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Note

Purification of anti-paraquat monoclonal antibodies by affinity chromatography on immobilised hapten

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Monoclonal antibodies are currently of great commercial interest for their applications in the areas of both in vitro and in vivo diagnostic and therapeutic agents[1–3]. Many purification methods have been successfully applied. Initial enrichment steps such as ultrafiltration are used to concentrate the product. Purification, however, is generally carried out by a chromatographic technique[4]. Many such techniques have been used; ion exchange[5], affinity chromatography using immobilised Protein A[6], and hydroxyapatite[7]. None of these methods has a truly general application, however, due to the heterogeneity in isoelectric point, hydrophobicity and biological activity of different monoclonal antibodies[8].

Purification of monoclonal antibodies by affinity chromatography on the immobilised antigen is possible in situations where the antigen is cheap, in ready supply, and capable of being immobilised on an inert support in a stable form. Often in such cases the antigen is a small molecule or "hapten", rather than a complex protein. Affinity purification is particularly advantageous when it exploits the variable region of the antibody, as it enables antigen-specific antibody to be separated from other antibodies. Affinity purification utilising the interaction at the antigen binding site also allows active antibody to be separated from antibody in which the binding site has been denatured.

In this paper, a paraquat derivative (Fig. 1) immobilised on AH-Sepharose 4B is employed for the purification of antiparaquat monoclonal antibodies from ascites fluid. The purified antibody was required for the preparation of immobilised antibody affinity adsorbents, part of a larger research program studying the characteristics of such adsorbents[9], so it was particularly important that the antigen-specific antibody was selected by the purification procedure. Successful affinity purification relies on the absence of any non-specific interactions, or at least their reduction to an insignificant level. Non-specific binding is a particular problem in the case of the

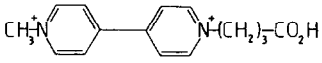

Fig. 1. The chemical structure of paraquat propionate.

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paraquat ligand as it is positively charged and therefore has ion-exchange properties. In the presence of salt, however, such ion-exchange interactions are reduced to an insignificant level and two different protocols for carrying out the chromatography were investigated.

(i) The ascites fluid is applied to the column, the column is washed briefly with buffer, and then more extensively with 1 *M* NaCl. After further washing with buffer the antibody is eluted at low pH.

(ii) The ascites fluid is applied to the column in the presence of 1 *M* NaCl. After washing with buffer the antibody is eluted at low pH.

The advantage of the second strategy over the first is that material that would bind to the column via ion exchange interactions never gets the chance to bind, so it is probable that the capacity of the column for the specifically bound antibody would be greater. It is also much faster, as the need for a separate salt wash and subsequent washing step is removed. On a larger scale difficulties might arise with the addition of large quantities of salt to the crude starting material as this would involve an extra process step.

In order to produce monoclonal antibodies hybrid cells are prepared by fusing lymphocyte (antibody producing) cells with myeloma cells to give hybridoma cells. The hybridoma cells secrete not only heavy and light chains of the required antibody, but may secrete heavy and light chains from the myeloma. The anti-paraquat monoclonal antibodies were prepared by fusion with the NS-1 mouse myeloma cell line[6,10], which secretes only light chains. Once cloned, cell lines were selected for antibody activity. In the selected cell line, however, some light chain from the parent NS-1 myeloma was secreted, so that as the heavy and light chains combine to form the antibody molecules, there will be some antibody with two light chains from the NS-1 myeloma, which will not bind antigen, some mono-valent antibody which contains one light chain from the NS-1 myeloma, but will bind antigen, using the other light chain, and the required antibody with two antigen-binding light chains. The purification procedure must therefore be capable of separating the mono- and bi-valent antibody.

EXPERIMENTAL

Materials

Paraquat propionate and mouse ascites fluid containing antiparaquat monoclonal antibody were a kind gift of ICI Corporate Bioscience Group. AH-Sepharose 4B was obtained from Pharmacia Biotechnology International, Uppsala, Sweden. 1-Ethyl-3-(3 dimethylaminopropyl)carbodiimide and papain immobilised on carboxymethyl cellulose beads were obtained from Sigma (U.K.).

Methods

Preparation of paraquat–Sepharose. In order to attach paraquat covalently to the Sepharose matrix, a derivative of paraquat, paraquat propionate (Fig. 1), which contains a carboxyl groups, and AH-Sepharose 4B which contains free primary amino groups on 6-carbon spacer arms were used. The spacer arm was employed to reduce the possible steric hindrance to the access of antibody from the surface of the support. Steric hindrance often results if a small antigen is bound too closely to the surface of the support[11].

A carbodiimide coupling procedure was employed. A 1-g amount of dry AH-Sepharose 4 was allowed to swell then washed with 200 ml of 0.5 *M* NaCl according to the manufacturers instructions, then washed with distilled water at pH 4.5, and added to a solution of 50 mg paraquat propionate in 3 ml water at pH 4.5. A solution of 100 mg 1-ethyl-3-(3 dimethylaminopropyl)carbodiimide was added dropwise to the suspension whilst the pH was maintained between 4.5 and 6. The suspension was left gently shaking overnight. Non-coupled material was removed by five alternate washings with 0.1 *M* sodium acetate buffer, pH 4 and 0.1 *M* buffer, pH 8.3 each containing 0.5 *M* NaCl.

Purification of antibody from ascites fluid. The purification runs were carried out using an automated chromatography system as described previously[12]. Micro-computer controlled valves allow buffer, sample, salt and eluent in turn to be pumped through the column. The effluent from the column passes through a UV spectrophotometer, which measures the optical density at 280 nm, and is then collected in a fraction collector.

In each case a 1-cm diameter column containing 3 ml of adsorbent was used and run at a flow-rate of 0.5 ml/min.

For the method in which a separate salt wash step was used, ascites fluid was diluted × 5 with 10 m*M* sodium phosphate buffer containing 0.65% sodium chloride, pH 7.5 (phosphate-buffered saline, PBS) and applied to the column. The column was washed briefly with PBS, and then more extensively with 1 *M* NaCl buffered with 10 m*M* sodium phosphate, pH 7.5. After a further PBS wash, the antibody was eluted with 0.2 *M* glycine, pH 2.5 (the optimum pH was determined by lowering the pH in steps until no further antibody was removed). The eluted fractions were neutralised immediately, and dialysed into a suitable buffer.

For the adsorption method carried out in the presence of salt, the ascites fluid was diluted × 5 with 10 m*M* sodium phosphate buffered salt solution, pH 7.5, and NaCl added to a final concentration of 1 *M* NaCl, before being applied to the column. After the application, the column was washed with PBS, and then eluted as above.

In each case the collected fractions were reduced with 2-mercaptoethanol and run on 10% polyacrylamide gels in the presence of sodium dodecyl sulphate (SDS). The method used was that published by Chase[12]. The quantity of pure antibody in the eluted peaks was determined from optical density measurements of the pooled fractions. An extinction coefficient of 1.4 was assumed[13].

The purity of the eluted material was also assessed by using a fast protein liquid chromatography system (FPLC; Pharmacia). A total of approximately 1 mg of protein was applied to a 1 ml Mono Q column in 0.1 *M* sodium acetate, pH 5, and then eluted with a 0–1 *M* NaCl gradient.

Papain digestion of antibody. The digestion was carried out following the method of Kaye and Janeway[14]. 10 U of papain immobilised on carboxymethyl cellulose beads was added to approximately 18 mg of antibody in 25 ml PBS containing 0.1% sodium azide, 4 m*M* EDTA and 0.01 *M* 2-mercaptoethanol. The solution was stoppered to exclude air, and incubated at 37°C for 18 h. The immobilised papain was then removed by centrifugation.

Separation of papain-digested fragments. A 10-ml volume of the digested antibody was applied at 0.25 ml/min to the immobilised paraquat column described

above. Buffer, separate salt wash, and elution were carried out as for the purification runs.

RESULTS AND DISCUSSION

The results of the two purification runs are shown in Figs. 2 and 3. Figs. 2a and 3a show the spectrophotometer signal as a function of time, which is an indication of the protein content of the column outlet. For a mixture of proteins the optical density cannot be readily converted into a protein concentration, so the signal is left in arbitrary units for convenience. From this trace, the adsorption, washing and elution phases of the purification run can be seen. The SDS–polyacryl amide gel electrophoretic (PAGE) analysis (Figs. 2b and 3b) shows that the starting material contains a number of different proteins.

For the purification using a separate salt wash step (Fig. 3), some proteins start to appear in the outlet stream as the adsorption phase progresses. During the salt wash, many proteins are eluted. The eluted peak, however, contains only three bands. The SDS-PAGE analysis is carried out in the presence of 2-mercaptoethanol, under which conditions, the antibody is broken down into two heavy chains with a molecular weight of 50 000, and two light chains with a molecular weight of approximately 25 000. The track on the gel corresponding to the eluted peak shows one band in the heavy chain region, and, somewhat unexpectedly, two in the light chain region.

For the run in the presence of salt (Fig. 2), breakthrough of unbound proteins is immediate and a great number of components pass straight through the column. It can be seen from the optical density trace that there is a rise near the end of the application stage, suggesting that the antibody is beginning to break through. This is confirmed by the corresponding gel tracks (7 and 8), where bands corresponding to the eluted peak appear (it is the appearance of the light chain that is observed; the appearance of the heavy chains is masked by the albumin (molecular weight 66 000) passing through the column). So, the effective capacity of the adsorbent under these conditions has been reached, and was estimated to be about 6 mg/ml of adsorbent. The amount of antibody recovered was greater for the run in which the adsorption was carried out in the presence of salt (6 mg/ml adsorbent as opposed to 4,3 mg/ml adsorbent).

The gels from both purification protocols show that although all the contaminating material has been removed, the eluted peak shows not just one antibody light chain, but two different ones. Only one of these can be due to the bi-valent paraquat-specific antibody. The other one is present due to the existence of antibody molecules which in addition to the heavy chains contain one paraquat specific light chain and one light chain from the myeloma parent. These "monovalent" molecules contain one paraquat specific "arm", and therefore bind to the affinity column, but their presence in the affinity product may be undesirable. If the antibody is itself to be used as an affinity ligand, then the presence of antibody with only one specific site may reduce the potential capacity of the immunoadsorbent.

To determine which of the two light chains corresponds to the paraquat-specific arm, papain digestion of the eluted antibody was carried out. The action of papain on immunoglobulin G is highly specific. It divides the antibody into three sections, two fragments, known as Fab fragments, and one Fc fragment, with the latter having no

Fig. 2. Purification of anti-paraquat antibody on an immobilised paraquat column. Adsorption carried out in the presence of salt. A buffer wash was started at 90 min, followed by acid elution at 150 min. (a) Spectrophotometer reading at 280 nm as a function of time (arbitrary units). (b) Gel electrophoresis of samples corresponding vertically to the respective position of the optical density trace. M = Molecular weight markers 205, 116, 97, 66, 45 and 29 kilodaltons; S = Starting material; 1–8 = unadsorbed material; 11,12 = eluted peak. Antibody at 0.5 mg/ml was applied at a flow-rate of 0.5 ml/min to a 3-ml column of paraquat immobilised on Sepharose 4B. The eluted peak contained 18 mg of purified antibody.

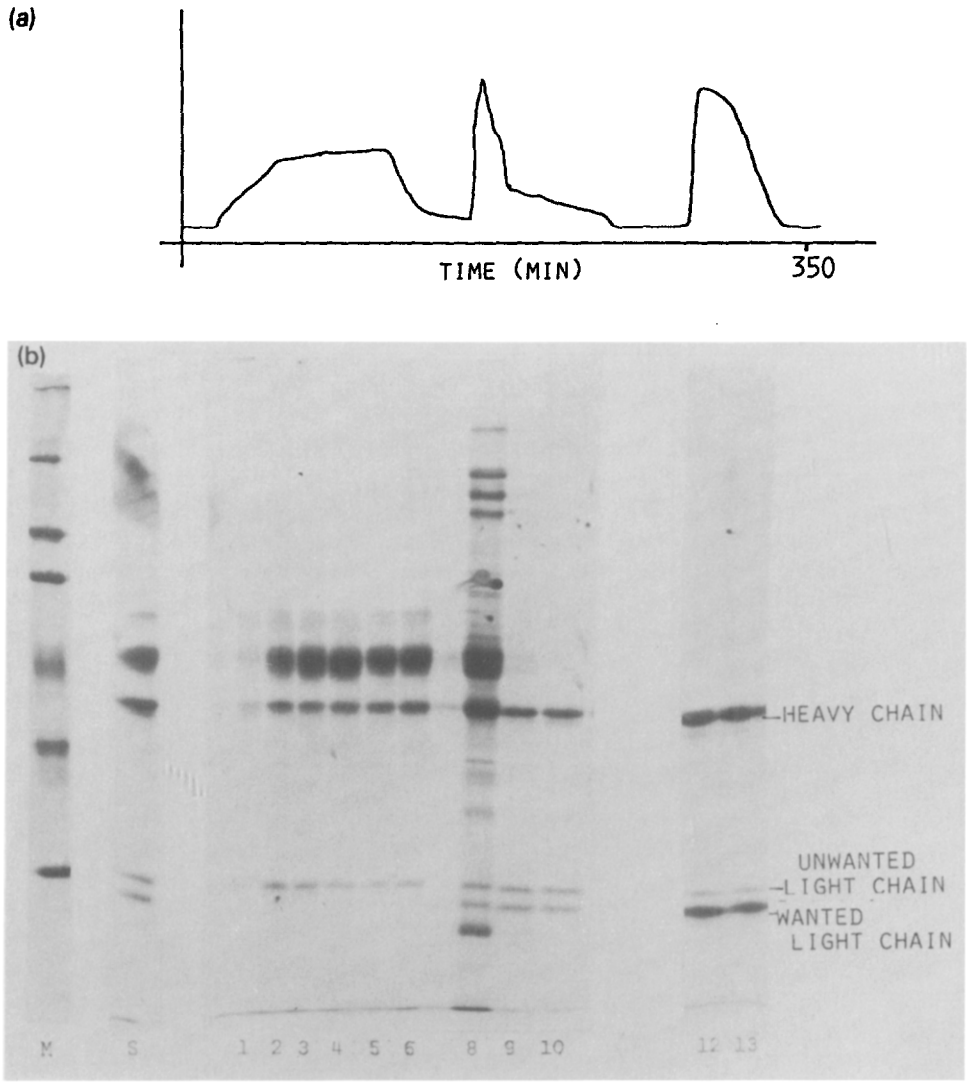

Fig. 3. Purification of anti-paraquat antibody on an immobilised paraquat column. Adsorption carried out in phosphate-buffered saline, followed by washing with 1 *M* NaCl. A buffer wash was started at 90 min, followed by the salt wash at 135 min, a futher buffer wash at 195 min, and acid elution at 255 min. (a) Spectrophotometer reading as a function of time (arbitrary units). (b) Gel electrophoresis of samples corresponding vertically to the respective position on the optical density trace. M = Molecular weight markers, 205, 116, 97, 66, 45 and 29 kilodaltons; S = starting material; 1–6 = unadsorbed material; 8 = components removed immediately by salt wash; 9, 10 = components removed by extended salt wash; 12, 13 = eluted peak. Antibody at 0.5 mg/ml was applied at a flow-rate of 0.5 ml/min to a 3-ml column of paraquat immobilised on Sepharose 4B. The eluted peak contained 13 mg of purified antibody.

recognition site for antigen. The Fab fragments still retain their specific binding sites, and can still bind antigen, but Fab fragments that originate from the myeloma parent of the monoclonal will not recognise paraquat. Therefore, if the mixture of digested antibody is applied to a column of immobilised paraquat, then the Fc fragments and the Fab fragments corresponding to the myleoma parent will pass straight through, and only the paraquat specific Fab fragment will bind. As the light chains are unaffected by the papain digestion, comparison of the tracks corresponding to purified antibody with those for unadsorbed and eluted material from the digestion, on an SDS-PAGE gel, shows which light chain is which. Such analysis with this antibody showed that it is the smaller of the two light chains that is paraquat specific (result not shown).

Comparison of the size of the eluted peaks for the two purification runs on SDS-PAGE shows that the quantity of the unwanted light chain is considerably reduced during the method where a separate salt wash step was used. This is because monovalent antibody molecules are removed from the column during the salt wash and are no longer adsorbed on the column at the start of elution. From Fig. 3a it can be seen that as the salt wash is applied to the column, initially there is a large peak which indicates that most contaminants are removed immediately by the salt, but the peak has a "shoulder" showing that some material is removed only slowly. Examination of the corresponding gel tracks, track 8 (the initial peak) shows that indeed many proteins are eluted at the start of the salt wash. The tracks corresponding to the shoulder (tracks 9 and 10), however, contain only three bands, and represent pure antibody. As in the eluted peak, there are two bands indicating two different light chains, but here they are equal in intensity, suggesting that only the monovalent antibody with one light chain of each type is being removed by the prolonged salt wash. Affinity adsorption between paraquat and a paraquat binding site via a single monovalent interaction does not appear to be particularly strong, as paraquat-specific Fab fragments adsorbed to the column could also be removed by an extended salt wash. In the absence of salt, antibody molecules are adsorbed to the column by a combination of affinity and ion exchange interactions. In the presence of salt, the ion exchange interactions are abolished, and the strength of the adsorption is consequently weakened. The bivalent antibody binds more strongly than the monovalent antibody in the presence of salt, as the former species is not eluted with a salt wash. This suggests that the bivalent antibody is actually bound to the column using both antigen binding sites. Although the monovalent antibody is removed from the column by a salt wash, it is still significantly retarded due to the affinity interactions involving the single antigen-binding site. When ascites fluid is applied to the column in the presence of salt followed by a buffer wash, a considerable amount of monovalent antibody is still retained by the column, as this protocol does not contain a long enough irrigation of the column with salt to result in complete removal of the monovalent antibody. The monovalent antibody is then adsorbed more strongly during the subsequent buffer wash as additional ion-exchange interactions can now occur which also prevent its removal from the column. The purified antibody still contains some monovalent antibody. In order to effect complete removal of the monovalent antibody, a longer salt wash would be required. As a result of improved cell-line selection methods, in subsequent batchs of ascites fluid, the contamination with light chain variants was not as great, and this protocol proved to be sufficient. The apparent increased

recovery of antibody observed during the method involving adsorption in the presence of salt may be due substantially to the presence of increased quantities of the adsorbed monovalent antibody, rather than resulting from an increase in the capacity of the column for antibody in the absence of ion-exchange interactions.

The analysis by FPLC (result not shown) also confirmed the complete separation of the purified antibody from other proteins, as only a single peak was observed. The monovalent antibody molecule does not appear to be separated from the bivalent antibody during ion-exchange chromatography. The presence of monovalent antibodies in ascites fluid has been noted elsewhere[7]. In order to effect resolution of the light chain variants, high performance liquid chromatography on hydroxyapatite and gradient elution were employed.

It was possible to effect elution of anti-paraquat antibodies from the immobilised antigen simply by lowering the pH, without resorting to the use of chaotropic salts, or other methods. Even at pH 2.5, the elution process is slow, requiring about three column volumes of eluent. Despite being broad the eluted peak does not display the long tailing usually observed in such systems, and the tailing edge of the peak was observed to drop fairly sharply (see Figs. 2 and 3).

To ensure that the antibody is not denatured by the elution conditions, a purification run was carried out and then the eluted peak dialysed against PBS and re-applied to the column. No protein passed straight through the column, and only a tiny fraction was eluted with a salt wash, the remainder being eluted with acid as before. This shows that the antibody still retains its full antigen binding activity.

Immobilised paraquat also proved to be very stable, as during this work a single column of paraquat–Sepharose was used repeatedly (approx 50 times) and over the course of several years without a noticeable drop in performance.

CONCLUSION

Affinity purification of monoclonal anti-paraquat from ascites fluid using immobilised antigen proved to be a successful single step method for purification to protein homogeneity. As salt wash was required, during the protocol, due to the presence of positive charges on the paraquat molecules, giving the adsorbent ion-exchange properties. The purification method had the added advantage that it resulted in separation of antibody that had two paraquat-specific binding sites from antibody that had only one. Such separation would not have been possible by methods such as ion-exchange chromatography.

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