FITC-labelled antibody on human monocytes isolated and treated as described under Experimental. Cellular fluorescence calculated from the gated volume/scatter dot plot; amount of FITC-labelled antibody was plotted against the total mean fluorescence values of each cell population. •, Total binding of the antibody to cells preincubated for 48 h in RPMI containing 10% LPDS; ○, values of cells preincubated for 48 h in RPMI containing 10% FCS.

blood monocytes are shown in Fig. 7. Cells preincubated for 48 h in RPMI containing 10% LPDS showed saturation at a concentration of 0.5 μg/ml of monoclonal antibodies. In cells preincubated for the same time in RPMI containing 10% FCS, no high-affinity binding of the antibody was observed. There was only a linear binding which parallels the nonspecific binding component in the LPDS-treated cells, owing to a down-regulation of the LDL receptor expression.

DISCUSSION

We have shown that preparative ITP is a suitable method for the separation of immunoglobulins and monoclonal antibodies from a protein pool in liquid media. The separation conditions are determined by the nature of the solvent, the total protein concentration and the relative content and the chemical characteristics of the protein that has to be isolated. As it is possible to control the instrumental parameters such as current, voltage and sample flow-rate over wide ranges, a suitable isotachophoretic separation can be achieved [36]. Moreover, the selection of the electrolyte and the spacer is of great importance in obtaining a high discrimination of the individual sample components. Under the conditions described in this paper, it is possible to purify IgGs from sera, tissue culture media and mouse ascites fluid.

The IgG concentration (0.8 mg/ml) and the IgG/protein ratio (860 μg of IgG per mg of protein) in ITP-purified mouse ascites fluid were very high, in contrast to the amounts determined in tissue culture medium after purification by ITP. In this instance another purification procedure is not necessary.

In addition to the isolation of monoclonal antibodies against the LDL receptor from mouse ascites fluid, we also used ITP in this study for the separation of these monoclonal antibodies from tissue culture medium. Because of the low mouse IgG/total protein ratio in tissue culture medium (0.22 μg of IgG per mg of protein), the purification of the monoclonal antibodies needs a preconcentration prior to the ITP separation. However, compared with DEAE-Trisacryl chromatography, ITP of tissue culture medium yielded a mouse IgG/protein ratio and a purification factor 2–3 times higher than those after DEAE-Trisacryl chromatography (ITP, 5 μg of IgG per mg of protein; DEAE-Trisacryl, 1.9 μg of IgG per mg of protein), and the yield was much better (ITP, 77%; DEAE-Trisacryl, 59%). The reason for these results is that DEAE-Trisacryl chromatography needed two more purification steps than ITP.

The concentration procedure for tissue culture medium before application to the anion-exchange column was not essential, but immunoglobulins in a high dilution cannot be separated from other proteins efficiently bound to the gel matrix. Therefore, omission of one concentration step results in a reduced discrimination and a longer duration of the chromatographic procedure.

The separation of monoclonal antibodies from mouse ascites fluid can be performed by ITP in a single-step procedure, just as Protein A chromatography. Because of the specific binding characteristics of immobilized Protein A it is possible to obtain a highly purified IgG fraction, which may be separated into several IgG subclasses by applying elution buffers of different pH or a pH gradient [35]. Isolation of monoclonal antibodies by Protein A chromatography may alter the

binding properties of the antibody, as a low pH often has to be used for elution [11]. The greatest advantage of ITP over Protein A chromatography is the mild elution conditions, and the piurification rates are comparable. If it is taken into consideration that the novel technique of preparative ITP has not yet exhausted its potential the separation of IgG subclasses could become possible. When choosing appropriate elution conditions, the isolation of any protein fraction can be performed based on their different net electric mobility and with the addition of discrete spacer compounds. It is concluded that preparative ITP is a simple and rapid method for the continuous mild purification of monoclonal antibodies with a high recovery rate.

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