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CONCANAVALIN A-REACTIVE PROTEIN OF RABBIT THYMOCYTE PLASMA MEMBRANES: ANALYSIS BY CROSSED IMMUNE ELECTROPHORESIS AND SODIUM DODECYLSULFATE/POLYACRYLAMIDE GEL ELECTROPHORESIS

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SUMMARY

1. Thymocyte plasma membrane extracts, prepared with the non-ionic detergent Triton X-100, show 10 major protein components upon sodium dodecylsulfate/polyacrylamide gel electrophoresis and at least 11 immunologic components upon crossed immune electrophoresis.

2. Concanavalin A reactive membrane proteins have been identified using crossed immune electrophoresis with receptor-ligand interaction.

3. These proteins are adsorbed from Triton X-100-solubilized membranes onto immobilized concanavalin A. They are eluted in stepwise fashion, using increasing concentrations of α-methyl-D-glucoside, between 0.0004 M and 0.1 M. The predominant proteins eluted in each step are components with high electrophoretic mobility in crossed immune electrophoresis and are identical with a glycosylated component in sodium dodecylsulfate/polyacrylamide gel electrophoresis with a molecular weight of 55 000.

4. This component forms multimers in the presence of Triton X-100 which are not totally dissociated in sodium dodecylsulfate.

5. Neuramidase treatment followed by crossed immune electrophoresis of total plasma membrane isolates, as well as the purified glycoprotein fraction, indicates that the concanavalin A-reactive proteins are sialoglycoproteins.

6. Sodium dodecylsulfate component 5.1 comprises at least two different populations of glycoproteins (6 and 9) in crossed immune electrophoresis, one of which exclusively exhibits heterogeneous carbohydrate antigenic sites (component 9).

7. Present data, taken together with previously published experiments, indicate that concanavalin A binding to intact thymocytes induces an increased turnover and release of the receptor protein(s).

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INTRODUCTION

Stimulation of thymocytes with concanavalin A in vitro constitutes a useful system for the study of biochemical events in the early stage of blastogenic lymphocyte transformation. Concanavalin A or other mitogens very rapidly induce the formation of ion-permeable junctions between contacting lymphocytes [1, 2], increase the uptake of ions [3], nucleosides [4], carbohydrates [5] and amino acids [6] from the medium and stimulate phosphatide metabolism [7, 8]. Enhanced protein, RNA and DNA metabolism occurs somewhat later [9–11].

Our previous studies show that the turnover of several plasma membrane components, defined by sodium dodecylsulfate/polyacrylamide gel electrophoresis, increases upon in vitro treatment of rabbit thymocytes with concanavalin A [12]. One of these components stands out in particular; it exhibits a decrease in its half-life from about 24 h to 10 h. We have suggested that this protein (or its multimers) may constitute a plasma membrane concanavalin A receptor. We have accordingly solubilized thymocyte membrane proteins using Triton X-100 and have purified these by affinity chromatography on Sepharose 4B-immobilized concanavalin A. We then identify the concanavalin A-reactive components using sodium dodecylsulfate/polyacrylamide electrophoresis and further characterize these proteins by crossed immune electrophoresis.

MATERIAL AND METHODS

Chemicals

Unless stated otherwise, all chemicals used are of analytical purity grade. Triton X-100, α-methyl-D-glucoside and dithiothreitol were obtained from Sigma (St. Louis, Mo., U.S.A.); concanavalin A-Sepharose and Dextran-150 from Pharmacia (Sweden); concanavalin A from Miles Laboratories (Elkhart, Ind., U.S.A.); agarose (charge 102 D) from Litex, Denmark. Pig immunoglobulins to rabbit serum from Dako Immunoglobulins (Copenhagen, Denmark). Neuraminidase from *Clostridium perfringens* was from Worthington Biochemical Corporation (Freehold, N.J., U.S.A.), and complete Freund Bacto Adjuvant from Difco Laboratories (Detroit, Mich., U.S.A.). Acrylamide, *N,N'*-methylenebisacrylamide, *N,N'*-diallyltartardiamide, *N,N,N',N'*-tetramethylethylenediamine (TEMED) and ammonium persulfate were obtained from BioRad Laboratories (Richmond, Calif., U.S.A.).

Membrane material

White New Zealand rabbits (female, 2 months old) were employed. Thymocyte microsomes and plasma membranes were prepared as described in refs 13 and 14. Plasma membranes purified on dextran gradients were washed once in 1 mM HEPES/1 mM $MgCl_2$, pH 8.2, by pelleting at an average of $2 \cdot 10^7$ $g \cdot$ min. Extraction of microsomes and plasma membranes with Triton X-100 was done in two steps: first, the pelleted membrane material was suspended in 0.038 M Tris/0.1 M glycine, pH 8.7, containing 1 % Triton X-100 to a final concentration of 2 mg protein/ml. Non-solubilized material was then pelleted at an average of $1.2 \cdot 10^7$ $g \cdot$ min, extracted with 1.0 % Triton in the same buffer followed by centrifugation at an average of $1.2 \cdot 10^7$ $g \cdot$ min. The supernatant fluids were combined and concentrated

to a final protein concentration of 3 mg/ml. Triton X-100 extracts were frozen in liquid N_2 and stored at −70 °C.

Antisera against membrane proteins

Antisera against the membrane proteins in rabbit thymocyte microsomal membranes were produced in guinea pigs according to the following immunization scheme (Hammer, D. K., personal communication): the guinea pigs were first immunized with 50 μg membrane protein/animal (in complete Freund adjuvant) by injecting the antigen mixture into the foot pads. A first booster was given after two weeks, injecting 500 μg/animal intramuscularly and subcutaneously, and this was followed by two other boosters using the same amount of protein at six week intervals. 10 days after the third booster, the animals were exsanguinated by heart puncture and their serum stored in 0.5 ml portions at −70 °C after adding NaN_3 to a final concentration of 15 mM. Two batches of anti-microsomal sera were used, one was non-pooled, the other one was pooled from two guinea pigs.

Crossed immunoelectrophoresis

In this method [16, 17] the Triton X-100 solubilized membrane proteins were first separated in one direction according to their charge. The strip containing the separated proteins was left on the plate and the rest of the agarose substituted by an antibody-containing agarose layer. After turning the plate by 90°, the separated proteins were electrophoresed into the antibody-containing agarose. This was done at pH 8.7 to immobilize the immunoglobulins.

For our experiments 1 % agarose containing 1 % Triton X-100 was cast on a 50 × 50 mm glass plate as a 1.5 mm thick layer. The buffer was 0.038 M Tris/0.1 M glycine, pH 8.7. Firstly 100–150 μg of Triton X-100-solubilized membrane protein was separated electrophoretically for 60 min using a voltage of 10 V/cm of separation distance (a total of 40 V). The agarose on the plate was then substituted by agarose containing 25 μl anti-microsomal serum/ml and 1 % Triton X-100. The pre-separated membrane proteins were then electrophoresed at right angles to the first dimension into the immobilized immunoglobulins. To avoid any diffusion or migration of proteins into the electrode wicks, dialysis membranes were placed between these and the anodal and cathodal end of the agarose plate. After electrophoresis, the plates were washed twice for 20 min in 0.9 % NaCl, dried between by compressing with one water-wetted and several dry sheets of electrophoresis paper, rinsed with distilled water and dried at room temperature. Staining of the dry plates was performed with 0.5 % Coomassie solubilized in 25 % ethanol/10 % acetic acid/65 % water for 1 min, and for destaining 25 % methanol/10 % acetic acid/65 % water was used. Plates were dried in cold air.

Crossed immunoelectrophoresis with receptor-ligand interaction

For this we use an alternative design to that recently described [18]. Triton X-100-solubilized membrane proteins were separated in the first dimension as described above. For the second dimension, the 12 mm wide agarose strip containing the pre-separated membrane proteins was moved 8 mm to the anodal end of the plate and on its cathodal site an 8 mm wide agarose strip containing 750 μg of concanavalin A/ml is cast. The anodal space of the plate is filled with agarose containing 25 μg anti-microsomal serum/ml. The second dimension was run as described above.

Concanavalin A-sepharose affinity chromatography

Before use, concanavalin A-Sepharose was washed at 0–4 °C to remove all loosely bound concanavalin A. 10 ml of concanavalin A-Sepharose sediment was washed four times using 80 ml each time of 1.0 M NaCl/0.1 M sodium acetate/ 0.001 M $CaCl_2$/0.001 M $MnCl_2$, pH 4.0, and thereafter 1.0 M NaCl/0.1 M Tris · HCl/ 0.001 M $CaCl_2$/0.001 M $MnCl_2$, pH 8.5. After this concanavalin A-Sepharose was washed three times in the buffer used for affinity chromatography, namely 0.075 M Tris · HCl/0.0002 M $CaCl_2$/0.0002 M $MnCl_2$, pH 7.5/Triton X-100 (0.2 %, v/v). All the following procedures were done in a cold room at 6 °C. The concanavalin A-Sepharose is packed into a column (1 cm inner diameter, 15 cm long) and rinsed with another 50 ml of chromatography buffer. This final washing eluted no more protein from the column, as determined by chemical assay, and sodium dodecylsulfate/ polyacrylamide gel electrophoresis.

The membrane protein (7–10 mg/4 ml) was now slowly pumped into the column and equilibrated for 90 min. The non-specifically adsorbed protein was eluted using 50 ml of 0.075 M Tris · HCl/$CaCl_2$ 0.0002 M/$MnCl_2$ 0.0002 M/0.2 % Triton X-100, pH 7.5. Thereafter, the specifically bound membrane material was eluted in stepwise fashion using 0.075 M Tris · HCl/0.0002 M $CaCl_2$/0.0002 M $MnCl_2$/0.2 % Triton X-100, pH 7.5, containing increasing concentrations of α-methyl-D-glucoside. The elution was begun with 0.0004 M α-methyl-D-glucoside, continued with 0.0016 M and 0.01 M and the very tightly bound proteins were released with 0.1 M. Protein still adsorbed thereafter was removed using 1.0 M NaCl. All the column eluates are concentrated 10-fold using an Amicon Diaflo (PM 10) membrane. Aliquots of these concentrates, further concentrated to a protein concentration of about 3 mg/ml, were used in crossed immunoelectrophoresis. For these experiments, the pooled anti-microsomal serum was used. The other material is prepared for analysis using sodium dodecylsulfate/polyacrylamide gel electrophoresis. It was washed twice with 30 ml 0.0075 M Tris · HCl/0.001 M EDTA/0.1 % (v/v) sodium dodecylsulfate, pH 7.5 using a Diaflo PM 10 ultrafiltration membrane. This was to remove interfering excess Triton X-100. Sodium dodecylsulfate was now added to the concentrated and washed eluates to a final concentration of 1 %. The samples were then heated for 2 min at 100 °C and dialysed overnight against 4000 ml of 0.0075 M Tris · HCl/0.001 M EDTA/ 0.2 % sodium dodecylsulfate, pH 7.5, at room temperature. After dialysis the samples were concentrated to a protein level of 1.5 mg/ml, sodium dodecylsulfate was added to a final concentration of 5 % and dithiothreitol to 0.04 M. The samples were heated for 5 min at 100 °C.

In some experiments, the membrane proteins released by 0.0016 M α-methyl-D-glucoside from the first column were adsorbed into a second concanavalin A column. For this, the non-concentrated eluate was slowly adsorbed into the second column and then released using 0.1 M α-methyl-D-glucoside. The unadsorbed and adsorbed fractions were analysed by crossed immunoelectrophoresis and sodium dodecylsulfate/ polyacrylamide gel electrophoresis.

Neuraminidase treatment of α-methylglucoside eluate

80 μg of the 0.0016 M eluate and 100 μg of the 0.01 M eluate in 70 μl each were incubated with 200 μg of neuraminidase at 37 °C for 30 min, after adjusting to a pH of about 6 using 0.1 M HCl. After the reaction, the samples were titrated back to

pH 7.5 using 0.1 M NaOH. Thereafter, the samples were compared to untreated controls by crossed immunoelectrophoresis and in sodium dodecylsulfate/polyacrylamide gel electrophoresis to exclude proteolytic activity in the neuraminidase preparation.

Sodium dodecylsulfate/polyacrylamide gel electrophoresis

For sodium dodecylsulfate/polyacrylamide gel electrophoresis, the procedure described in ref. 19 was followed using 7 % acrylamide, cross-linked with 3.75 % *N,N'*-diallyl-tartardiamide, containing 1 % sodium dodecylsulfate. The electrophoresis buffer was Tris acetate/EDTA (0.04 M, 0.02 M, 0.002 M), pH 7.4. Coomassie brilliant blue staining for proteins and the reaction with periodic acid Schiff reagent were done as described in ref. 19. For the latter staining 6 % acrylamide gels, cross-linked with 2.5 % *N,N'*-methylene-bisacrylamide, were used.

Chemical determinations

Protein concentration was determined according to Lowry et al. [20] using bovine serum albumin as standard.

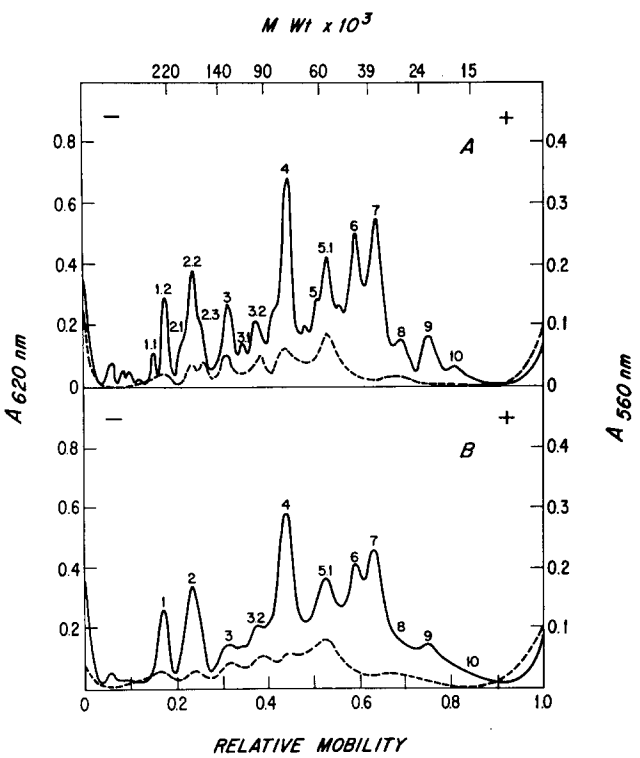


Fig. 1. Sodium dodecylsulfate/polyacrylamide gel electrophoretograms of rabbit thymocyte plasma membrane proteins. The abscissa gives the relative mobility and the molecular weight; the ordinate the absorbance, at 620 nm for Coomassie blue and at 560 nm for periodic acid/Schiff stain, respectively. (A) Sodium dodecylsulfate-solubilized membranes. (B) Membrane proteins extracted twice with 1 % Triton X-100 equilibrated with 1 % sodium dodecylsulfate/40 mM dithiothreitol and subjected to electrophoresis in presence of 1 % sodium dodecylsulfate.

RESULTS

Solubilisation of membrane proteins using Triton X-100

The non-ionic detergent Triton X-100 solubilizes more than 90 % of the membrane proteins, i.e. they become non-sedimentable at an average $1.2 \cdot 10^7$ $g \cdot$ min. The proteins solubilized from microsomes and plasma membranes (Figs 1A and 1B) show the same qualitative protein and glycoprotein pattern as sodium dodecylsulfate-solubilized membranes when analysed in sodium dodecylsulfate/polyacrylamide gel electrophoresis. Some smaller quantitative differences in the electrophoretograms are observed; these are in part due to lesser resolution in the presence of Triton X-100.

Identification of thymocyte plasma membrane proteins by means of crossed immuno-electrophoresis

The solubilized membrane proteins give at least 11 individual immunoprecipitates when electrophoresed against an antiserum pooled from two guinea pigs (Fig. 2A). The precipitates are numbered from 1 up according to increasing electrophoretic mobility (Fig. 2B). Multiple precipitates of the same electrophoretic mobility are additionally assigned by big letters, e.g. 9A, 9B etc. The strong, poorly defined precipitates near the well (precipitates 1 and 2) are probably due to aggregates, because several other immunoprecipitates originate in this area, indicating identical antigenic

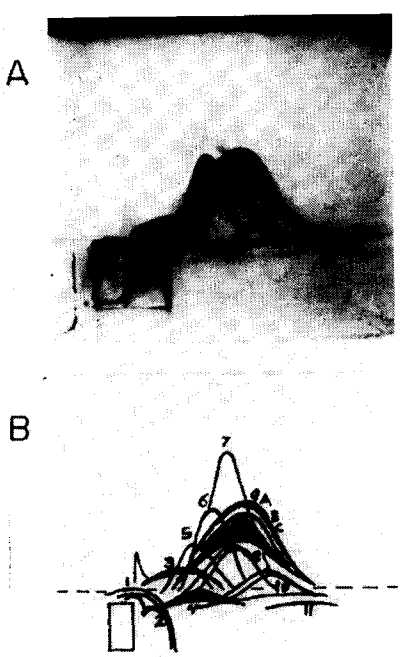


Fig. 2. Crossed immune electrophoretogram of rabbit thymocyte plasma membrane Triton X-100 extract. 100 μg plasma membrane protein were separated in the first dimension and precipitated by pooled anti-microsomal serum (25 μl/ml agarose) in the second dimension. (A) Immunoplate. (B) Scheme of the plate shown in A. The precipitates are numbered according to increasing electrophoretic mobility.

sites. These precipitates do not resolve well even when agarose with lower electroendosmosis is used. We have excluded the possibility that any of the immunoprecipitates shown in Fig. 2 arise from rabbit serum protein contaminants; upon electrophoresis in the second direction against pig immunoglobulins to rabbit serum, no immunoprecipitate was obtained. The well-defined immunoprecipitates indicate a high-titre antiserum and intact antigens. Two immunoprecipitates are split on their cathodal side, probably due to antigenic heterogeneity of those components. The strongest

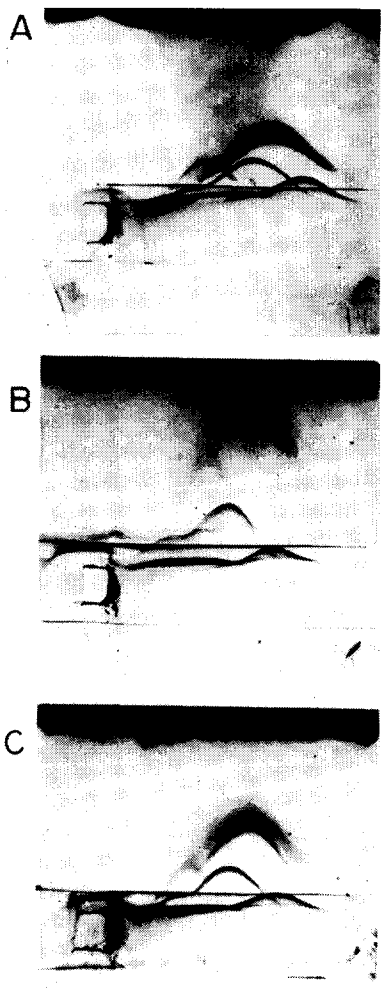


Fig. 3. Crossed immunoelectrophoretograms with receptor-ligand interaction. About 100 μg of plasma membrane proteins were separated in the first dimension. In the second dimension, 500 μg concanavalin A were subjected to electrophoresis behind the membrane proteins which form immunoprecipitates with unpooled anti-microsomal serum (25 μl/ml agarose). (A) Control without concanavalin A in the agarose strip placed cathodally from the strip containing pre-separated membrane proteins. (B) Second dimension, run with 500 μg concanavalin A. (C) Second dimension, run with 500 μg concanavalin A in the presence of 0.1 M α-methyl-D-glucopyranoside.

precipitate arises from material with high electrophoretic mobility and consists of several lines having an identical relative mobility in the first dimension (component 9). The immunoprecipitation pattern shown in Fig. 2 is highly reproducible and can be used as a sensitive immunological method to identify rabbit thymocyte plasma membrane proteins. The amount of protein producing an immunoprecipitate is too small to identify the membrane proteins involved, by re-electrophoresis on polyacrylamide laden with sodium dodecylsulfate (unpublished observation). However, application of both techniques at varying states of our affinity purification procedure, allows their close correlation.

Crossed immunoelectrophoresis with receptor-ligand interaction

In these experiments, antiserum from one guinea pig was used, to avoid multiple precipitates due to the antibody diversity between different serum donors (Fig. 3A). This serum yields the same concanavalin A-reactive components as the pooled serum used in Fig. 2. The membrane proteins are separated electrophoretically in one direction. The concanavalin A is electrophoresed at right angles through the agarose strip containing the pre-separated membrane proteins. These also migrate in the same direction into the antibody-containing zone. If the lectin reacts with any membrane proteins during this second electrophoresis, one might expect an altered immunoprecipitation for these components. This could be due to steric hindrance by the lectin, structural alterations induced by the lectin or a combination of these effects.

Our experiments (Figs 3A–3C) show that four of the seven major immunoprecipitates do not change, but three are displaced anodally upon electrophoresis in the second direction. This means that the concanavalin-A-antigen complex must migrate further through the antibody-containing agarose before immune precipitation occurs. Of the components involved, the principal one (9A, 9B) consisting of two precipitates is markedly displaced (Fig. 3B). The effect is clear-cut for a component of lower initial electrophoretic mobility (component 6) which disappears and one just above the well (component 1) (Fig. 3B). These concanavalin-A induced effects are not observed when the procedure is carried out in the presence of 0.1 M α-methyl-D-glucopyranoside, one of the lectin's hapten sugars. The three components can thus be identified as membrane proteins reacting specifically with concanavalin A.

Affinity chromatography using concanavalin A immobilized on sepharose

When microsomal Triton X-100 extracts are applied to immobilized concanavalin A, about 60 % of the protein is eluted from the column with the chromatography buffer. As shown in Fig. 4A, mainly the lower molecular weight, non-carbohydrate components are released in this step; most of the higher molecular weight membrane proteins are bound to the concanavalin A. This adsorbed material can be released either in bulk (Fig. 5E) using 0.1 M α-methyl-D-glucoside, or stepwise, using increasing concentrations of the hapten sugar (Figs 5A–5D). The latter approach also gives information about the affinity of the different protein components. After the last (0.1 M) α-methyl-D-glucoside elution, prior to the 1.0 M NaCl step, more than 90 % of the membrane material bound to the column is eluted.

In evaluating the immunoplates of the column eluates, one has to consider that the electrophoretic mobility of the proteins is significantly reduced if the protein samples contain an excess of Triton X-100. This is because the eluates must be exten-

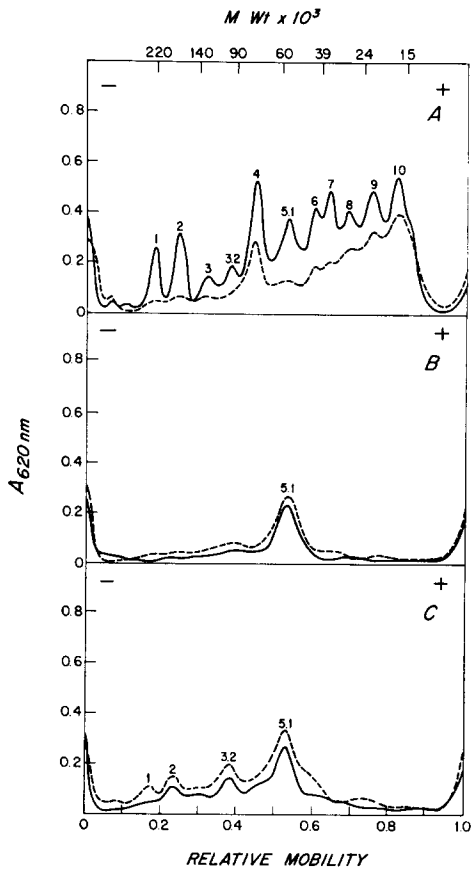
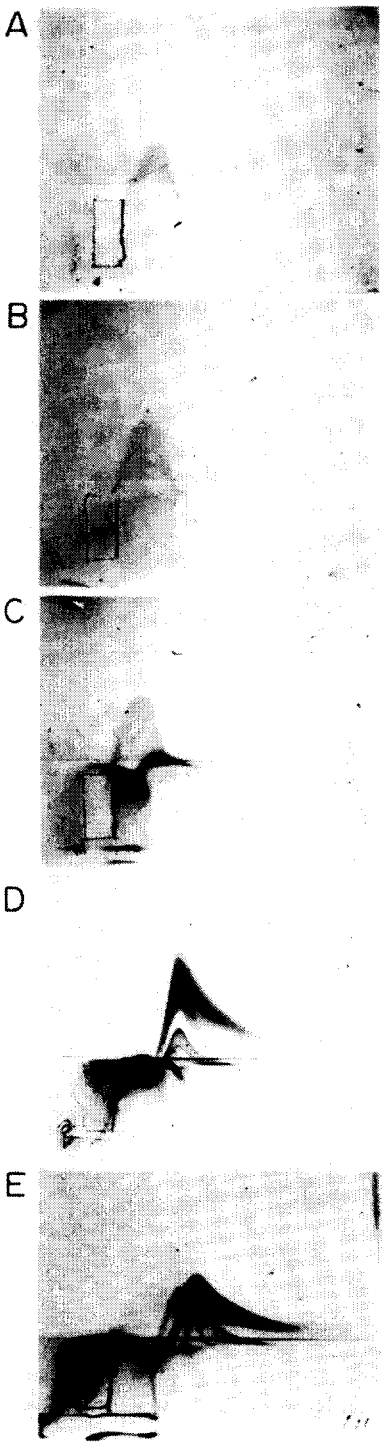


Fig. 4. Sodium dodecylsulfate/polyacrylamide gel electrophoretograms of the thymocyte microsomal Triton X-100 extract and concanavalin A affinity chromatography eluates. The abscissa gives the relative mobility and molecular weights, respectively; the ordinate gives the absorption for Coomassie blue at 620 nm. (A) —, rabbit thymocyte microsomes, Triton X-100 extract; - - -, microsomal proteins not bound to immobilized concanavalin A. (B) —, membrane proteins eluted with 0.0004 M α-methyl-D-glucoside; - - -, 0.0016 M α-methyl-D-glucoside eluate. (C) —, 0.01 M α-methyl-D-glucoside eluate; - - -, 0.1 M α-methyl D-glucoside eluate.

sively concentrated to yield satisfactory protein concentration.

Triton X-100-solubilized microsomal membrane proteins adsorbed on immobilized concanavalin A can be specifically eluted with a 'hapten sugar', α-methyl-D-glucopyranoside (0.0004–0.1 M). When 0.0004–0.0016 M 'hapten sugar' is used, a protein is released which gives a single band upon polyacrylamide electrophoresis in sodium dodecylsulfate (Fig. 4B). It has an apparent molecular weight of 55 000, identical with that of component 5.1 (Figs 4B and 4C), previously shown to exhibit high turnover after concanavalin A addition [13]. Crossed immune electrophoresis of the 0.0004 M eluate yields one immunoprecipitate (Fig. 5A) which is identical to component 6 on Fig. 2. The 0.0016 M eluate produces two precipitates of identical electrophoretic mobility (Fig. 5B); one of these is strong, but the one below is very faint and probably derives from aggregated material.


A
B
C
D
E

Elution of the affinity column with 0.01 M α-methyl-D-glucopyranoside releases three components identifiable by sodium dodecylsulfate/polyacrylamide gel electrophoresis. These correspond to membrane sodium dodecylsulfate components 5.1, 3.2 and 2 (apparent mol. wts: 55 000, 106 000 and 160 000, respectively) (Figs 4C and 5C). Components 5.1 and 3.2 predominate (Fig. 4C). Crossed immune electrophoresis also reveals a major component identical to that eluted with 0.0004 M and 0.0016 M 'hapten sugar' (component 6) and two less predominant ones, with lower electrophoretic mobility (Fig. 5C). The strong precipitate with high electrophoretic mobility derives from component 9.

Polyacrylamide gel electrophoresis in sodium dodecylsulfate of the 0.1 M α-methyl-D-glucopyranoside eluate reveals the components eluted with 0.01 M

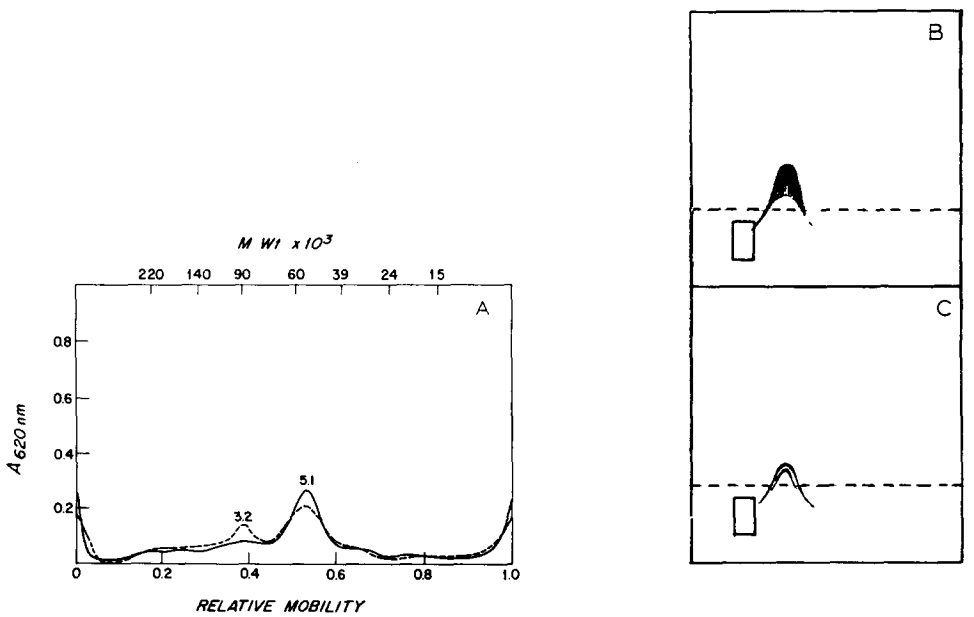


Fig. 6. Affinity chromatography with immobilized concanavalin A using two columns. The membrane proteins eluted by 0.0016 M α-methyl-D-glucoside from the first column are concentrated in presence of concanavalin A on the second column and then released by 0.1 M α-methyl-D-glucoside. (A) Protein composition of the eluate from the first column (—) compared with that from the second column (- - -). Sodium dodecylsulfate/polyacrylamide gel electropherograms. The abscissa gives the relative mobility and the molecular weights, respectively; the ordinate gives the absorption for Coomassie blue at 620 nm. (B) Proteins eluted from the first column, analysed by crossed immune electrophoresis. (C) Proteins eluted from the second column analysed by crossed immune electrophoresis. For B andC a pooled anti-microsomal serum was used.

Fig. 5. Concanavalin A-reactive thymocyte membrane proteins eluted with different concentrations of α-methyl-D-glucoside defined by crossed immune electrophoresis. In the second dimension run, the receptor proteins were precipitated by pooled anti-microsomal serum (25 μl/ml agarose). (A) Membrane protein eluted with 0.0004 M α-methyl-D-glucoside. (B) Membrane proteins eluted with 0.0016 M α-methyl-D-glucoside. (C) Membrane proteins eluted with 0.01 M α-methyl-D-glucoside. (D) Membrane proteins eluted with 0.1 M α-methyl-D-glucoside. (E) Membrane released proteins by one-step elution with 0.1 M α-methyl-D-glucoside.

'hapten sugar' plus membrane component 1 (Figs 4C and 5D), with an apparent molecular weight of 230000. Periodic acid/Schiff-stained polyacrylamide gel electrophoretograms of whole membranes and of column eluates again reveal component 5.1 as the predominant component. Crossed immune electrophoresis of the 0.1 M α-methyl-D-glucopyranoside eluate shows component 9 predominating; component 6, which exhibits lower affinity for concanavalin is completely eluted after 0.01 M α-methyl-D-glucoside. The strong immunoprecipitates at low electrophoretic mobility are the same as in the 0.01 M α-methyl-D-glucoside eluate. Crossed immune electrophoresis of the eluate obtained by a one-step elution with 0.1 M α-methyl-D-glycopyranoside yields a pattern almost as complex as that obtained with whole plasma membrane extracts (Fig. 5E) and components 6 and 9 are released simultaneously. The step-wise release of membrane components eluted with increasing concentrations of α-methyl-D-glucopyranoside is due to the hapten sugar, a stepwise increase in buffer osmolarity using increasing concentrations of Tris · HCl does not release the concanavalin A-bound membrane proteins.

85 % of the material eluted from immobilized concanavalin A with 0.0016 M α-methyl-D-glucopyranoside, can be readsorbed onto a second column of immobilized concanavalin A, and eluted out by excess hapten sugar (0.1 M). An analysis of the concentrated eluate from the first column, the material not absorbed to the second column, and the protein eluted from the second column using 0.1 M α-methyl-D-

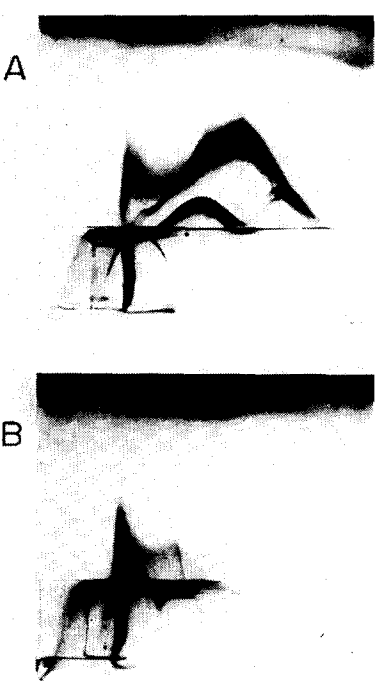


Fig. 7. Crossed immune electrophorograms of rabbit thymocyte plasma membrane proteins (Triton X-100 solubilized) treated with neuraminidase. Between 100 and 150 μg membrane proteins in 70 μl of chromatography buffer, containing at least 1 % Triton X-100, were treated with 200 μg neuraminidase at pH 6.0. The proteins were run in the second dimension against unpooled anti-microsomal serum (25 μl/ml agarose). (A) Control. (B) Neuraminidase treated plasma membrane proteins.

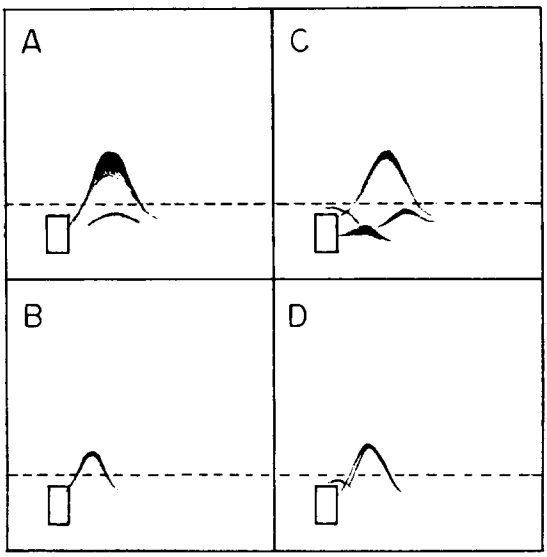


Fig. 8. Crossed immune electropherograms of thymocyte membrane proteins eluted from immobilized concanavalin A treated with neuraminidase. Between 30 and 70 μg membrane proteins in 70 μl of chromatography buffer, containing at least 1 % Triton X-100, were treated with 200 μg neuraminidase at pH 6.0. The proteins were run in the second dimension against a pooled anti-microsomal serum (25 μl/ml agarose). (A) Untreated membrane proteins eluted by 0.0016 M α-methyl-D-glucoside. (B) Neuraminidase-treated membrane proteins eluted by 0.0016 M α-methyl-D-glucoside. (C) Untreated membrane proteins eluted by 0.01 M α-methyl-D-glucoside. (D) Neuraminidase-treated membrane proteins eluted by 0.01 M α-methyl-D-glucoside.

glucopyranoside by electrophoresis in polyacrylamide laden with sodium dodecylsulfate and by crossed immune electrophoresis was performed. Material not adsorbed to the second column yields no immunoprecipitate. The material eluted from the second column with hapten sugar gives essentially the same immunoprecipitation pattern as the material loaded onto the column (Figs 6A–6C), except that the original diffuse single precipitate exhibits a doubled line in its top area.

To obtain more information about the homo- or heterogeneity of the fractions eluted with 0.0016 M and 0.01 M α-methyl-D-glucopyranoside, we treated these fractions with neuraminidase. Crossed immune electrophoresis then reveals both qualitative and quantitative deviations from the patterns observed with control samples. This is shown in Figs 7A and 7B, which demonstrates the effect of neuraminidase on Triton X-100 extracts of whole plasma membrane. In both column eluates, one observes a sharpening of the immunoprecipitates but a decrease in their heights (Figs 8A–8D). Moreover, the precipitates indicate reduced electrophoretic mobility in the first direction. This would suggest that sialic acid contributes to the antigenicity of these components and also to their electrophoretic mobility. Moreover, in the case of the material eluted with 0.01 M 'hapten sugar' two diffuse bands of low initial electrophoretic mobility disappear completely and the remaining immunoprecipitate sharpens and resolves into two components (Figs 8C and 8D). Any protease contamination of the neuraminidase is excluded because enzyme treatment does not change the membrane protein pattern upon sodium dodecylsulfate/polyacrylamide gel electrophoresis.

DISCUSSION

Fractionation of the concanavalin A-reactive proteins, by affinity chromatography on immobilized concanavalin A, was undertaken and these proteins were characterized using sodium dodecylsulfate/polyacrylamide gel electrophoresis and crossed immune electrophoresis. The joint use of these techniques to monitor the stepwise release of the membrane proteins from the concanavalin A-Sepharose column, with increasing concentrations of α-methyl-D-glucoside, has allowed us to correlate the data provided by the two electrophoresis systems.

Using 0.0004 M α-methyl-D-glucoside, an immunologically homogeneous protein was obtained which corresponds to the periodic acid/Schiff-positive component 5.1 (apparent mol. wt 55 000) seen in sodium dodecylsulfate/polyacrylamide gel electrophoresis. In crossed immune electrophoresis both the electrophoretic mobility and the precipitate height of this protein decrease after neuraminidase treatment. The data show that 5.1 is a sialoglycoprotein, and that sialic acid contributes to the charge and is involved in the antigenicity of this protein. Moreover, crossed immune electrophoresis suggests that component 5.1 comprises at least two different glycoprotein populations. The fact that these populations show no immunological cross reactivity suggests that we may be dealing with a protein of identical polypeptide chain but different carbohydrate residues; this could explain the different antigenicities, the different mobilities in crossed immune electrophoresis and the different affinities for immobilized concanavalin A.

The two immune components corresponding to protein 5.1 are component 6 and 9. The properties of component 6 are less influenced by its carbohydrate than component 9. It has a lower affinity for concanavalin A and its electrophoretic mobility is only slightly reduced by neuraminidase. In contrast, the immunoprecipitates of component 9 are due primarily to carbohydrate antigens. Moreover, component 9 is unique in crossed immune electrophoresis analysis in that it exhibits several precipitation lines; this also suggests a molecular heterogeneity with respect to the carbohydrate residues. All of the immunoprecipitates appear to involve sialic acid as part of the antigenic determinant because only one sharp line persists after membrane Triton extracts are treated with neuraminidase (Figs 7A and 7B). This effect of neuraminidase in the case of unfractionated membrane is greater than that observed with the immune component 6 eluted with 0.0004 M α-methyl-D-glucoside; the latter does not show a comparable loss in the height of the immunoprecipitate. This effect of neuraminidase, as well as the disappearance of component 9 in the 0.01 M α-methyl-D-glucoside eluate after enzyme treatment (Figs 8C and 8D), again suggest that component 5.1 may constitute a class of proteins that are heterogeneous with respect to their carbohydrate and that the material released at low hapten sugar concentrations differs in carbohydrate composition and/or content from that eluted by higher α-methyl-D-glucoside concentrations.

The two-column experiment, in which we concentrate component 5.1 in presence of concanavalin A, demonstrates the tendency of this protein to form multimers; whereas only one component, 5.1, was applied to the second column, 0.1 M α-methyl-D-glucoside elutes both this component and a smaller proportion of component 3.2. This has an apparent molecular weight of 106 000, i.e. about double the molecular weight of component 5.1, suggesting that 3.2 is a dimer of 5.1 and that

the dimeric form is not fully dissociated by sodium dodecylsulfate/polyacrylamide gel electrophoresis [21]. Immune component 6 of protein 5.1 has the lowest binding affinity of the concanavalin A-reactive membrane material. When the α-methyl-D-glucoside concentration in the elution buffer is increased, we release more tightly associated membrane proteins and get a correspondingly more complex pattern in crossed immune electrophoresis; however, component 5.1 in sodium dodecylsulfate/polyacrylamide gel electrophoresis, and components 6 and 9 in crossed immune electrophoresis always predominate.

In accordance with the other evidence presented, the proteins eluted from immobilized concanavalin A by α-methyl-D-glucoside, components 1, 6 and 9 in crossed immune electrophoresis, are the same as those of which the precipitation pattern is modified by electrophoresing concanavalin A behind the pre-separated proteins.

Our results indicate that the concanavalin A affinity of the protein may depend on its degree of polymerization as well as the possible carbohydrate micro-heterogeneity already discussed. Only four sodium dodecylsulfate/polyacrylamide electrophoresis components, probably consisting of identical polypeptide chains, appear involved in concanavalin A binding. The discrepancy between the rather simple pattern obtained, using sodium dodecyl sulfate/polyacrylamide gel electrophoresis, and the complicated one found in crossed immune electrophoresis might be explained by persistence or formation of protein-protein complexes in Triton X-100. We have some evidence for this from the neuraminidase experiments, because this enzyme reduces the electrophoretic mobility of all protein yielding immunoprecipitates (Figs 7A and 7B).

In contrast to other investigations [22, 23] for which pig lymphocyte membranes solubilized in deoxycholate were used, it was found here that one can elute a very homogeneous receptor molecule from immobilized concanavalin A, using low concentrations of the hapten sugar. The reason why Allan et al. [22] find a heterogeneity in the material eluted from immobilized concanavalin A might be that they release all the protein in a single step, using an α-methyl-D-glucoside concentration of 0.1 M. It is also conceivable that lymph node lymphocytes are more heterogeneous in terms of their concanavalin A receptors.

Our present data demonstrate that component 5.1 is a concanavalin A receptor. Together with previous results [13] showing enhanced turnover and release of this protein after addition of concanavalin A to intact cells, our results indicate that the lectin induces the turnover of this receptor. These data are in agreement with data presented by Jones [24] who found concanavalin A, concanavalin A-receptor complexes and increased amounts of receptor in the cultivation media obtained from stimulated mouse lymphocytes. Together with recent observations on the turnover of immunoglobulins on mouse B lymphocytes [25, 26], our experimentation and the work of Jones [24] suggest that the release of receptor molecules after ligand binding may constitute a general lymphocyte response.

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