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THE PURIFICATION OF MEMBRANE-ASSOCIATED TUMOUR ANTIGENS BY PREPARATIVE POLYACRYLAMIDE GEL ELECTROPHORESIS

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SUMMARY

The technique of preparative polyacrylamide gel electrophoresis has been employed to purify solubilized membrane-associated tumour antigens from two aminoazo dye-induced rat hepatomas.

Limited papain digestion and EDTA treatment of subcellular membrane fractions or viable ascites hepatoma cells was found to liberate soluble antigenic material suitable for further fractionation. The heterogeneity of initial extracts was revealed by analytical polyacrylamide gel electrophoresis. This technique was used to monitor fractions obtained throughout the preparative electrophoresis in order to assess the purification obtained.

The value of preparative polyacrylamide gel electrophoresis is discussed in relation to other methods currently available for the purification of solubilized membrane-associated antigens.

INTRODUCTION

The isolation of tumour-specific antigens has, in the main, followed closely the concepts developed for the study of mouse H-2 and human HL-A histocompatibility antigens. The proteolytic enzyme papain has been used by several groups to release cell surface antigens from a variety of normal and neoplastic cell types (Mann *et al.*[1], Shimada and Nathenson[2], Yamane and Nathenson[3]) and has recently been employed to release tumour-specific antigen from rat hepatoma D_{23} by Baldwin and Glaves[4]. Fractionation of the extracted antigens was performed by DEAE-cellulose column chromatography and a single hepatoma D_{23} antigenic region was defined in the effluent. Analytical polyacrylamide gel electrophoresis has shown this region to be heterogeneous (Baldwin *et al.*[5]) and further purification was therefore attempted by rate zonal centrifugation followed by preparative polyacrylamide gel electrophoresis.

To avoid this overall lengthy procedure efforts have been made to perform

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a single step purification of hepatoma-specific antigen by preparative polyacrylamide gel electrophoresis. The results of this study are to be described and will be compared with those obtained when partially purified hepatoma-specific antigen from DEAE-cellulose chromatography and rate zonal centrifugation was used as the starting material for the preparative electrophoresis. Because of the sensitivity of the analytical polyacrylamide gel electrophoresis procedure in resolving the initially very complex papain extract from rat hepatoma D_{23} and its ability to reveal the heterogeneity of the partially purified antigen obtained following DEAE-cellulose chromatography, it can reasonably be concluded that when put onto a preparative scale the technique might have the ability to separate the many components in the initial papain extract directly. The method has in fact been successfully used to purify prolactin and growth hormone by Groves and Sells[6], and to purify HL-A antigens by Reisfeld and Kahan[7].

Throughout this study hepatoma-specific antigen was extracted by limited papain digestion from suspensions of rat ascites hepatoma cells (D_{23} and D_{31}) and also from an 'extranuclear' membrane fraction obtained following homogenization of rat hepatoma D_{23} tissue. Preliminary experiments have also been performed with protein extracted by EDTA treatment of the membrane fraction. Tumour-specific antigenicity was detected throughout the purification schemes (a) by the capacity of fractions to neutralise antibody in syngeneic immune serum, thereby preventing the reaction of this antibody with tumour-specific antigen at the surface of viable hepatoma cells, as detected by the indirect membrane immunofluorescence test (Baldwin and Barker[8]), and (b) by a simple immunodiffusion system using rabbit anti-rat hepatoma serum that has been absorbed with normal liver cells and normal rat serum to remove antibodies directed against normal tissue and serum antigens.

The results presented will be discussed in relation to current concepts of the meaning of 'homogeneity' with regard to solubilized membrane antigens. The technique of preparative polyacrylamide gel electrophoresis will be critically evaluated and compared with other purification methods.

MATERIALS AND METHODS

Rats and tumours

Rat hepatomas D_{23} and D_{31} were originally induced by oral administration of 4-dimethylaminoazobenzene and have been maintained by serial transplantation in syngeneic Wistar rats. The ascites variants of hepatomas D_{23} and D_{31} were established by intraperitoneal injection of single cell suspensions obtained from solid tumour mince by trypsin digestion. Ascites hepatoma D_{23} was grown for 9 or 10 days and D_{31} for 7 days; $2 \cdot 10^8$–$3 \cdot 10^8$ D_{31} cells and $1 \cdot 10^8$–$2 \cdot 10^8$ D_{23} cells were harvested per rat.

Homogenization of hepatoma D_{23} tissue

Hepatoma D_{23} tissue was homogenized by controlled mechanical disruption using an Ultraturrax homogenizer and a crude 'extranuclear' subcellular membrane fraction (the $78\,000 \times g$ sediment of the $600 \times g$ supernatant from the homogenate) was prepared according to previously reported methods (Baldwin and Glaves[4], Baldwin *et al.*[5]).

Papain extraction of hepatoma D_{23} 'extranuclear' membrane

Crude hepatoma D_{23} membrane in 5 mM Tris–HCl buffer, pH 8.0, prepared as above, was incubated with papain (Sigma) at a concentration of approximately 1 mg enzyme per 30 mg membrane suspension. The papain was activated by the addition of L-cysteine to give a concentration of 5 mM. The stirred suspension was then incubated at 37 °C for 60 min and the membrane fragments sedimented at $78\,000 \times g$ for 90 min. The supernatant was then dialysed overnight against 5 mM Tris–HCl buffer (pH 8.0) at 4 °C to remove the L-cysteine and thereby inactivate the papain. This dialysis was followed by concentration using either rapid pervaporation or Aquacide II (Calbiochem.) and the solution was then redialysed against 5 mM Tris–HCl buffer (pH 8.0) at 4 °C. The solution was finally clarified by centrifugation at $165\,000 \times g$ for 10 min and the small pellet discarded. The supernatant was used immediately for the fractionation procedures or was stored at 4 °C with 0.01% sodium azide present until required.

EDTA extraction of hepatoma D_{23} 'extranuclear' membrane

Crude hepatoma D_{23} membranes prepared as above were stirred at 4 °C for 60 min following the addition of EDTA solution (pH 9.0) to give a final concentration of 20 mM EDTA. The suspension was then centrifuged at $78\,000 \times g$ for 30 min and the supernatant removed, concentrated as above, dialysed overnight against 5 mM Tris–HCl buffer (pH 8.0) at 4 °C and finally clarified by centrifugation at $165\,000 \times g$ for 10 min. The solution was used immediately or stored at 4 °C with 0.01% sodium azide present.

Papain extraction of ascites hepatoma cells

Ascites hepatoma D_{23} and D_{31} cells were collected in heparinised medium 199 and washed in Tris–NH_4Cl buffer (pH 7.2) to haemolyse and remove the red blood cells. The ascites hepatoma cells were then washed three times with medium 199. D_{23} cells were then washed three times with calcium-free Lockes solution. This step was omitted with the D_{31} cells owing to the fact that the cells clumped extensively if it was included. L-Cysteine was then added to the cells following suspension in medium 199 to give a 5 mM concentration. A measured volume of papain suspension (Sigma) was then added (1 mg papain/$5 \cdot 10^8$ cells) and the cells incubated at 37 °C for 60 min with stirring. Following the incubation the cells were sedimented at 3000 rev./min for 10 min, the supernatant decanted and recentrifuged at $78\,000 \times g$ for 30 min. The supernatant from this centrifugation was then dialysed overnight at 4 °C against deionized water to reduce the salt concentration and then reduced in volume by rapid pervaporation. This was followed by overnight dialysis against 5 mM Tris–HCl buffer (pH 8.0) and the solution finally clarified by centrifugation at $165\,000 \times g$ for 10 min.

Analytical polyacrylamide gel electrophoresis

Analytical polyacrylamide gel electrophoresis was performed on gels composed of 7.0% acrylamide (Kodak), 0.1% *N,N'*-methylenebisacrylamide (B.D.H.) in 0.05 M Tris–HCl buffer (pH 8.0), polymerized by 0.1% *N, N, N', N'*-tetramethylethylenediamine (Koch-Light) and 0.05% $(NH_4)_2S_2O_8$ (B.D.H.). Gels 7.0 cm in length and 0.5 cm in diameter were formed and left overnight before use. 0.05 M

Tris–HCl buffer (pH 8.0) was used in both the anode and cathode compartments. Gels were routinely pre-run before application of protein samples to remove possible impurities from the system and the electrolysis buffer replaced.

Electrophoresis was performed using approximately 50-μl quantities of the protein solutions per tube, at a current of 3 mA per tube. Current was passed until bromophenol blue, added as a tracked dye, had passed to the bottom of the gels. Gels were then removed from their tubes and stained in 0.01% Coomassie blue in acetic acid–methanol–water (10:20:70, by vol.) and destained by washing in the same solvent mixture, in which the gels were stored without appreciable fading of the stained bands of protein. Photography was performed using Kodak Tri-X Pan 35 mm film with gels positioned over a Shandon Illuminated Viewer.

Preparative polyacrylamide gel electrophoresis

Preparative polyacrylamide gel electrophoresis was performed using the Quickfit Instrumentation apparatus with tap water cooling. 30-ml gels were formed using the same acrylamide composition as for the analytical polyacrylamide gel electrophoresis system, above. The gel was left overnight before use and was pre-run until a sample of bromophenol blue had been eluted before the protein sample was applied. The electrolysis buffers were replaced at this stage. Samples of approximately 3.0 ml containing up to 90 mg of protein were applied to the region just above the gel surface. The current was gradually increased from 10 mA to the running current of 80 mA. The eluant buffer (0.05 M Tris–HCl, pH 8.0) was monitored at 280 nm. Fractions of 1.5 ml were taken and the material thus collected was used immediately or stored at -20 °C.

Immunodiffusion analysis of tumour antigen

Antiserum against ascites hepatomas D_{23} and D_{31} were prepared in rabbits by weekly injecting subcutaneously $1 \cdot 10^8$ cells in Freunds Complete Adjuvant, over a period of 8 weeks. After preliminary immunodiffusion testing had been performed the rabbits were bled out and the serum stored at -20 °C. This serum was absorbed with $5 \cdot 10^6$ normal liver cells per ml and also with 1.0 ml normal rat serum per ml to produce an antiserum reacting primarily with tumour antigens.

Agar immunodiffusion plates were prepared on 3 inches $\times$ 1 inch microscope slides and wells cut in the conventional hexagonal pattern. The centre wells were filled with the absorbed rabbit anti-hepatoma antiserum and the outer wells with sequential fractions from preparative polyacrylamide gel electrophoretic tumour antigen purifications. In this way the fractions exhibiting tumour (specific) antigenic activity were rapidly located, following overnight development of precipitin lines.

Membrane immunofluorescence assay of tumour specific antigen

The indirect membrane immunofluorescence test was performed with viable hepatoma D_{23} cells in suspension as previously described[8], using sera from syngeneic rats sensitised to hepatoma D_{23} cells. Fluorescence indices were calculated for test serum samples by determining the percentage of cells unstained with control normal rat serum *minus* the percentage of cells unstained with the test serum divided by the former figure.

Individual or pooled soluble fractions, dialysed against phosphate–buffered saline, pH 7.3, were assayed for antigenic activity by determining their capacity to neutralise the reaction of antibody in hepatoma D_{23} immune serum with tumour-specific antigens on viable hepatoma D_{23} cells as assessed using membrane immunofluorescence (Baldwin and Glaves[4], Baldwin *et al.*[5]). Thus, antigenic activity associated with soluble fractions was denoted by a reduction of fluorescent staining with absorbed immune serum as compared with immune serum diluted with equivalent volumes of phosphate-buffered saline. The percentage inhibition of the fluorescence index with absorbed serum was defined as a measure of antigenic activity.

Electron microscopy

Electron microscopic studies of rat ascites hepatoma D_{23} and D_{31}, and hepatoma D_{23} tissue employed conventional procedures. Fixation was performed in phosphate-buffered 2.0% glutaraldehyde solutions at room temperature overnight, and was followed by staining with 1.0% osmic acid in phosphate buffer for 2 h. Samples were then dehydrated by taking them through a series of graded ethanol solutions at 4 °C and finally epoxy propane before embedding in TAAB epoxy resin. Thin sections were cut with an LKB Ultratome 111 using glass knives and were mounted on Micron Type 400 grids. Post staining was performed using 2.0% uranyl acetate in 50% ethanol. Specimens were viewed in a Philips E.M. 300 and photographs were taken on Ilford E.M. 6 plates.

RESULTS

The purification of extracts from cell suspensions of ascites hepatoma D_{23} and D_{31}

Electron microscopy of the cells. Figs 1 and 2 show representative electron

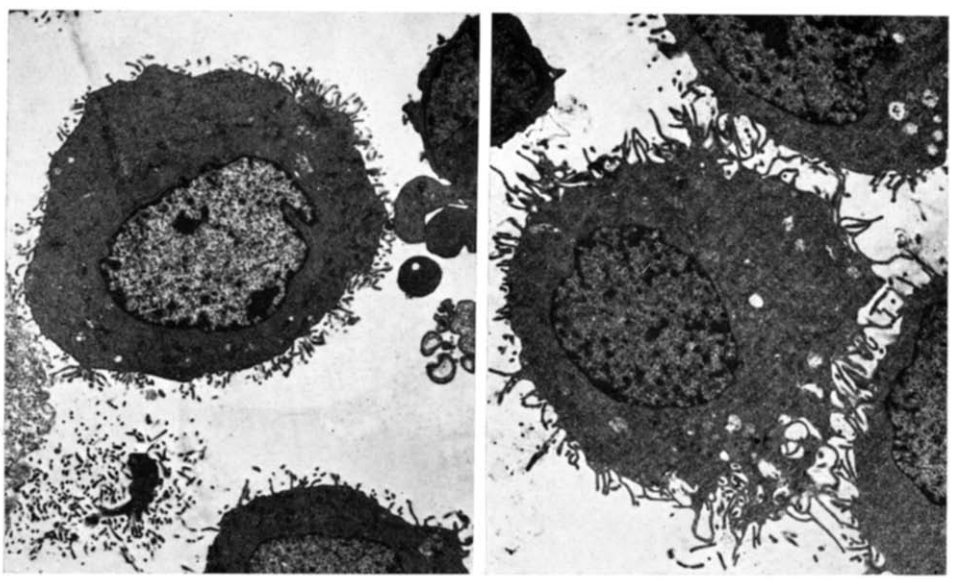

Fig. 1. Rat ascites hepatoma D_{23} cells. × 2800.

Fig. 2. Rat ascites hepatoma D_{31} cells. × 4000.

micrographs of ascites hepatoma D_{23} and D_{31}, respectively. The cells in both cases are coated with microvilli and have extensive endoplasmic reticulum. The two cell types are easily distinguished as ascites hepatoma D_{31} has a larger number of lipid containing vesicles within the cytoplasm. Following papain digestion for 60 min at 37 °C the cells remain viable as judged by their exlusion of methylene blue, but in the electron microscope it has been found that the microvilli are less pronounced, indicating the possibility that they have been broken off and that the cell surface has resealed during the incubation, see Fig. 3.

Analytical polyacrylamide gel electrophoresis of ascites hepatoma extracts. The release of protein from ascites hepatoma cells by papain digestion was as follows: D_{23} ascites; 0.6 mg protein per 10^8 cells (mean of 11 extractions). D_{31} ascites; 0.8 mg protein per 10^8 cells (mean of 16 extractions). These extracts showed tumour-specific antigenicity when tested by the indirect membrane immunofluorescence technique and by the production of precipitin lines when analysed by immuno-diffusion. Representative analytical polyacrylamide gel electrophoresis runs performed with ascites hepatoma D_{23} and D_{31} papain extracts are shown in Fig. 4. The complexity of the protein solutions is immediately apparent. It should be mentioned at this stage that papain itself is electropositive at the pH at which the electrophoresis is performed (pH 8.0), and therefore it can be concluded that all the protein bands seen on the gels are derived from the ascites hepatoma cells.

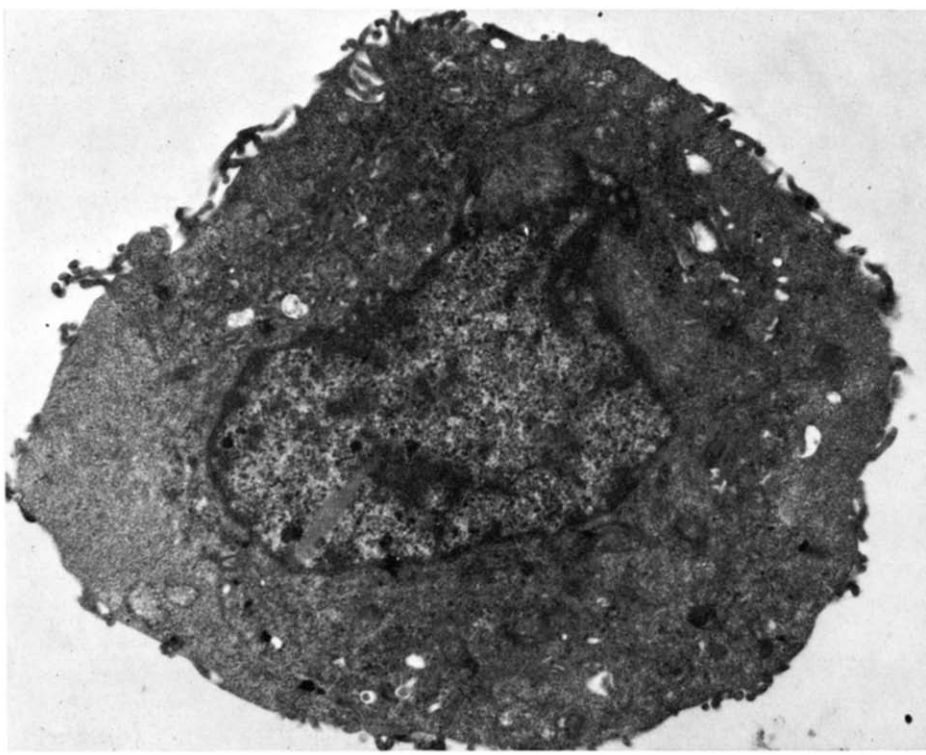

Fig. 3. A rat ascites hepatoma D_{23} cell following proteolytic digestion with papain for 60 min at 37 °C. Many of the surface microvilli appear to have been lost. × 6000.

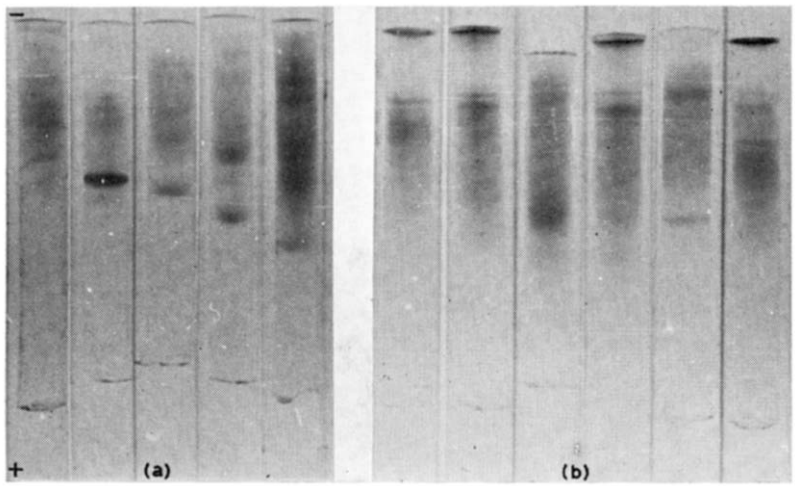

Fig. 4. Analytical polyacrylamide gel electrophoresis of the papain-extracted protein obtained from rat ascites hepatoma; (a) D_{23} cells. (b) D_{31} cells.

Preparative polyacrylamide gel electrophoresis of extracts from ascites hepatoma D_{23} and D_{31}. Fig. 5 shows representative profiles of the protein content of the eluant from preparative polyacrylamide gel electrophoresic separation performed on papain extracts from rat ascites hepatoma D_{23} and D_{31}, respectively. The region between the arrows indicates the range of fractions containing tumour-associated antigen, as assessed by the production of immunodiffusion precipitin lines. Analytical polyacrylamide gel electrophoresis performed with samples of the preparative polyacrylamide gel electrophoresis fractions reveals the separation obtained, see Figs 6 and 7. In the case of the D_{31} extract the purification obtained was not very good over the range where the antigenic activity was detected.

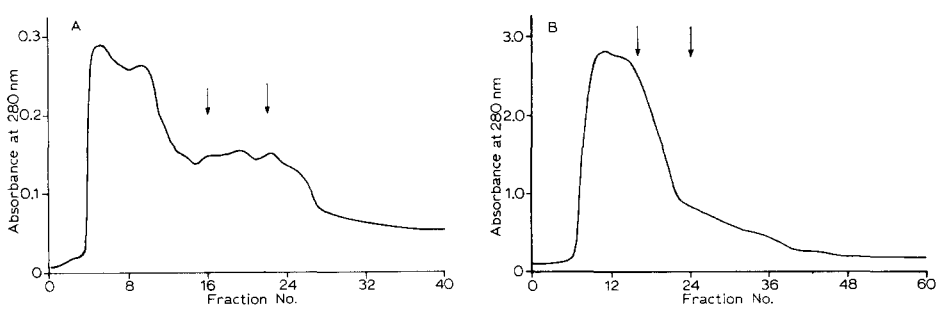

Fig. 5. Preparative polyacrylamide gel electrophoresis elution profiles given by papain extracted protein from rat ascites hepatomas D_{23} and D_{31}, respectively. The region between the arrows indicates in each case the fractions containing tumour-associated antigen, detected by immunodiffusion.

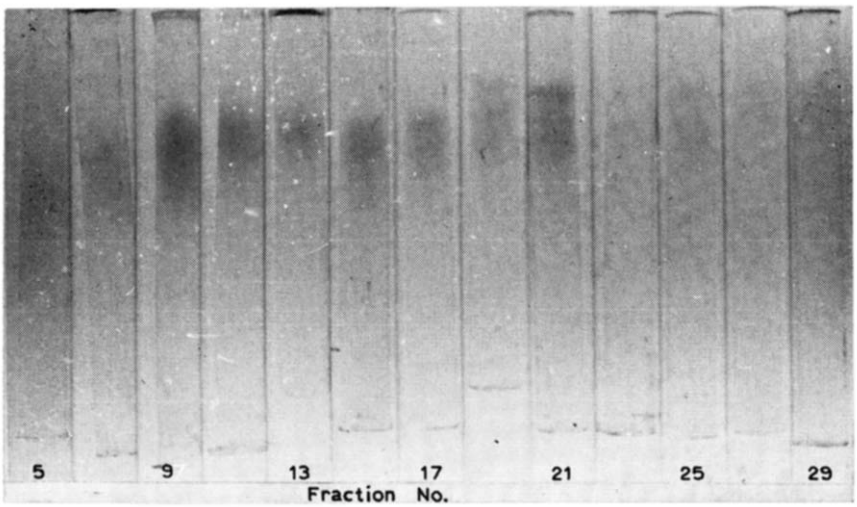

Fig. 6. Analytical polyacrylamide gel electrophoresis corresponding with the preparative electrophoretic separation of rat ascites hepatoma D_{23} papain extracted protein shown in Fig. 5A.

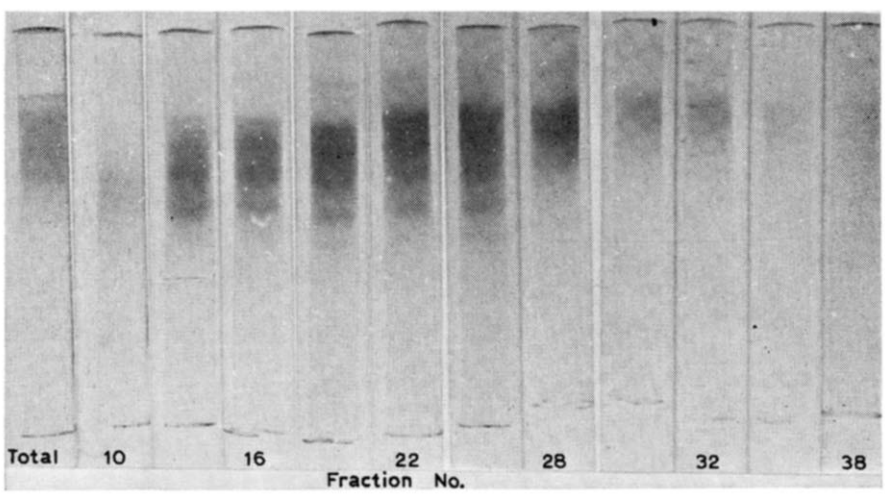

Fig. 7. Analytical polyacrylamide gel electrophoresis corresponding with the preparative electrophoretic separation of rat ascites hepatoma D_{31} papain extracted protein shown in Fig. 5B.

Extracts from rat hepatoma D_{23} 'extranuclear' membrane

Electron microscopy of the intact tissue. The cells within rat hepatoma D_{23} tissue are extremely irregular in shape and many show surface microvilli, see Fig. 8. The tissue abounds with collagen and as with the ascitic hepatoma, the endoplasmic reticulum of the cells is very well developed, as expected in rapidly growing tissue.

Analytical polyacrylamide gel electrophoresis of hepatoma D_{23} 'extranuclear' membrane extracts. Figs 9 and 10 show representative analytical polyacrylamide gel electrophoretic separations of papain and EDTA extracts obtained from

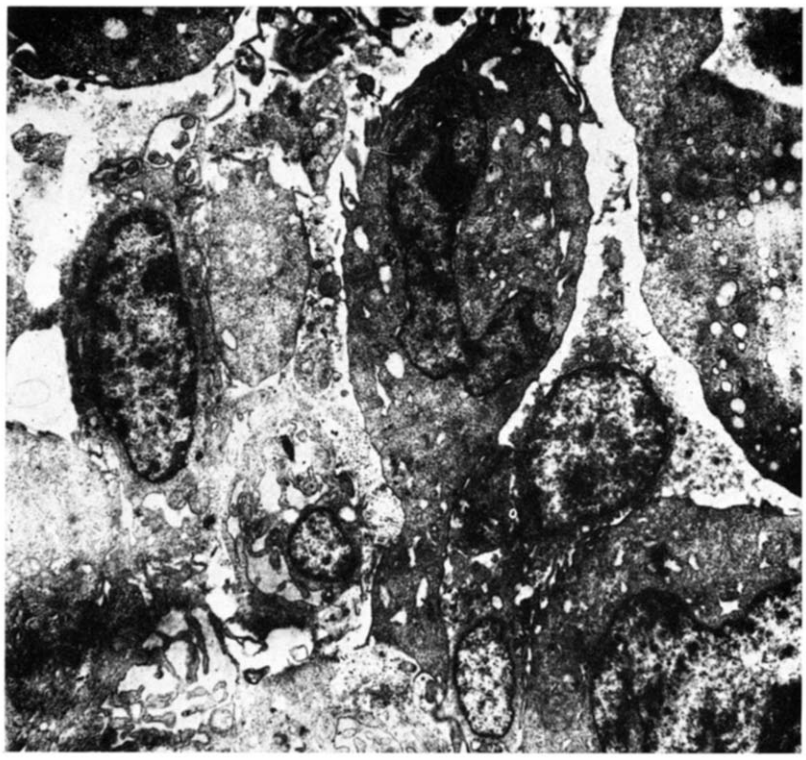

Fig. 8. An electron micrograph of rat hepatoma D_{23} tissue. The bundles of collagen lying in the extracellular spaces are very apparent as are the varied cell chapes and the microvilli. ×4200.

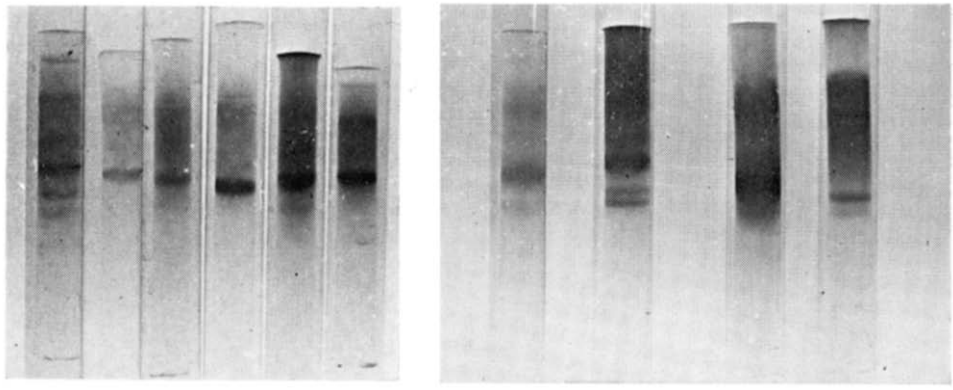

Fig. 9. Analytical polyacrylamide gel electrophoresis of extracts obtained from rat hepatoma D_{23} 'extranuclear' membrane by papain digestion.

Fig. 10. Analytical polyacrylamide gel electrophoresis of extracts obtained from rat hepatoma D_{23} 'extranuclear' membrane by EDTA treatment (20 mM at pH 9.0).

hepatoma D_{23} 'extranuclear' membrane. Both types of extract exhibit hepatoma-specific antigenicity when tested by indirect membrane immunofluorescence and immunodiffusion.

Preparative polyacrylamide gel electrophoresis of papain and EDTA extracts from hepatoma D_{23} 'extranuclear' membrane. Representative profiles of the preparative polyacrylamide gel electrophoretic purifications of hepatoma D_{23} papain and EDTA extracts are shown in Figs 11 and 12. Included in Fig. 11 is a curve showing the results of the indirect membrane immunofluorescence test, which compares favourably with the immunodiffusion analysis for the localisation of fractions

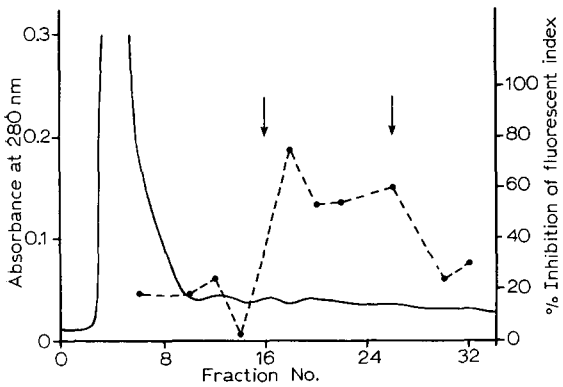

Fig. 11. A preparative polyacrylamide gel electrophoretic elution profile given by papain extracted protein from rat hepatoma D_{23} 'extranuclear' membrane. The broken line indicates the presence of tumour-specific antigen as assessed by indirect membrane immunofluorescence and the region between the arrows indicates the presence of tumour-associated antigen as detected by immunodiffusion.

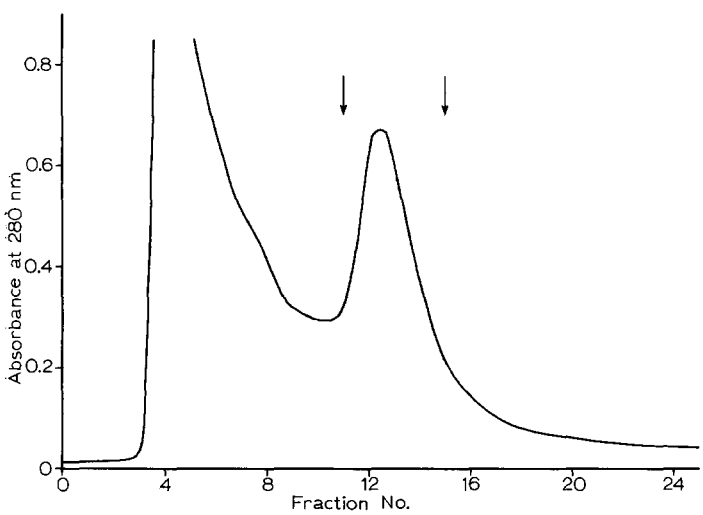

Fig. 12. A preparative polyacrylamide gel electrophoretic elution profile given by EDTA-extracted protein from rat hepatoma D_{23} 'extranuclear' membrane. The region between the arrows indicated the presence of tumour-associated antigen as detected by immunodiffusion.

containing tumour-specific antigen. Analytical polyacrylamide gel electrophoretic separations of the purified fractions from the two preparative polyacrylamide gel electrophoretic runs are shown in Figs 13 and 14.

Preparative polyacrylamide gel electrophoresis of partially purified hepatoma D_{23} specific antigen. Pooled fractions from DEAE-cellulose chromatography and rate zonal centrifugation, which showed hepatoma D_{23} specific antigenicity[5], located by both the detection methods, were applied as the starting sample in the preparative polyacrylamide gel electrophoresis apparatus. The protein elution

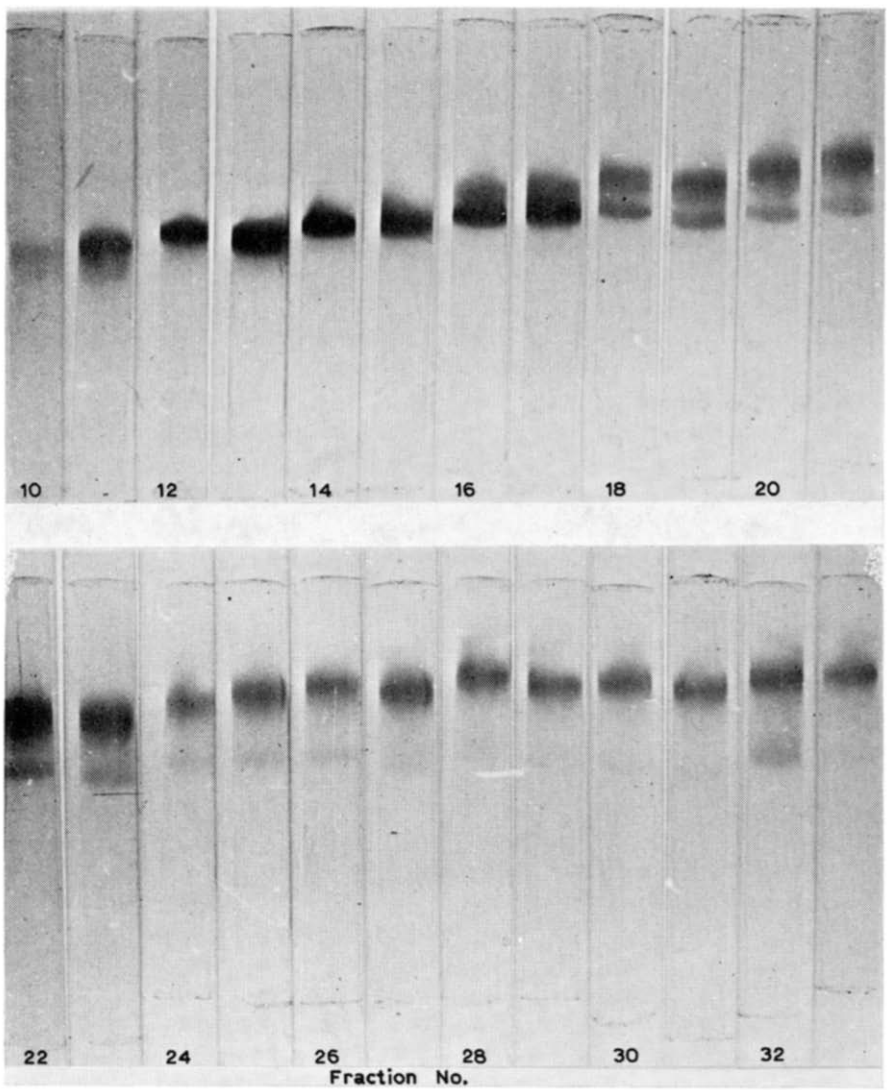

Fig. 13. Analytical polyacrylamide gel electrophoresis of the fractions obtained following preparative electrophoresis of the proteins released from rat hepatoma D_{23} 'extranuclear' membrane by papain digestion, shown in Fig. 11.

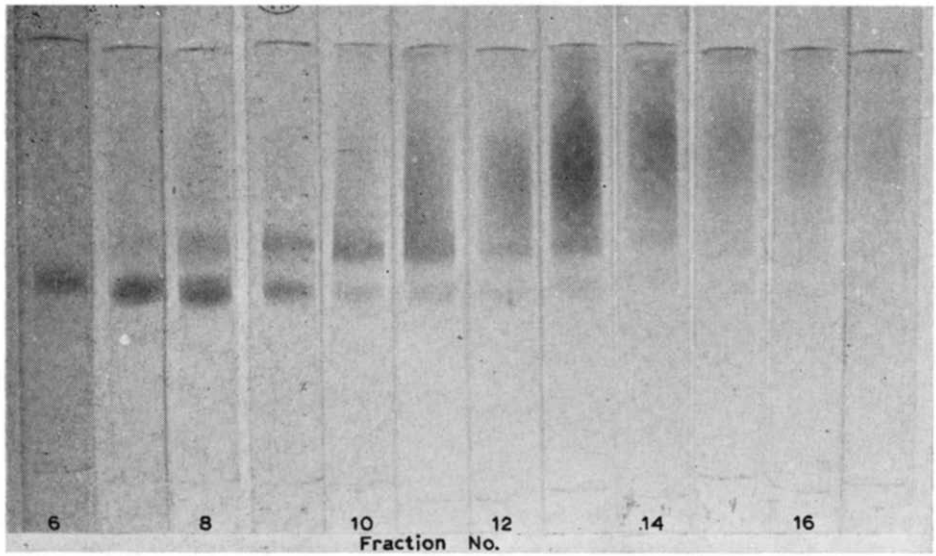

Fig. 14. Analytical polyacrylamide gel electrophoresis of the fractions obtained following preparative electrophoresis of the protein released from rat hepatoma D_{23} 'extranuclear' membrane by EDTA treatment, shown in Fig. 12.

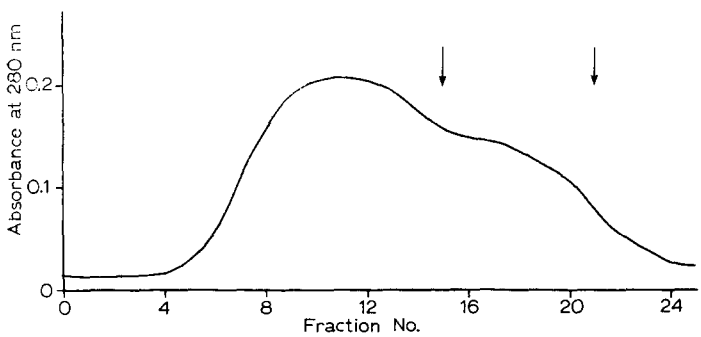

Fig. 15. A preparative polyacrylamide gel electrophoretic elution profile given by hepatoma D_{23} tumour-specific antigen that had been partially purified by DEAE-cellulose column chromatography. The region between the arrows indicates the fractions containing purified tumour-associated antigen as detected by immunodiffusion.

profile from a representative separation is shown in Fig. 15. The corresponding analytical polyacrylamide gel electrophoretic and immunodiffusion analysis performed in the purified fractions are shown in Figs 16 and 17.

DISCUSSION

The results presented indicate that the technique of preparative polyacrylamide gel electrophoresis has the ability to separate purified fractions containing tumour-specific antigen from the many components present in the initial extracts

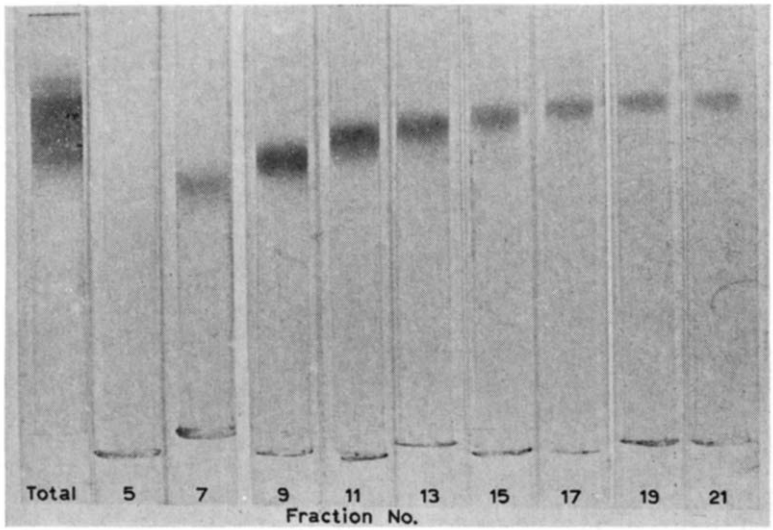

Fig. 16. Analytical polyacrylamide gel electrophoresis of the fractions obtained following preparative electrophoresis of hepatoma D_{23} tumour-specific antigen that had previously been partially purified by DEAE-cellulose chromatography, as shown in Fig. 15.

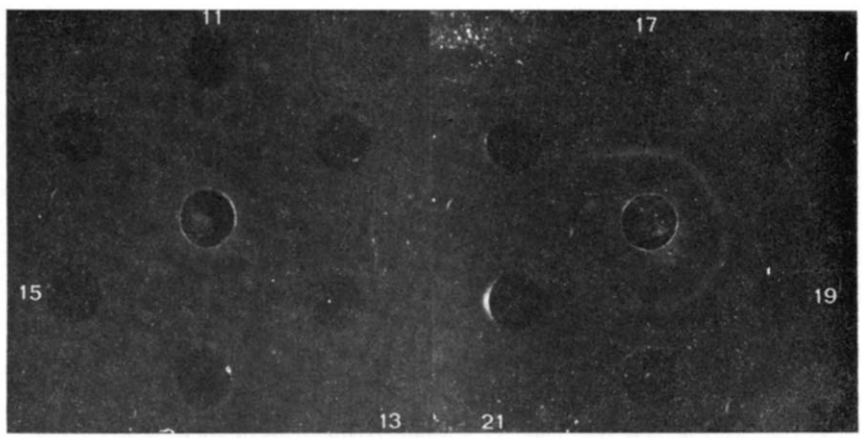

Fig. 17. Immunodiffusion analysis of the fractions obtained following preparative electrophoresis of rat hepatoma D_{23} tumour-specific antigen that had previously been partially purified by DEAE-cellulose chromatography, as shown in Fig. 15.

made from ascites hepatoma cells and hepatoma cell membrane. The major limitation of the method is that the upper limit to the amount of protein that can conveniently be applied to the electrophoresis apparatus is in the region of 200 mg. This immediately indicates that a preliminary purification prior to preparative electrophoresis is highly desirable to remove some of the non-tumour-specific components of the protein extracts. Thus, the most successful preparative polyacrylamide gel electrophoretic purifications have been obtained when material from DEAE-cellulose chromatography was taken for preparative electrophoresis[5].

Nevertheless, it is also shown that a very significant purification can be obtained directly from the initially complex extracts by the single step preparative electrophoresis procedure.

There has recently been much discussion and criticism of the use of papain as a solubilisation agent for membrane antigens (Reisfeld and Kahan[7]). This is directed firstly at the low percentage release of antigens by papain digestion, together with the fact that many non-antigenic components are released. Secondly, there is the possibility that destruction of the antigens might occur and thirdly, since papain is a proteolytic enzyme of broad specificity, antigenic fragments of varying molecular weight might be obtained. The concept of antigenic 'homogeneity' thus becomes rather vague if fragmentation of the true antigenic molecule has occurred, though it is likely that the part of the molecule acting as the antigenic determinant remains the same in the fragments of varying size.

Along with the possible loss of antigenic activity during the papain extraction, it must be borne in mind that the antigen purification methods may themselves inactivate the antigen under investigation. There is evidence that ion-exchange chromatography does lead to a large loss of membrane antigen (Reisfeld and Pellegrino[9]), but the situation with regard to preparative electrophoresis appears to be less worrying. With the tumour-specific antigens under investigation it has not been possible to quantify the loss of antigenicity throughout the extraction and purification schemes owing to the essentially qualitative nature of the antigen-assay methods employed (indirect membrane immunofluorescence and immunodiffusion). It must be stated that the rabbit antisera to rat hepatomas D_{23} and D_{31} are not absolutely monospecific after the absorption with normal rat serum and normal rat liver cells. They probably detect a range of components which are expressed on hepatoma and not on normal liver; these components being immunogenic in the rabbit. However, on the basis of the inhibition of membrane immunofluorescence data, it can reasonably be said that one of these components is the tumour-specific antigen. Quantitative information would be extremely valuable and it is hoped that by developing a radioimmunoassay system for hepatoma D_{23}-specific antigen a more precise determination of antigenicity will be possible in the future.

The preliminary experiments performed using material extracted from hepatoma D_{23} 'extranuclear' membrane by EDTA treatment in general follow a similar pattern to the papain extract when purified by preparative polyacrylamide gel electrophoresis. With EDTA antigenic degradation is less likely to occur than with papain, since it has been used by several groups of workers to solubilise membrane proteins (Tillack *et al.*[10]; Tanner and Gray[11]), without producing any denaturation, though dissociation due to the removal of calcium is likely.

Analytical polyacrylamide gel electrophoresis is considered to be a very sensitive method for detecting heterogeneity in a protein solution, but with regard to the purified hepatoma-specific antigen it has always been found that a rather diffuse band of stained protein was detected on the gels[5]. This could be because the material consists of a range of almost identical molecules rather than a homogeneous solution, due to the degradative effects of papain action of the antigen, as mentioned above. On the other hand, the purified antigen extracted initially by EDTA likewise gave a rather broad band, as did the H-LA antigen extracted

by KCl and purified by preparative electrophoresis by Reisfeld and Kahan[7], so it may be that on the cell surface there is a spectrum of similar molecules all carrying the same antigenic determinant.

Recently the technique of isoelectric focusing in polyacrylamide gel has been developed which increases still further the resolution obtainable with the analytical polyacrylamide gel system. Preliminary results have indicated that the purified material which gives a single diffuse band on the simple analytical polyacrylamide gel electrophoresis system does in fact give rise to more than one band of protein when ampholines are incorporated into the gel. Preparative isoelectric focusing may be of use for purifying membrane antigens, though in preliminary experiments it has been found that the antigenic material underwent severe aggregation in the electrofocusing column. In this context it should be mentioned that O'Neil and Davies[12] considered that ordinary electrophoresis was superior to isoelectric focusing for separating mouse H-2 specificities.

The importance of obtaining tumour-specific antigen in a highly purified form for molecular characterisation studies is considered to be paramount in relation to the understanding of the changes that occur at the cell surface during carcinogenesis. These studies may also contribute to the development of cancer immunotherapy and to the understanding of the fundamental genetic lesions which are responsible for cancerous changes. Progress along this line will require a detailed comparison of normal tissue antigens with tumour specific antigens at the molecular level.

ACKNOWLEDGEMENTS

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