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COMPARISON OF PROTEIN A, PROTEIN G AND COPOLYMERIZED HYDROXYAPATITE FOR THE PURIFICATION OF HUMAN MONOCLONAL ANTIBODIES

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SUMMARY

Protein A Superose, protein G Sepharose fast flow and copolymerized hydroxyapatite were used for the purification of human monoclonal antibodies against HIV 1. Both desalted culture supernatant and a prepurified protein solution were used as starting materials. The different runs were compared with respect to yield and recovery of biological activity. The biological activity (specific reactivity) was checked by antigen enzyme-linked immunosorbent assay with recombinant antigen. The human monoclonal antibodies could not be selectively eluted from the hydroxyapatite but elution could be effected from the protein A Superose at pH 4.0 and from protein G at pH 3.0. The eluted immunoglobulin G was distributed over a broad pH range when protein G Superose was used. Biologically active material could be obtained from protein A Superose and protein G Sepharose fast flow.

INTRODUCTION

Various methods have been described for the purification of monoclonal antibodies of murine origin. In general, the methods involve two major steps. First, a prepurification step, *i.e.*, a precipitation step, can be carried out (ammonium sulphate, caprylic acid, polyethylene glycol or isoelectric precipitation) or a concentration step can be performed by ultrafiltration[1–6]. Second, various chromatographic methods are used, such as ion-exchange chromatography or affinity chromatography on protein A, immunoligands or synthetic antigen analogues[7,8]. For murine monoclonal antibodies (G and M isotypes), Pavlu *et al.*[9] and Chen *et al.*[10] compared different purification methods.

The pretreatment steps depend on the starting material if ascites or culture supernatant is used. Clezardin and co-workers[11,12] used Mono Q and gel chromatography on Superose 6. Methods have been described for immunoglobulins M (IgM) and G (IgG) from ascites fluid or culture supernatant. Nearly all workers apply the purification mode to accomplish the purification of the monoclonal antibody. How-

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ever, for preparative purposes, especially starting from the diluted culture supernatant, the concentration mode[13] is to be preferred. In this mode, the advantage is in the reduction of the column size, resulting in a high product concentration in the eluate.

Few papers have described purification methods for human monoclonal antibodies. In this paper, different purification strategies for human monoclonal antibodies are compared. In all experiments the concentration mode was applied. Human and murine monoclonal antibodies differ in many ways. The most important difference with respect to purification is the binding behaviour on protein A and protein G[14,15]. The affinity purification of human monoclonal antibodies is the method of choice.

Three different methods were investigated in these comparative experiments. Starting from the desalted culture supernatant or a concentrated eluate from a CM-Sepharose fast flow step, the purification power of protein A Superose, protein G Sepharose fast flow and hydroxyapatite Ultrogel were tested. The following criteria were investigated in this comparative study: purity [checked by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)], capacity and recovery (yield and avidity). Using protein A or protein G, a highly pure and biologically active protein could be obtained.

EXPERIMENTAL

Human hybridoma cells producing humAb against HIV 1, as described by Grunow *et al.*[16], were mass cultivated in an airlift fermenter, using RPMI[a] 1640 cell culture medium (Gibco, Paisley, U.K.) supplemented with 5% foetal calf serum as substrate. The culture broth was clarified by means of microfiltration on Pellicon cross-flow membranes (Millipore, Bedford, MA, U.S.A.). The clear supernatant was used for the chromatographic experiments.

The starting material was either a desalted culture supernatant or a highly concentrated material, as described in detail elsewhere[17]. Briefly, the culture supernatant was desalted and applied to a CM-Sepharose fast-flow column. The column was equilibrated with 100 m*M* histidine–hydrochloric acid buffer (pH 6.5) and elution was effected by a step gradient with 50 m*M* sodium chloride. The fractions containing the human monoclonal antibody were concentrated 10-fold on a 30 000-dalton cut-off polysulphone membrane (PTTK; Millipore).

The chromatography on protein A, protein G and hydroxyapatite Ultrogel (IBF, Paris, France) was performed on an fast protein liquid chromatography apparatus (Pharmacia, Uppsala, Sweden). The column effluent was monitored continuously with a UV monitor and a pH/ion monitor (2195 pH/ion monitor, Pharmacia-LKB). The ion monitor was calibrated with 1 *M* hydrochloric acid (highest value) and distilled water (lowest value).

Protein A Superose (Pharmacia) was used in this experiment. Similar conditions were used to those described in detail by Martin[18] for Rhesus monkey IgG. Briefly, the protein solution was desalted on Sephadex G-25 (coarse) to increase the pH to 9.0 as the protein A Superose was equilibrated with 0.1 *M* citric acid–0.2 *M*

[a] RPMI = Rosevelt Park Memorial Institute.

sodium phosphate buffer (pH 9.0). Then a step gradient from pH 6.0 to 3.0 was applied to elute the human monoclonal antibody. Immediately after elution, the humAb solution was desalted.

Protein G Sepharose fast-flow (Pharmacia) was used. The recombinant protein G without the albumin-binding region was immobilized. Similar conditions to those described for protein A were used. The culture supernatant or the concentrated material was desalted. The equilibration buffer was 0.1 *M* glycine–sodium hydroxide (pH 9.0). Elution was performed by a step gradient from pH 5.0 to 2.5, and a step gradient with two steps, pH 3.0 and 2.5. Immediately after elution, the samples were desalted.

The capacity was determined by a dynamic method. At least twice the amount of IgG was percolated over the gel. The difference between the amount in the original sample and the flow through was defined as the capacity.

SDS-PAGE was carried out in a Phast System (Pharmacia). According to the manufacturer's recommendations, the desalted samples were applied to a gradient gel (from 8 to 25%T). The gels were silver-stained and both reduced and non-reduced samples were applied.

IgG determination

The human monoclonal antibody was determined by enzyme-linked immusorbent assay (ELISA) as described by Grunow *et al.*[16]. Human monoclonal IgG (Sigma, St. Louis, MO, U.S.A.) was used as a standard protein. As the first antibody, goat anti-human gamma-chain was coated on microtitre plates. After coating and washing, samples and the calibration proteins were applied to the wells. The standard proteins were diluted (eight 2^n dilutions from 250 ng/ml to 1.953 ng/ml). Eight equal dilutions (2^n dilutions) were made from each sample. As the second antibody, goat anti-human gamma-chain conjugated with horseradish peroxidase was used. After staining, the absorption at 455 nm was measured. The results were evaluated with a fourth-degree polynomial.

Specific reactivity

The same sample dilutions as used for the determination of IgG content were tested for specific reaction to recombinant gp 160 (recombinant envelope glycoprotein from HIV 1 was a gift from Immuno, Orth, Austria). Instead of the first antibody, recombinant gp 160 was coated on microtitre plates. The subsequent steps were performed in the same manner as described for the IgG determination. The different chromatographic fractions were related to the main fraction or the culture supernatant.

RESULTS

A 1-ml volume of gel (protein G Sepharose fast flow) was packed into a column of 0.6-cm^2 cross-sectional area. A desalted culture supernatant was loaded on the column. To minimize loss of biological activity caused by denaturation at low pH during elution, a step gradient was used. The antibody was eluted over a broad pH range, as indicated in Fig. 1. The purity of the recovered antibody was shown by SDS-PAGE (Fig. 2).

Prepurified material (eluate from CM-Sepharose fast flow) was also loaded on

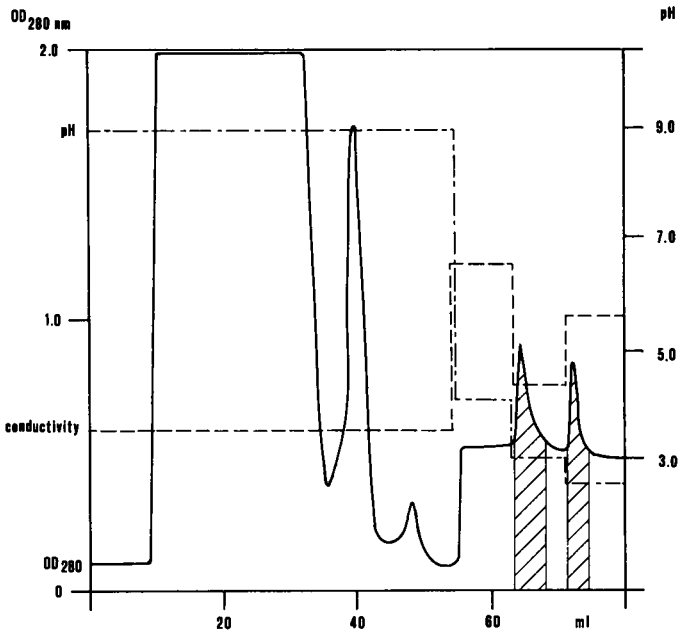

Fig. 1. Chromatography of desalted culture supernatant on protein G Sepharose fast flow. Sample, 30 ml of a concentrated (5-fold, by ultrafiltration) and desalted (on Sephadex G-25 coarse) culture supernatant; column, 1.3 ml; linear velocity, 150 cm/h. The column was loaded at pH 9.0 and a step gradient from pH 4.0 to 2.5 was used. The hatched area indicates the eluted antibody.

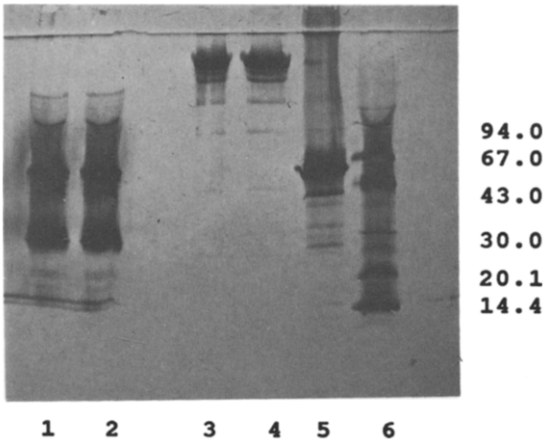

Fig. 2. SDS-PAGE from the chromatogram in Fig. 1. The samples were separated on a 8–25% polyacrylamide gradient gel. Samples: lane 1, eluate pH 2.5 (reduced); lane 2, eluate pH 3.0 (reduced); lane 3, eluate pH 2.5; lane 4, eluate pH 3.0; lane 5, culture supernatant, 5-fold concentrated; lane 6, molecular weight marker.

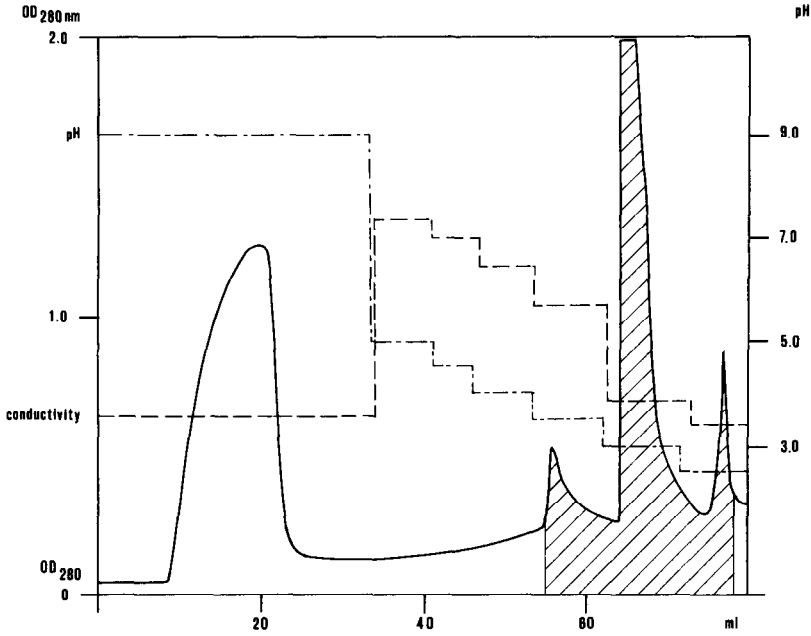

Fig. 3. Chromatogram of protein G Sepharose fast flow. Sample, 14 ml of desalted eluate from CM-Sepharose fast flow was loaded at pH 9.0; column, 1.3 ml; linear velocity, 150 cm/h. Elution was effected by a step gradient from pH 5.0 to 2.5 in 0.5 pH intervals. The hatched area indicates the eluted antibody.

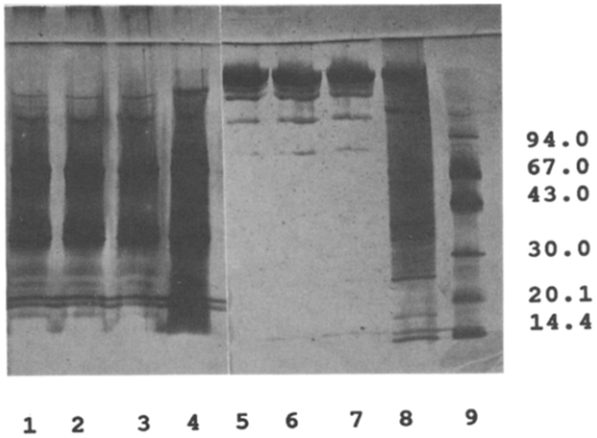

Fig. 4. Electropherograms referring to Fig. 3. Samples: lane 1, eluate pH 2.5 (reduced); lane 2, eluate pH 3.0 (reduced); lane 3, eluate pH 3.5 (reduced); lane 4, concentrated ion-exchange eluate (reduced); lane 5, eluate pH 2.5; lane 6, eluate pH 3.0; lane 7, eluate pH 3.5; lane 8, concentrated ion-exchange eluate; lane 9, molecular weight marker.

TABLE I

SPECIFIC REACTIVITY TO gp 160 OF THE DIFFERENT CHROMATOGRAPHIC STEPS AND PEAKS

The specific reactivity of the culture supernatant was set arbitrarily at 100%.

Gel	*Concentrated culture supernatant*	*CM-Sepharose eluate*	*Peak 1*	*Peak 2*	*Peak 3*
Protein G Sepharose (Fig. 1)	98.4	–	92.0	95.3	–
Protein G Sepharose (Fig. 3)	–	95.3	97.6	95.4	98.1
Protein G Sepharose (Fig. 5)	–	95.3	93.6	97.2	–
Protein A Superose (Fig. 6)	–	95.3	98.7	–	–
Protein A Superose (Fig. 7)	98.4	–	95.3	–	–

the column. To investigate the elution conditions, a step gradient with smaller pH intervals (0.5 pH unit from pH 5.0 to 2.5) was applied. The antibody was eluted in three fractions at pH 3.5, 3.0 and 2.5. The main fraction was eluted at pH 3.0. On applying more concentrated material (eluate from the CM-Sepharose fast flow) to the column (Fig. 3), the same purity (Fig. 4) and biological reactivity (Table I) were obtained. Moreover, a higher concentration of the final purified material could be achieved (Table II). To avoid incomplete elution from the protein G Sepharose fast flow, the step gradient was reduced to one step (Fig. 5). Again, the biological activity was checked by antigen ELISA (Table I). It is important that the eluted antibody is immediately transferred into phosphate-buffered saline (PBS), glycine buffer or equivalent buffer, because a low pH destroys the antibody completely within 24 h (results not shown).

A prepacked 1-ml protein A Superose column was used at half the linear flow-rate used for protein G; chromatograms are shown in Figs. 6 and 7. The purity of the

TABLE II

SUMMARY OF ALL CHROMATOGRAPHIC EXPERIMENTS WITH THE HUMAN MONOCLONAL ANTIBODY

Gel	*Starting material*	*Capacity (mg/ml gel)*		*Recovery (%)*	*Concentration factor*
		Manufacturer's declaration	*Experimental results*		
Protein A	Culture supernatant	12	12	70	5.0
	Ion-exchange eluate	12	12	76	5.9
Protein G	Culture supernatant	17	n.d.[a]	50[b]	5.0
	Ion-exchange eluate	17	n.d.	60[b]	1.2
	Ion-exchange eluate[c]	17	n.d.	70	n.d.
Hydroxyapatite	Culture supernatant	–	n.d.	n.d.	n.d.

[a] Not determined.
[b] Poor recovery owing to the broad elution zone.
[c] Elution at pH 2.5 instead of a step gradient.

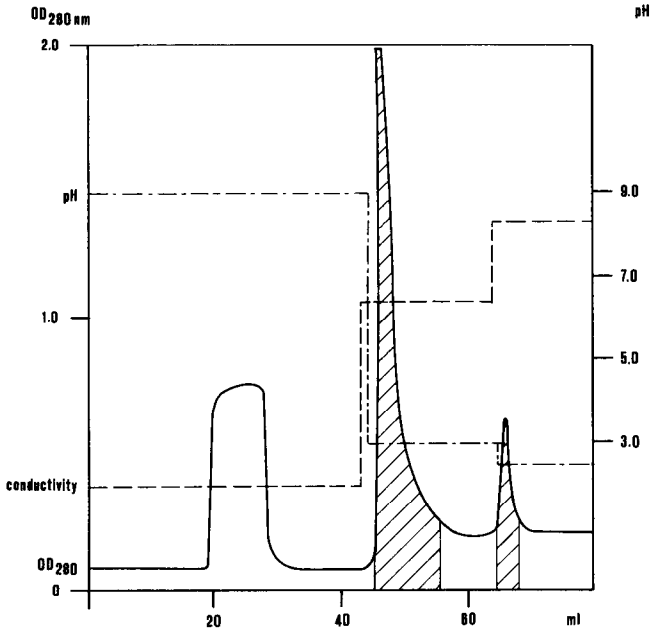

Fig. 5. Chromatogram of protein G Sepharose fast flow. Conditions as in Fig. 3; the step at pH 3.5 was neglected to reduce the broad elution. The hatched area indicates the eluted antibody.

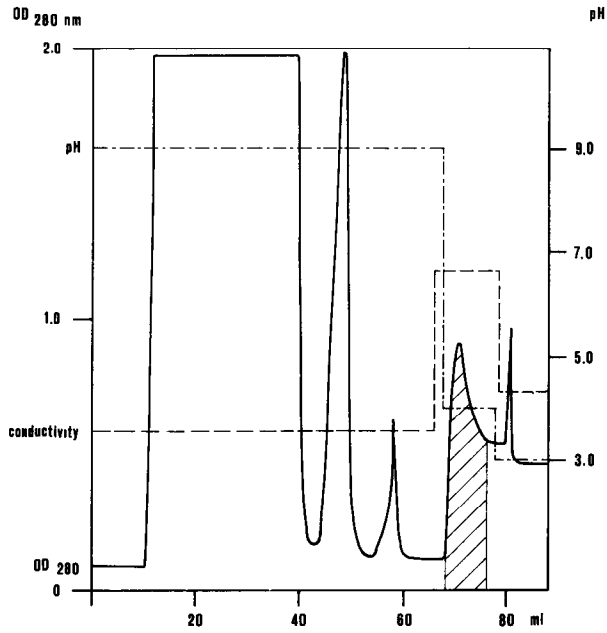

Fig. 6. Chromatogram of desalted culture supernatant on protein A Superose. Sample, 30 ml of concentrated (5-fold by ultrafiltration) and desalted culture supernatant (on Sephadex G-25 coarse); column, 1 ml; linear velocity, 75 cm/h. Elution was effected by a step gradient. The sample was loaded at pH 9.0, eluted at pH 4.0 and regenerated at pH 3.0. The hatched area indicates the eluted antibody.

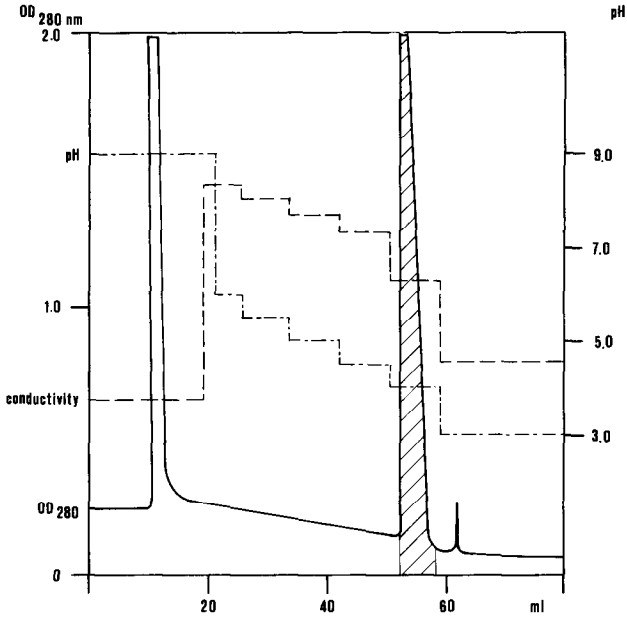

Fig. 7. Chromatogram of protein A Superose. Sample, eluate from CM-Sepharose fast flow; 1 ml of desalted (Sephadex G-25 coarse) ion-exchange eluate was loaded on a 1-ml column. Linear velocity, 75 cm/h. The sample was loaded at pH 9.0, a step gradient was used; elution was effected at pH 4.0; regeneration at pH 3.0. The hatched area indicates the eluted antibody.

eluted material was checked by SDS-PAGE (Figs. 8 and 9). The results were similar to those with protein G and the same purity was obtained. However, the human monoclonal antibody was eluted sharply at higher pH (4.0) and was not distributed over a broad pH range. The yield in the main fraction was twice that with protein G

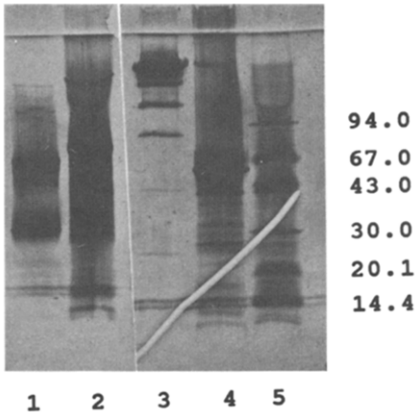

Fig. 8. Electropherograms referring to Fig. 6. The eluates at pH 3.0 contained undetectable amounts of protein. Samples: lane 1, eluate pH 4.0 (reduced); lane 2, culture supernatant, 5-fold concentrated (reduced); lane 3, eluate pH 4.0; lane 4, culture supernatant, 5-fold concentrated; lane 5, molecular weight marker.

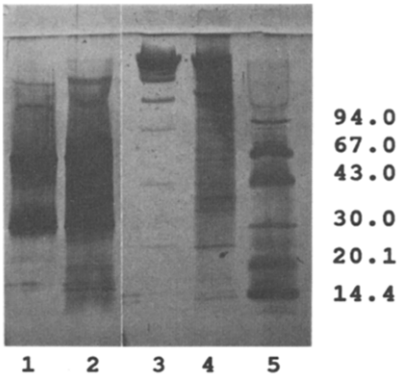

Fig. 9. Electropherograms referring to Fig. 7. Samples: lane 1, eluate pH 4.0 (reduced); lane 2, concentrated ion-exchange eluate (reduced); lane 3, eluate pH 4.0; lane 4, concentrated ion-exchange eluate; lane 5, molecular weight marker.

(Table II). The biological activity was checked by an antigen ELISA with recombinant gp 160 as antigen (Table I).

A 5-ml volume of hydroxyapatite Ultrogel was packed into a column of 5.3-cm^2 cross-sectional area. The desalted concentrated ion-exchange eluate was pumped over the column and equilibrated with 10 m*M* sodium phosphate (pH 6.8). The

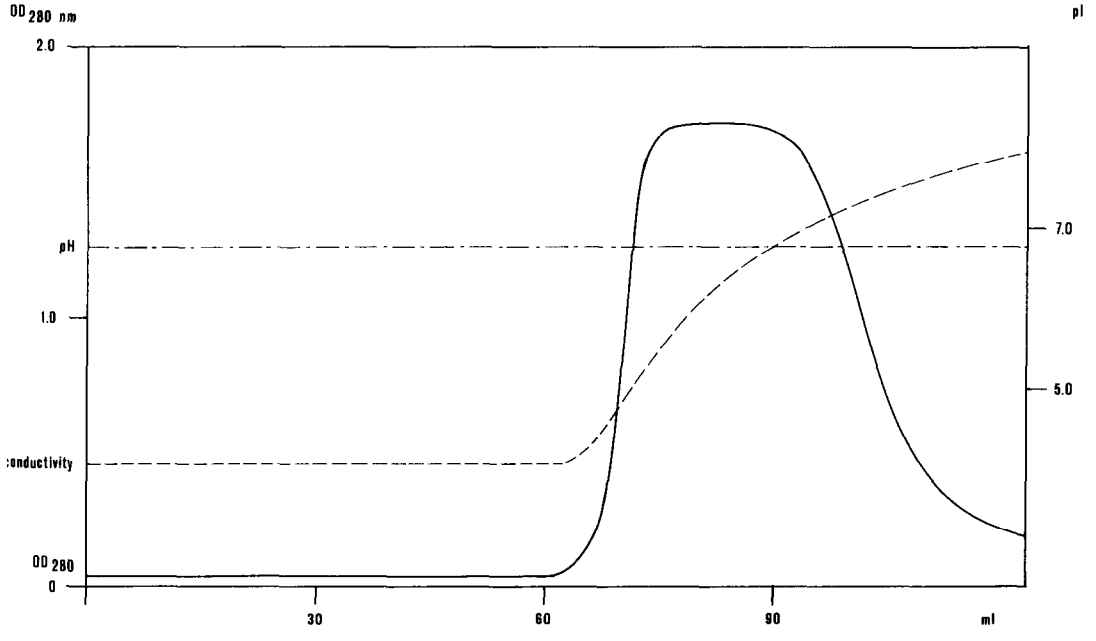

Fig. 10. Chromatogram of hydroxyapatite Ultrogel. Sample, eluate from CM-Sepharose fast flow; 20 ml of desalted (Sephadex G-25 coarse) concentrated (by ultrafiltration) ion-exchange eluate was loaded on a 14-ml column; linear velocity, 11 cm/h. The sample was transferred into the equilibration buffer (10 m*M* phosphate buffer, pH 6.8) and elution was effected by a linear gradient from 10 to 500 m*M* phosphate (gradient volume = 8.5 × the total column volume).

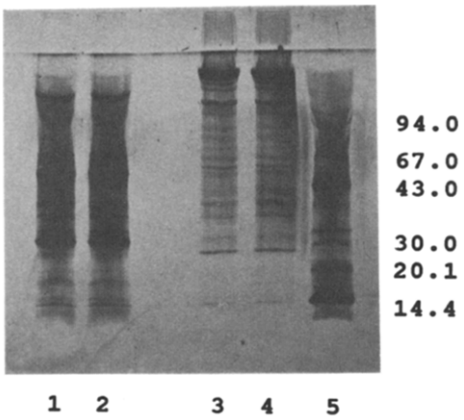

Fig. 11. Electropherograms referring to Fig. 10. Samples: lane 1, eluate (reduced); lane 2, concentrated ion-exchange eluate (reduced); lane 3, eluate; lane 4, concentrated ion-exchange eluate; lane 5, molecular weight marker.

elution was effected by a linear gradient. As shown in Fig. 10, nearly 100% of the total protein was bound to the hydroxyapatite, and could not be selectively eluted from the gel. Varying the gradient did not change this behaviour (results not shown). SDS-PAGE applied to the eluted proteins is shown in Fig. 11.

DISCUSSION

The aim of these experiments was to compare different methods of purification for human monoclonal antibodies with respect to purity and recovery. The biological activity has often been neglected by other workers describing methods for the purification of monoclonal antibodies[9,19–23]. In many instances, the use of affinity chromatography for the isolation of monoclonal antibodies requires rigid elution conditions to break the tight binding of the protein to the affinity matrix. It often happens that such conditions can destroy the biological activity (in general measured as avidity by ELISA or similar immunological assays). Many workers consider only the purity and recovery (yield) and not the biological activity.

Protein A Superose is preferred to protein G Sepharose because elution can be effected at pH 4.0 instead of 3.0, although the biological activity could be preserved in all experiments. A step gradient with narrow pH steps (Fig. 3) was tried in order to optimize the elution conditions. The elution profile from protein G Sepharose fast flow was similar to that from protein A Superose if monoclonal antibody was eluted at pH 3.0 instead of with a step gradient. The broad elution (Fig. 3) observed when using a step gradient with narrow pH steps is not caused by the human monoclonal antibody itself, because in all three fractions the antibody displays similar specific reactivity to the antigen gp 160 (Table I). This incomplete elution might be effected by the elution buffers. As already reported, protein G binds polyclonal IgG more tightly than does protein A.

As already pointed out, the low pH denatures the antibody, which therefore must be transferred immediately after elution to an appropriate buffer. The addition

of solid Tris to increase the pH in the eluate has the disadvantage that local over-concentrations can occur and the antibody can be denatured. Desalting by gel filtration instead of addition of solid Tris preserves the biological activity.

SDS-PAGE and an immunochemical technique such as antigen ELISA with the appropriate antigen are suitable methods for checking the biological activity of monoclonal antibodies. The use of SDS-PAGE alone is not sufficient as it shows only if the antibody molecule is complete, and provides no information on the biological activity. The antigen ELISA shows the biological activity, also called specific reactivity. Recombinant gp 160 was used only for safety reasons to avoid handling complete HIV 1 virus. When the antibody is used in a broad diagnostic or research programme, such as the presently described antibody, it must be proved that the isolation and purification procedure does not influence the properties of the antibody[24].

In this instance, binding of the monoclonal antibody to hydroxyapatite is very tight and the antibody could not be separated from other impurities. Albumin was present in the protein solution. The separation of IgG and albumin was not investigated because, in general, the removal of albumin from the monoclonal antibody solution causes no problems.

Therefore, we conclude that both protein A Superose and protein G Sepharose fast flow are suitable for the purification of human monoclonal antibodies. It is possible to start with either the desalted culture supernatant or a prepurified material. Both techniques lead to satisfactory results. The purified antibody was pyrogen-free and could be used for *in vitro* cell culture assays (results not shown). The techniques used with protein A and protein G are simple compared with methods described by Clezardin and co-workers[11,12], who used Mono Q and Superose 6. They are also easier to perform than either precipitation or ion-exchange methods described by other investigators.

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