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Selective high-performance liquid chromatographic purification of bispecific monoclonal antibodies

Laura Tarditi, Maria Camagna, Anna Parisi and Cristina Vassarotto

Biochemical Oncology Laboratories, SORIN Biomedica, 13040 Saluggia (VC) (Italy)

Lucia B. DeMonte

Department of Genetics, Biology and Medical Chemistry, University of Turin, Turin (Italy)

Michelle Letarte

Hospital for Sick Children, Toronto (Canada)

Fabio Malavasi

Department of Genetics, Biology and Medical Chemistry, University of Turin, Turin and Centre for Immunogenetics and Histocompatibility, CNR, Turin (Italy)

Massimo Mariani*

Biochemical Oncology Laboratories, R & D Diagnostic Laboratories, SORIN Biomedica, 13040 Saluggia (VC) (Italy)

ABSTRACT

The recent development of improved production techniques for bispecific monoclonal antibodies (biMAbs) has significantly increased interest in specific purification procedures. In this investigation, a general high-performance liquid chromatographic (HPLC) purification method is proposed that allows highly purified biMAbs to be obtained from mouse ascites fluid containing a mixture of different antibodies, *i.e.*, parental MAbs, active biMAb and a mixture of randomly assembled heavy and light chains. Proteins from ascites fluid were precipitated with ammonium sulphate and applied to a high-performance protein A column to separate the total immunoglobulin fraction. BiMAbs were isolated from other immunoglobulins by two subsequent passages through a high-performance hydroxyapatite (HPHT) column. This purification protocol combines specificity of protein A for immunoglobulin G (IgG) and high selectivity of hydroxyapatite for different IgG idiotypes. All purification steps were performed rapidly and reliably by HPLC. This method was applied to the purification of six different biMAbs with consistently high yields, purity and homogeneity. This general purification method may prove extremely valuable when highly pure preparation of biMAbs is required, as for *in vivo* use.

INTRODUCTION

Bispecific murine monoclonal antobodies (biMAbs) have been developed in the last decade [1] and have found widespread use only recently, mainly in the study of cell–cell interactions and in lymphocyte retargeting. In the former instance, biMAbs can simultaneously recognize molecules expressed on tumour cells and on cytotoxic effector cells. Other models include biMAbs as targeting agents for toxins, drugs or radiopharmaceuticals. BiMAbs are becoming a widely used tool in applied cell biology; in functional studies and in diagnostic and therapeutic applications.

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BiMabs have been obtained both by immunoglobulin G (IgG) heterocross-linking [2] and by the production of hybrid hybridomas [1,3]. The latter method can be achieved either by fusing a hybridoma with splenocytes of mice immunized with the selected antigen or by direct fusion of two hybridomas [4]. Both methods involve cumbersome procedures and show low efficiency. As an alternative, a high-efficiency biological protocol for producing hybrid hybridomas has been devised recently [5]. This approach includes conventional somatic fusion between two hybridoma cell lines, previously made resistant to different metabolic drugs by means of retrovirus-derived shuttle vectors carrying the relevant resistance genes. The resulting hybrid hybridomas secrete a mixture of antibodies, including biMabs, parental MAbs and several inactive or partially active (monospecific) antibodies due to randomly assembled heavy and light parental chains.

Therefore, the critical problem in the production of biMAbs by hybrid hybridomas is purification of active biMAb from other contaminant immunoglobulins. Purification protocols have so far exploited the difference in the isoelectric points of individual immunoglobulins in ion-exchange chromatography [4]. An alternative method is the use of anti-idiotype MAbs to parental MAbs in double affinity chromatography [6]. Neither of the above-mentioned techniques has found general application. In several instances, monoclonal IgGs show differences in isoelectric points that are not sufficient to allow their separation by ion-exchange chromatography. Detailed studies using different stationary phases and elution gradients are essential. Affinity chromatography with anti-idiotype MAbs requires the availability of anti-idiotype reagents to all parental MAbs. Moreover, Regulatory Authorities suggest avoiding affinity chromatography as the last purification step owing to possible leakage of ligands from the columns when the product is intended for *in vivo* use [7,8].

The scope of this work was the definition of the parameters of a purification protocol able to yield large amounts of pure biMAbs to be used as *in vitro* reagents and *in vivo* immunopharmaceuticals. Experience indicated a three-step high-performance liquid chromatographic (HPLC) purification method using a combination of well defined antibody purification procedures: protein A [9–11] and hydroxyapatite [12–14] chromatography. As described previously [15], such a combination resulted in a generally applicable method for MAb purification. This protocol includes (i) protein A affinity chromatography to isolate the total IgG fraction, (ii) a first passage through hydroxyapatite to fractionate different MAb idiotypes and to evidence the biMAb peak and (iii) a second hydroxyapatite chromatography to remove inactive contaminants completely from biMAb preparations.

The selected method has been succesfully tested in the purification of six different biMAbs. The results indicate that the requirements of general application, high yields and product purity have been fully met.

EXPERIMENTAL

Monoclonal antibodies

A set of hybridomas of known specificity were used to obtain hybrid hybridomas. These included F023C5 (anti-CEA IgG_1) [16], Ep2 (anti-HMW-MAA IgG_{2a}) [17], 44C10 (anti-CALLA IgG_{2b}) [18], CBT3 (anti-CD3 IgG_{2a}) [19], AB8.28 (anti-FcR-like molecule IgG_{2a}) [19], CBT11 (anti-CD2 IgG_1) [20], A10 (anti-CD38 IgG_{2a}) [21] and 1LF5 [anti-diethylenetriaminepentaacetic acid (DTPA) metal chelating agent IgG_1] [22].

Generation of hybrid hybridomas

Resistance to geneticin (G418) or methotrexate (MTX) was conferred on hybridoma cells by means of retroviral shuttle vectors carrying the relevant resistance genes. Details were given by DeMonte *et al.* [5]. Hybridomas were infected by co-cultivation with irradiated psi-2 packaging cell lines, previously transfected with the appropriate vectors. The psi-2 packaging cell line, carrying the pMV7 vector [23] or the pSDHT vector [24], was used to confer either G418 or MTX resistance on the parental hybridomas, respectively. PEG-mediated somatic cell fusion and hybrid hybridoma selection were performed according to the literature [5].

The biMAbs produced included AB8.28 × Ep2 [5], CBT3 × Ep2 [5], A10 × Ep2 [25], CBT3 × CBT11 [20], CBT3 × 44C10 [20] and F023C5 × 1LF5 [26]. Massive production of biMAb was obtained by growing the hybrid hybridomas *in vivo* as tumour ascites in pristane-primed BALB/c mice.

Purification of biMAbs

Purification was performed by HPLC with a MAPS 800 system (Bio-RAD Labs., Richmond, CA, USA), equipped with UV–visible and conductivity detectors. The same instrument, equipped with analytical and preparative injection loops, can be used both for analytical and process-scale purification.

Ascites fluid was pretreated with 1 m*M* $CaCl_2$ (final concentration) (2 h at 18–25°C and 16–22 h at 2–8°C) to allow conversion of fibrinogen present in the sample to fibrin. After removing the fibrin clot, ascites specimens were centrifuged at 10 000 *g* and then heat-inactivated at 56°C for 40 min. The samples were further clarified by ultracentrifugation at 100 000 *g* for 60 min at 2–8°C and then precipitated by adding an equal volume of saturated ammonium sulphate solution at pH 6.5.

Pellet was collected after centrifugation (10 000 *g*, 25 min at 2–8°C) and subsequently dissolved in water and dialysed *versus* protein A binding buffer, namely 1.5 *M* glycine–3.0 *M* NaCl (pH 9.0). Dialysed solution was applied to a 100 × 25 mm I.D. Affi-Prep protein A column (Bio-Rad Labs.) at a flow-rate of 5 ml/min. The total IgG fraction was eluted with 0.1 *M* citrate buffer (pH 3.0) and immediately neutralized with 1 *M* Tris (pH 8.5).

After dialysis against 10 m*M* sodium phosphate buffer (pH 6.8), a trace amount of IgG was loaded on to a 100 × 7.8 mm I.D. hydroxyapatite (HPHT) column (Bio-Rad Labs.) to select the appropriate fractionation gradient for each biMAb. Purification was performed at a flow-rate of 0.4 ml/min using 10 m*M* phosphate buffer (pH 6.8) as buffer A and 350 m*M* phosphate buffer (pH 6.8) as buffer B.

After appropriate gradient selection, hydroxyapatite purification of biMAbs was linearly scaled-up and performed on a 50 × 25 mm I.D. HPHT preparative column (Bio-Rad Labs.) at a flow-rate of 4 ml/min. Identification of peaks containing biMAbs was achieved by analysis of eluted peak fractions according to biMAb specificity, *i.e.*, on antigen-coated plates by indirect radioimmunoassay or on effector and target cells by indirect immunofluorescence assay. The fractions corresponding to active biMAbs were dialysed *versus* 10 m*M* phosphate buffer (pH 6.8) and re-chromatographed through a hydroxyapatite column under the same conditions to separate biMAbs further from residual contaminant immunoglobulins. The fractions corresponding to pure biMAbs were finally dialysed *versus* 0.1 *M* phosphate-buffered saline (pH 7.4), sterile filtered and stored deep-frozen.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing

Purified bispecific and parental MAbs were analysed by 12.5% SDS-PAGE under reducing conditions (2% dimercaptoethanol) according to Laemmli [27]. Isoelectric focusing was performed with a PhastSystem using PhastGel IEF 3–9 (Pharmacia LKB, Uppsala, Sweden).

IgG determination

Immunoglobulin contents of the ascites fluid was evaluated by means of an in-house enzyme-linked immunosorbent assay (ELISA) method employing affinity-purified rabbit IgG to mouse IgG insolubilized on wells or conjugated to horseradish peroxidase [15]. Calibration graphs were obtained with an irrelevant affinity-purified MAb, class-matched with the antibody under analysis. With biMAbs derived from parental immunoglobulins of different isotypes, the graphs were derived from samples containing 50% mixtures of class-matched irrelevant antibodies.

Purified bispecific and parental MAbs concentrations were evaluated by measuring the UV absorbance at 280 nm using $E^{1\%}_{1\,\mathrm{cm}}$ = 14.0 [11].

RESULTS AND DISCUSSION

Pretreatment of ascites before chromatography appears to be a critical step in the whole purification process. Indeed, fibrin removal by $CaCl_2$, lipid and mineral oil (pristane) removal by ultracentrifugation and partial protein purification by ammonium sulphate precipitation proved to be of high relevance in preserving the column lifetime.

In order to evaluate purification yields, immunoglobulin contents of ascites fluids were checked by ELISA. Mean values of the estimated total IgG are reported in Table I. Ammonium sulphate precipitation and protein A purification make it possible to obtain the whole IgG fraction from ascites, with yields ranging from 65 to 75% (see Table III).

HPHT chromatography separates IgGs according to their net charge; this feature confers on hy-

TABLE I

IMMUNOGLOBULIN CONCENTRATIONS IN HYBRID HYBRIDOMA ASCITES FLUID

Immunoglobulin concentrations were evaluated by an ELISA method after ascites fluid ultracentrifugation.

biMAb	Ascites fluid IgG content (mg/ml)
AB8.28 × Ep2	9.2
CBT3 × CBT11	4.3
CBT3 × Ep2	7.5
F023C5 × 1LF5	11.4
A10 × Ep2	3.5
CBT3 × 44C10	2.7

droxyapatite its unique selectivity for IgG idiotypes [13–15]. As the active biMAb secreted by one hybrid hybridoma is formed of two IgG moieties, each corresponding to half of the parental IgG, this combination will have a total net charge intermediate between those of the parental MAbs. Therefore, the biMAb will be eluted from the HPHT column with an ionic strength intermediate between those of the parental MAbs. Thus, a consistent gap between the retention times of parental MAbs becomes a condition that cannot be overlooked in order to obtain a satisfactory separation of biMAbs. The choice of appropriate linear gradients for each biMAb appears to be critical to obtain a significant difference in HPHT retention times of parental MAbs and consequently to achieve pure preparations of biMAbs.

Gradients were selected after evaluating the retention times of parental MAbs on a 100 × 7.8 mm I.D. HPHT column (10–20 mg loading capability). The general gradient range was 0–80% buffer B. The next step was a linear scale-up of selected gradients to a 50 × 25 mm I.D. HPHT preparative column (about 500 mg loading capability) using a ten times faster flow-rate and a twice as long gradient, as reported in Table II.

The HPHT chromatographic elution profile of protein A-purified immunoglobulin is shown in Fig. 1 for each biMAb. The peak fractions corresponding to active biMAb were identified by appropriate double antigen specificity tests and by isoelectrofocusing. SDS-PAGE under reducing conditions was used to discriminate parental MAbs from bispecific IgGs when the two parental MAbs had differently migrating (fast and slow) light chains, as shown in Fig. 2 for MAbs AB8.28 and Ep2, and previously reported by other workers [13,14].

SDS-PAGE individually performed on peak fractions eluted from HPHT (Fig. 1A) showed that peaks 1 and 5 correspond to parental IgGs, each representing a differently migrating light chain. Peaks 2, 3 and 4 correspond to differently assembled parental heavy and light chains. Peak 3, which displayed light chain with a similar, if not identical, segregation, is the active bispecific fraction (activity data are reported elsewhere [5]).

Total IgG of the CBT3 × CBT11 hybrid hybridoma was also separated into five peaks by HPHT purification (Fig. 1B), two corresponding to parental MAbs (peaks 1 and 5), two to inactive products (peaks 2 and 4) and one to the active biMAb (peak 3). Under the conditions set up for this protocol, the other biMAbs studied were resolved into three main peaks by HPHT chromatography (Fig. 1C, D and E). CBT3 × 44C10 displayed a peculiar behaviour (Fig. 1F), which will be discussed later.

Middle peaks constantly included the active bispecific fraction. Parental Mabs were eluted in the first and third peaks (Fig. 1C, D and E). With A10 × Ep2 (Fig. 1E), the first peak was quantitatively lower than the others. The unbalanced proportion among the peaks probably reflects a different ex-

TABLE II

SELECTED GRADIENT FOR biMAb PURIFICATION ON A 50 × 25 mm I.D. HYDROXYAPATITE COLUMN

BiMAbs were purified through a 50 × 25 mm I.D. Preparative HPHT column at a flow-rate of 4 ml/min. Buffer A, 10 m*M* phosphate (pH 6.8); buffer B, 350 m*M* phosphate (pH 6.8).

biMAb	Phosphate linear gradient		
	Initial B (%)	Final B (%)	Time (min)
CBT3 × CBT11	10	70	110
CBT3 × 44C10	10	70	110
CBT3 × Ep2	20	80	110
A10 × Ep2	20	40	120
AB8.28 × Ep2	15	50	120
F023C5 × 1LF5	0	50	100

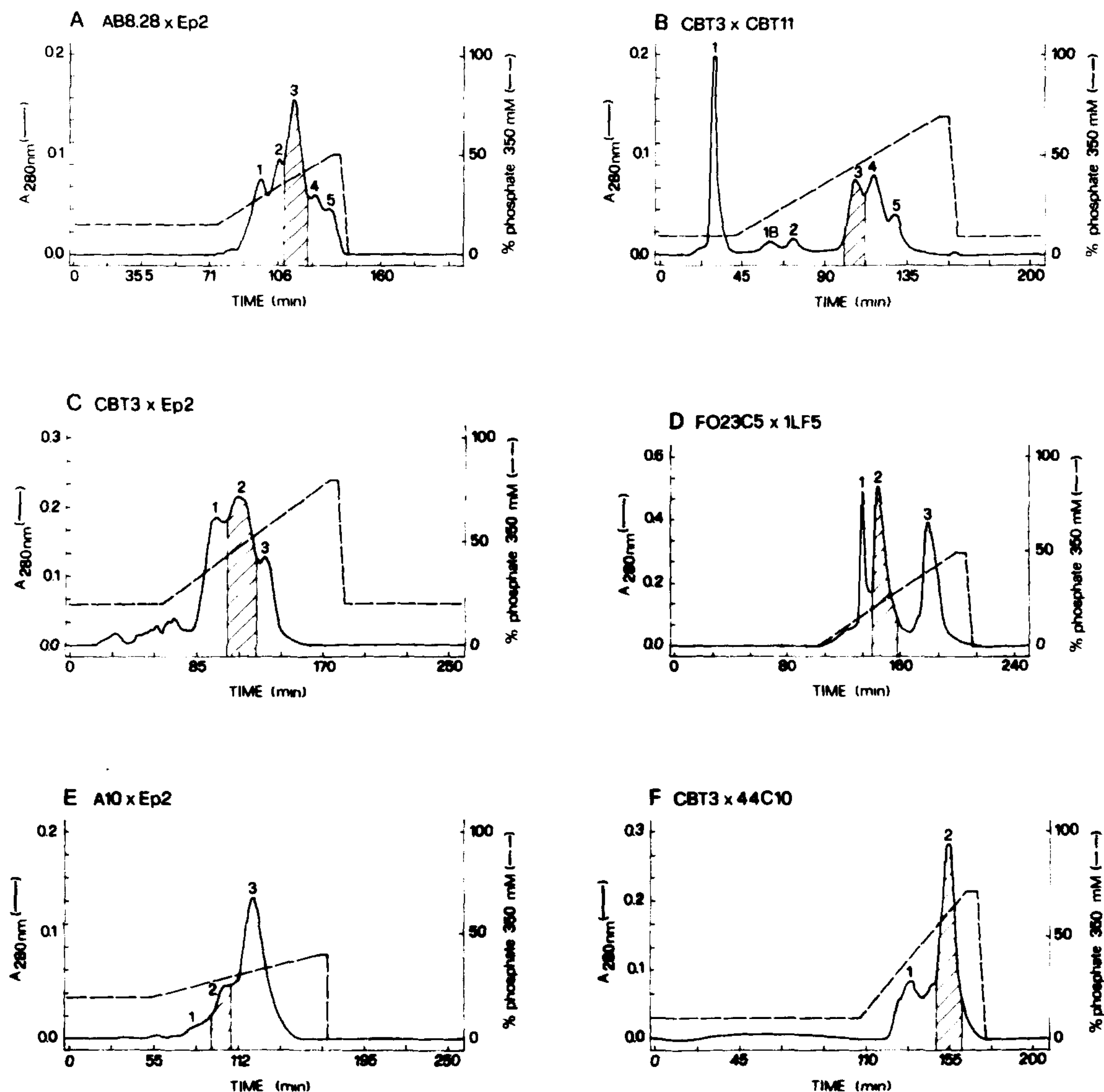

Fig. 1. Elution profiles of hydroxyapatite HPLC of immunoglobulins secreted by hybrid hybridomas. Purification was performed on a 50 × 25 mm I.D. HPHT column at a flow-rate of 4 ml/min. Buffer A, 10 m*M* phosphate (pH 6.8); buffer B, 350 m*M* phosphate (pH 6.8). Hatching indicates the peaks containing the active biMAb fractions. (A) AB8.28 × Ep2: 110-min linear gradient from 15 to 50% B. Peak 3 contains the biMAb; peaks 1 and 5 contain parental MAbs, AB8.28 and Ep2, respectively; peaks 2 and 4 contain inactive rearrangements of parental heavy and light chains. (B) CBT3 × CBT11: 110-min linear gradient from 10 to 70% B. Peak 3 contains the biMAb; peaks 1 and 5 contain parental MAbs, CBT11 and CBT3, respectively; peaks 2 and 4 contain inactive rearrangements of heavy and light parental chains. (C) CBT3 × Ep2: 110-min linear gradient from 20 to 80% B. The middle peak (hatched) contains the biMAb and peaks 1 and 3 correspond to parental MAbs Ep2 and CBT3, respectively. (D) F023C5 × 1LF5: 110-min linear gradient from 0 to 50% B. The middle peak (hatched) contains the biMAb and peaks 1 and 3 correspond to parental MAbs F023C5 and 1LF5, respectively. (E) A10 × Ep2. 120-min linear gradient from 20 to 40% B. The middle peak (hatched) contains the biMAb and peaks 1 and 3 correspond to parental MAbs A10 and Ep2, respectively. (F) CBT3 × 44C10: 110-min linear gradient from 10 to 70% B. Peak 2 corresponds to the active biMAb and peak 1 contains the parental MAb CBT3. The lack of resolution between the parental MAb 44C10 and the peak containing the biMAb is probably due to a characteristic of 44C10 hybridoma to secrete small amounts of IgG; a similar behaviour appeared on the A10 × Ep2 trace (E).

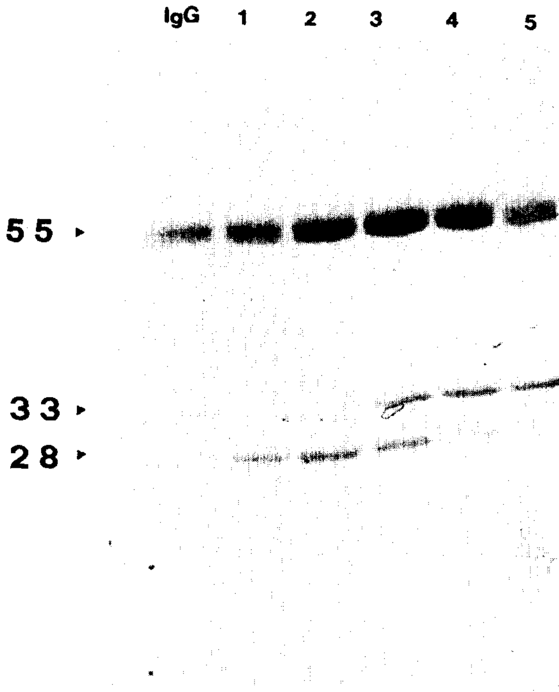

Fig. 2. SDS-PAGE under reducing conditions of the peak fractions derived from AB8.28 × Ep2 fractionation. Lanes 1–5 show the migration patterns of distinct peak fractions obtained by HPHT purification. Parental MAbs AB8.28 (1) and Ep2 (5) show differently migrating light chains. The marked lane shows IgG obtained by protein A affinity chromatography. Numbers on the left are molecular masses × 10^{-3}.

pression of heavy and light chains in the hybrid hybridomas. This evidence was further strengthened by the results of the HPHT purification of CBT3 × 44C10 (Fig. 1F), where only two detectable peaks were obtained. The first corresponds to the parental CBT3 MAb and the second contains the biMAb. A possible explanation of this observation may be the low IgG production of one parental hybridoma. Both A10 and 44C10 were found to be low IgG producers: selection of a high producer sub-clone of the anti-CD38 A10 hybridoma is in progress, in order to evaluate the influence of quantitative levels of immunoglobulin secretion by parental hybridomas in the generation of biMAbs and other molecular hybrid molecules.

IEF analysis of the peaks eluted after the first HPHT run showed low but significant peak-to-peak contamination. This prompted us to include a second application of peak fractions containing biMAbs on to a HPHT column in order to free biMAb preparations from any residual contamination. An example of chromatograms showing the second HPHT runs of peak fractions containing CBT3 × Ep2 and F023C5 × 1LF5 is given in Fig. 3. The retention time of the eluted peak is identical with the same parameter as recorded in the first HPHT run (*i.e.*, 105.3 ± 1.2 min for CBT3 × EP2 and 143.7 ± 0.8 min for F023C5 × 1LF5).

The final step was purity evaluation of biMAb preparations obtained following this protocol. The samples were submitted to SDS-PAGE and IEF, and the gels further tested by densitometric scanning.

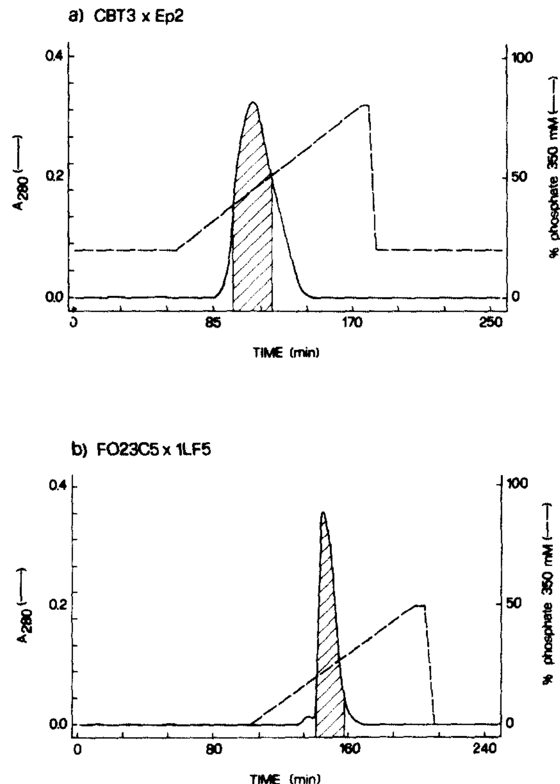

Fig. 3. HPHT second-run chromatographic profile of biMAb-containing fractions. BiMAbs obtained by first-run hydroxyapatite chromatography were re-loaded on a high-performance hydroxyapatite column (50 × 25 mm I.D.) at a flow-rate of 4 ml/min and eluted using the same phosphate gradients selected for the first run. (a) CBT3 × Ep2: 110-min linear gradient from 20 to 80% B. (b) F023C5 × 1LF5: 110-min linear gradient from 0 to 50% B.

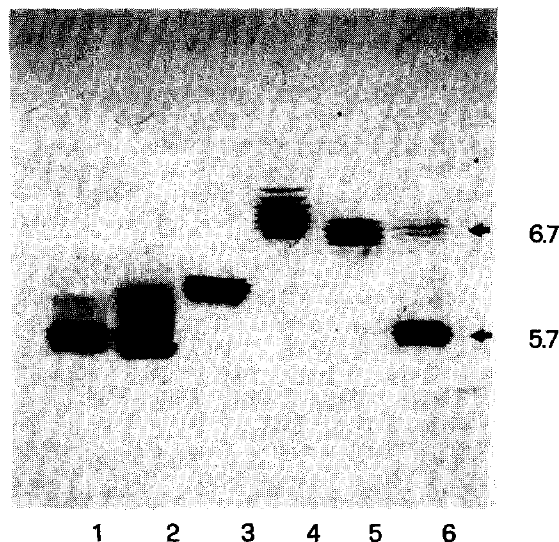

Fig. 4. Isoelectrofocusing of peak fractions collected after HPHT purification of F023C5 × 1LF5 biMAb. Lanes 1 and 5 show the IEF patterns of parental F023C5 and 1LF5 MAbs, respectively. Lane 6 contains an artificial mixture of the same parental MAbs. Lane 2 and lane 4 display an IEF pattern identical with that attributed to the parental MAbs with minimal contamination. The biMAb obtained after two HPHT purification runs (lane 3) is clearly distinguishable from the MAb mixture. It is also free from any apparent contaminating parental IgG. Numbers on the right are molecular masses $\times 10^{-3}$.

Fig. 4 reports the results of the IEF analysis of the peak fractions obtained after purification of F023C5 × 1LF5 biMAb. Peak 1 (lane 2) corresponds to the parental F023C5 MAb and, peak 3 (lane 4) is identical with the parental 1LF5 MAb. Peak 2, confirmedly containing the bispecific MAb (see above), displays bands the p*I* range of which is intermediate between those of the two parental MAbs (lane 3). The IEF pattern of purified biMAbs was unique and markedly different from that of the simple mixture of parental MAbs (lane 6).

A low peak-to-peak contamination was apparent in peak fractions 1 and 3: the highest degree of purification was achieved by the biMAb peak fraction after the second HPHT run (98.7% as highlighted by gel densitometric scanning). At the end of the purification procedure presented here, the purity obtained for each biMAb was >98%. This was achieved by submitting samples to repeated passages through chromatographic columns. However, high purity demands lower yields; in this instance, the yield of the process from ascites to pure biMAb was in the acceptable range 10–35% (Table III).

CONCLUSIONS

This paper has reported the experience acquired so far in the investigation of methods and conditions to be applied in the purification of murine biMAb.The proposed protocol is the result of comparative tests: here, only the final conditions are reported, which allow discrimination between biMAbs and contaminanting ascites IgG. In fact, parental MAb trace contamination may hamper biMAb functional acitivity or mislead its effects in *in vitro* and *in vivo* applications.

TABLE III

biMAb PURIFICATION YIELDS

biMAb	Protein A: total IgG yield[a] (%)	HPHT I			HPHT II: biMAb yield[a] (%)
		total IgG yield[a] (%)	biMAb yield[a] (%)	biMAb/IgG ratio[b] (%)	
AB8.28 × Ep2	61.0	45.7	20.9	45.7	17.7
CBT3 × CBT11	70.0	39.4	13.4	34.0	12.2
CBT3 × Ep2	74.0	59.0	34.0	57.6	25.5
F023C5 × 1LF5	56.1	40.4	16.7	41.3	14.3
A10 × Ep2	69.5	45.7	13.0	28.4	12.1
CBT3 × 44C10	69.4	44.4	33.0	74.3	31.3

[a] Total process yield determined as recovered IgG at the end of each chromatographic step with respect to initial total IgG contents in ascites fluid. Values include centrifugations, precipitation, dialysis and filtrations of processed samples.

[b] Determined as the ratio of mg of purified biMAb recovered to mg of total IgG recovered.

This work focused on an already validated method of IgG isolation [15], which combines IgG specificity of protein A and the peculiar ability of hydroxyapatite to discriminate IgG idiotypes. This two-step chromatographic purification method was also previously shown not to affect IgG-binding capability [15], and it has been successfully submitted to validation for endotoxin, murine DNA and virus removal [28]. In particular, hydroxyapatite was found to remove different viral agents with high efficiency, as suggested by FDA and EEC guidelines [29–31].

All purification steps were performed rapidly and reliably by HPLC. This purification procedure was selected to provide pure reagents meeting the requirements to become immunopharmaceuticals, to be used either *in vitro* or, mainly, *in vivo* for localization and/or the destruction of tumour cells.

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