Preliminary Characterization of Thy-1.1 and Ag-B Antigens from Rat Tissues Solubilized in Detergents

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1. A radioactive binding assay for Thy-1.1 alloantigen which functions in the presence of detergents was established by using glutaraldehyde-fixed thymocytes as target cells. Thy-1.1 activity in detergent extracts was then assayed by measuring inhibition of the binding assay. 2. Solubilization of Thy-1.1 from whole thymocytes, and their membranes by a large number of non-ionic detergents and deoxycholate was studied. In the same extracts Ag-B(4) histocompatibility antigenic activities were measured. With the exception of Nonidet P-40, the detergents did not affect the antigenicity of Thy-1.1, but only Lubrol-PX and deoxycholate gave effective solubilization as measured by activity remaining in the supernatant after centrifugation at 200000 *g* for 40 min. With Ag-B(4) antigen, Triton X-100, Triton X-67 and Nonidet P-40 gave effective solubilization as well as Lubrol-PX and deoxycholate. Solubilization of Thy-1.1 activity from leukaemia cells and a brain homogenate was also studied, but none of the non-ionic detergents gave satisfactory results with these tissues. 3. Extracts from thymocyte membranes were further examined by gel filtration and sucrose gradient centrifugation. The Thy-1.1 activity behaved as a single component in deoxycholate with a density similar to that of a globular protein, but in Lubrol-PX the antigen was contained in a low-density complex. In Lubrol-PX extracts Ag-B(4) was also found in aggregates not observed in deoxycholate. 4. The $s_{20,w}$ values for Thy-1.1 and Ag-B(4) antigens in deoxycholate were 2.4 and 4.4, and $\bar{v}$ values were 0.70 and 0.75 respectively. The Stokes radius observed for Thy-1.1 was 3.1 nm and for Ag-B(4) 5.3 nm. By using these values the molecular weights for the antigen-detergent complexes were calculated to be 28000 for Thy-1.1 and 100000 for Ag-B(4).

Thy-1.1 is a membrane-bound antigen found in large amounts only on thymocytes and brain of mice and rats (Reif & Allen, 1964; Douglas, 1973). The function of the antigen is unknown, and purification and characterization of the molecule may indicate its role and also show how it is integrated into the cell membrane.

It has been shown (Acton *et al.*, 1974) that there are approx. 500000 antigenic sites for Thy-1.1 per rat thymocyte, and that rat brain has an equivalent amount of antigen on the basis of wet wt. of tissue. Thus it should be possible to obtain enough antigen from these tissues for eventual purification.

The initial problem in the purification of membrane-bound molecules is that of solubilization; this has been successfully done by the use of detergents in the case of viral membrane proteins (Simons *et al.*, 1973), cell receptors (Cuatrecasas, 1972;

Meunier *et al.*, 1972; Bilezikian & Aurbach, 1973; Dufau *et al.*, 1973), and membrane enzymes (Levey, 1970; Allan & Crumpton, 1971; Neer, 1973; Umbreit & Strominger, 1973). With lymphocytes, detergents have proved useful in the solubilization of histocompatibility antigen (Schwartz & Nathenson, 1971; Snary *et al.*, 1974), and membrane-bound immunoglobulin (Vitetta *et al.*, 1972).

The most effective detergents are those of the non-ionic type, and the weakly ionic bile salts.

The use of detergents for the solubilization of cellular antigens creates the problem of assaying antigenic activity in their presence. If radioactive binding assays are used to detect antigen, the problem can be overcome by using glutaraldehyde-fixed target cells, and measuring inhibition of the binding of a specific antibody by detergent extracts (Williams, 1973).

In the present paper a binding assay was developed for the measurement of Thy-1.1 activity in the presence of various detergents and the solubilization of Thy-1.1 antigen from rat thymocytes, leukaemia cells and brain was examined. In the extracts from rat

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thymocytes, the activity of histocompatibility antigen Ag-B(4) was also assayed.

Under some conditions, both the Thy-1.1 and Ag-B(4) antigens were released into the supernatant, and remained in solution after centrifugation at 200 000 g. These extracts were further studied to determine if the antigens were in true solution, and to gain information about their molecular size. Sucrose gradient centrifugation was carried out to estimate apparent $s_{20,w}$ values and density, and gel filtration to measure Stokes radius.

Experimental

Materials

Miscellaneous. Na¹²⁵I (code number IMS 30) was from The Radiochemical Centre, Amersham, Bucks., U.K. ²H₂O was from Ryvan Chemical Co., Southampton, U.K.

Buffers. BBSS buffer, pH 7.4 (buffered balanced salt solution iso-osmotic for rats) was prepared as described by Williams *et al.* (1972). Tris-saline consisted of 0.01 M-Tris-HCl, pH 7.4, +0.15 M-NaCl. Buffered iso-osmotic bovine serum albumin was prepared as described by Legge & Shortman (1968).

Detergents. The following were used: Triton X-67, X-100, X-102, X-114, X-155, X-305, X-405, CF-21, CF-54, N-57 and N-101; Tween 20, 40, 60, 80 and 85; Lubrol PX and Lubrol WX; Brij 35, 56 and 58; deoxycholate [all from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.] and Nonidet P-40 (from BDH Chemicals Ltd., Poole, Dorset, U.K.).

The detergents were dissolved in BBSS buffer, Tris-saline buffer or Tris-saline buffer+sucrose, except for deoxycholate which was used in 0.01 M-Tris-HCl, pH 8.0, with or without sucrose.

Animals. DA and PVG/c rats were obtained from the MRC Cellular Immunology Unit, Oxford, U.K. Wistar rats, AKR, Balb/c and C₃H mice were obtained from Oxfordshire Laboratory Animal Colonies, Bicester, Oxon, U.K.

Methods

Unless otherwise stated all procedures were at 4°C or on ice.

Antibodies. Two types of antisera were used to assay Thy-1.1 alloantigen, C₃H anti(AKR thymocyte) serum (Reif & Allen, 1964) and Balb/c anti(Wistar rat brain) serum (Acton *et al.*, 1974). On AKR thymocytes these antisera recognize only Thy-1.1 antigen: this has been clearly established by the fact that antibody activity can be absorbed by thymocytes and brain from A/Thy-1.1 (A/θ-AKR) mice but not by the same tissues from A/Jax mice (Thy-1.2). These tests are described in detail elsewhere (Acton *et al.*, 1974). The A/Thy-1.1 and A/Jax mice which

are known to differ only at the Thy-1 locus (John *et al.*, 1972) were obtained from Dr. D. A. L. Davies of Searle Research Laboratories, High Wycombe, U.K.

Antisera against histocompatibility antigens were raised in PVG/c rats by two skin grafts from DA strain rats. DA strain rats carry Ag-B allele 4 whereas PVG/c strain rats have the Ag-B allele 5 (Festing & Staats, 1973). The PVG/c anti(DA alloantiserum) will thus recognize a number of specificities and these will be collectively referred to as Ag-B(4).

Purified ¹²⁵I-labelled F(ab')₂ rabbit anti(mouse Fab) and anti(rat Fab) antibodies were prepared as described by Jensenius & Williams (1974).

Preparation of cells. DA rat thymocytes and PVG/c rat leukaemic cells were prepared as described by Acton *et al.* (1974) and washed three times with BBSS buffer by centrifugation. With the leukaemic cells, dead cells were removed by the method of Von Boehmer & Shortman (1973).

Thymocyte membranes. Thymocytes were suspended in Tris-saline at a concentration of 10⁸ cells/ml and disrupted by extrusion through a small orifice under pressure as described by Snary *et al.* (1974). The broken cells were centrifuged at 4000 g for 20 min to remove nuclei and other debris. The supernatant was then centrifuged at 40 000 g for 30 min, and the pellet obtained (which is referred to as the crude membrane) was resuspended in Tris-saline+sucrose (2–15%, w/v) for solubilization. The yield of membrane was determined by measuring 5'-nucleotidase activity.

Brain homogenate. This was prepared by homogenization in BBSS buffer in a Potter-Elvehjem homogenizer and then washing the homogenate by centrifugation at 3000 g for 30 min.

Solubilization of cells, membranes and brain homogenate. Cells were suspended in Tris-saline or Tris-saline+sucrose (15%, w/v) at a concentration of 4 × 10⁸ cells/ml, and crude membranes from 10⁹ cells (1 mg of membrane protein) were suspended in 1.0 ml of Tris-saline+15% (w/v) sucrose (or 2%, w/v, sucrose when extracts were prepared for sucrose gradient centrifugation). As the yield of membrane was about 40% the membrane was equivalent to a cell suspension at 4 × 10⁸/ml. Brain homogenate was suspended in BBSS buffer at 30 mg of protein/ml.

In all cases an equal volume of detergent (usually 4%) was added in the same buffer, and the mixture was resuspended and incubated on ice for 2 h, and then centrifuged for 40 min at 200 000 g.

The activity of Ag-B(4) or Thy-1.1 antigens in the supernatant was assayed and the percentage solubilization was calculated as activity in the 200 000 g supernatant divided by the activity of the original preparation.

Binding assay for Ag-B(4) and Thy-1.1 antigens in the presence of detergents. Assays for determining Ag-B(4) antigen activity were carried out as described by Williams (1973). A similar assay involving the

binding of C₃H anti(AKR thymocyte), or Balb/c anti-rat brain) antisera to AKR thymocytes was developed for measuring Thy-1.1 antigen activity. Glutaraldehyde-fixed AKR thymocytes (10⁶) and 10⁷ fixed Balb/c erythrocytes (to improve pelleting in cell washing), in 50 μ l of 10% (w/v) bovine serum albumin in BBSS buffer, were incubated with 50 μ l of diluted antiserum in 2.5 ml plastic tubes for 60 min on ice. Cells were washed twice with 1.3 ml of 0.1% bovine serum albumin in BBSS buffer by centrifugation at 2000g. Then 100 μ l of ¹²⁵I-labelled anti(mouse Fab) (10⁶ d.p.m., sp. radioactivity 20 μ Ci/ μ g) in 0.5% bovine serum albumin in BBSS buffer was added and incubated with the cells for 60 min on ice. The cells were then washed as above, suspended in 0.6 ml of BBSS buffer, and transferred to 1 ml plastic tubes containing 0.1 ml of 5M-NaOH, for gamma counting (efficiency, 40%) in a Nuclear Enterprises Counter.

When inhibition of the assay was to be measured, the antibody solution was first incubated with cells or detergent extract, centrifuged to remove particulate matter, and then assayed as above. Details of the binding assay are further elaborated in the Results section.

Sucrose gradient centrifugation. Sedimentation on sucrose gradients was carried out in a Beckman L2 centrifuge with SW50L or SW50.1 rotors, and 10–30% or 5–20% sucrose gradients. Centrifugation was at 4°C for 20 or 40 h at 43000–45000 rev./min. The gradient volume was 5.1 ml and gradients were formed with a device similar to that described by Martin & Ames (1961). The linearity of the gradients was checked by reading refractive indexes of the fractions obtained with an Abbé refractometer. Gradients were run in 0.2% deoxycholate and 0.2% Lubrol PX, and the buffers used were 0.01M-Tris-HCl (pH 8.0)–0.02% NaN₃ and 0.025M-Tris-HCl (pH 7.5)–0.15M-NaCl–0.02% NaN₃ respectively; gradient centrifugation in ²H₂O (Meunier *et al.*, 1972) was also carried out, and for this all solutions and extracts were prepared in ²H₂O.

Membrane extract (0.25 ml) in 2.0% deoxycholate or Lubrol PX to which marker proteins had been added, was applied to the top of the sucrose gradients. The markers included 20 μ g each of rat IgG*, rat serum albumin, hen egg ovalbumin, and hen egg lysozyme.

Gradients were fractionated by inserting a long, thin needle to the bottom and pumping out with an LKB perflex pump. Fraction volume was 0.3 ml.

Antigen activity was assayed with gradient material at a dilution of 1/1 and 1/3, and expressed as a percentage of the activity of the starting extract assayed at the same time.

The position of marker proteins was determined by the use of specific antisera, and double-diffusion

* Abbreviations: IgG, immunoglobulin G; IgA, immunoglobulin A.

micro-analysis (Clausen, 1969). Although this method is only semi-quantitative, the peak of protein could be unequivocally determined.

Gel filtration. Upward-flowing columns (2.6 cm diam. $\times$ 87 cm long) of Sephadex-G200 and downward-flowing columns (0.9 cm diam. $\times$ 57 cm long) of Sepharose-6B were run in 0.2% deoxycholate–0.01M-Tris-HCl (pH 8.0)–5% (w/v) sucrose–0.02% NaN₃ and also in 0.2% Lubrol PX–0.01M-Tris-HCl (pH 7.5)–5% (w/v) sucrose–0.15M-NaCl–0.02% NaN₃.

The Sephadex-G200 columns had a flow rate of 8 ml/h, whereas that of the Sepharose-6B columns was 2 ml/h. Samples (5 ml or 0.5 ml) of membrane extract in 2% (w/v) detergent were loaded on to the Sephadex-G200 and Sepharose-6B columns respectively. Fraction volumes were also 5 and 0.5 ml respectively. Antigen activity was measured and expressed as described for sucrose gradients.

The column characteristics were determined by chromatography of marker proteins loaded in 2% (w/v) detergent, the position of elution being determined by measuring E_{280} in a 1 cm cuvette. Co-chromatography of markers with membrane extracts was also carried out and in this case the position of elution was determined by double-diffusion analysis with appropriate antisera. The markers used were as for sucrose gradients, plus rabbit IgA, rabbit IgG and whale myoglobin. The total volume of the columns was measured as the elution volume of Na¹²⁵I.

Marker proteins. All of the proteins (with the exception of myoglobin obtained from Miles Laboratories Ltd., Slough, U.K.) had been prepared in this laboratory by standard methods. The purity and size was checked by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate and the R_F of polypeptide chains fell on a straight line if plotted against log molecular weight (Weber & Osborne, 1969). The molecular parameters of the marker proteins were from the following sources: whale myoglobin, Crumpton & Polson (1965); lysozyme, Martin & Ames (1961), Ansari & Salahuddin (1973); ovalbumin, Smith (1970), Andrews (1970); rat serum albumin, Smith (1970); rat IgG, Bazin *et al.* (1974); rabbit IgG, Ansari & Salahuddin (1973), Noelken *et al.* (1965); rabbit IgA, Cebra & Small (1967). The Stokes radius for whale myoglobin, lysozyme, and rat serum albumin was not available, and was calculated from the other hydrodynamic parameters by using the equation, molecular weight = $6\pi\eta NaS/(1-\bar{v}\rho)$ (Siegel & Monty, 1966).

Analytical methods. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin [Sigma (London) Chemical Co. Ltd.] as standard.

5'-Nucleotidase (EC 3.1.3.5) activity was determined by the method of Michell & Hawthorne (1965)

and the P_i released was measured by the method of Fiske & SubbaRow (1925).

Results

Binding of anti(Thy-1.1) antiserum to AKR thymocytes

Fig. 1 shows the binding at various concentrations of C₃H anti(AKR thymocyte), Balb/c anti(Wistar rat brain) and normal Balb/c or C₃H serum to normal and glutaraldehyde-fixed AKR thymocytes. Both of the anti(Thy-1.1) antisera gave highly significant binding compared with normal serum, and the fixed cells were virtually indistinguishable from normal thymocytes in terms of their effectiveness as target cells. Williams (1973) reported that glutaraldehyde-fixed target cells gave high non-specific background binding compared with unfixed cells. This is not seen in Fig. 1, and the improvement is probably due to decreased numbers of thymocytes in the assay

(10^6 compared with 5×10^6), and to the addition of higher concentrations of bovine serum albumin.

The Balb/c anti(rat brain) antiserum had stronger binding activity than the C₃H anti(AKR thymocyte) serum.

Fig. 2 shows the binding of Balb/c anti(rat brain) and normal Balb/c serum to AKR thymocytes in the presence of detergents. Specific binding occurred to fixed cells in the presence of Lubrol-PX, Triton X-100, deoxycholate or Nonidet P-40 at 0.5%. Similar results were obtained with the other detergents listed in the Experimental section.

The binding of C₃H anti(AKR thymocyte) antiserum also occurred in the presence of detergents, but Balb/c anti (rat brain) was used as a routine since it was a stronger antiserum, and also gave better inhibition assays in the presence of deoxycholate [see under 'Solubilization of Ag-B(4) and Thy-1.1 antigens from DA membrane fractions'].

Fig. 1. *Binding of anti(Thy-1.1) antiserum to normal and glutaraldehyde-fixed AKR thymocytes*

Balb/c anti(rat brain) (●, ○), C₃H anti(AKR thymocyte) (■, □) and Balb/c or C₃H normal serum (▲, △) at the final concentration shown on the abscissa were incubated with 10^6 normal (●, ■, ▲) or glutaraldehyde-fixed (○, □, △) AKR thymocytes. The amount of antibody bound was measured with a second binding step by using 125 I-labelled anti(mouse Fab) antibody and the ordinate shows this binding as c.p.m./assay.

Fig. 2. *Binding of Balb/c anti(rat brain) antibody to fixed cells in the presence of detergents*

The specific binding of Balb/c anti(rat brain) antibody (—), and the control binding of normal Balb/c serum (---) to 10^6 glutaraldehyde-fixed AKR thymocytes was measured as described in Fig. 1. Binding was performed without detergents (○) or in the following detergents at 0.5%: (□) Lubrol-PX, (△) Triton X-100, (▲) Nonidet P-40, and (●) deoxycholate.

Fig. 3. Inhibition of the binding of anti(Thy-1.1) antibodies to fixed AKR thymocytes by DA thymocytes in buffer or detergents and by DA thymocyte detergent extracts

(a) Balb/c anti(rat brain) antiserum (25 μ l) at a concentration of 2.5% (v/v) was preabsorbed with DA thymocytes without detergents (○) or in 1% Lubrol-PX (△), or with C₃H thymocytes in buffer (●) or 1% Lubrol-PX (▲); also with Lubrol-PX extract (200000g supernatant) from DA thymocytes (□). Dilutions of cell suspension or cell extracts are shown on the abscissa and 1/2 corresponds to the addition of 2.5×10^6 cells in 25 μ l. The residual antibody activity in the absorbed antiserum was measured in the binding assay. (b) was as for (a) with the exception that Triton X-100 was the detergent used rather than Lubrol-PX.

Inhibition of the binding of anti(Thy-1.1) antiserum to AKR thymocytes

The binding of anti(Thy-1.1) antiserum to fixed AKR thymocytes can be specifically inhibited by pre-absorbing the antiserum with tissues that carry the Thy-1.1 allotype, but not by those of the Thy-1.2

type. Fig. 3 shows pre-absorption of a constant amount of Balb/c anti(rat brain) antibody with DA (Thy-1.1) and C₃H (Thy-1.2) thymocytes. The DA strain cells inhibit subsequent binding to AKR target cells whereas C₃H strain cells do not.

The presence of most detergents during absorption of Balb/c anti(rat brain) serum by DA or C₃H strain thymocytes does not affect the subsequent results in the binding assay. (Sections below deal with some exceptions.) This can be seen from Figs. 3(a) and 3(b) which show absorption and assays in the presence and absence of 0.5% Lubrol-PX and 0.5% Triton X-100. When solubilization is to be studied curves of this type are compared with inhibition curves obtained with supernatant extracts from the same preparations. For example Fig. 3(a) shows that Lubrol-PX at 2% released most of the Thy-1.1 activity into a 200000g supernatant, whereas Fig. 3(b) shows that 2% (w/v) Triton X-100 released very little.

It should be noted that for solubilization detergents were at a concentration of 2%, and then diluted to 1% for the assay resulting in a concentration of 0.5% for antibody absorption and 0.25% after target cells were added.

Solubilization of Ag-B(4) and Thy-1.1 antigens from DA thymocytes

In initial screening experiments, all of the non-ionic detergents listed in the experimental section were tested for their ability to release Thy-1.1 antigen into a 3000g supernatant. The most effective ones as well as some of the more commonly used detergents were then examined in more detail. Inhibition curves for whole cells, cells in detergent, and detergent extracts after centrifugation at 200000g were determined for Ag-B(4) and Thy-1.1 antigens (Table 1). The detergent/buffer ratio is low if any detergent has a detrimental effect on the antigenicity of Thy-1.1 or Ag-B(4). In only one case did a detergent markedly diminish the antigen activity, and this was with Nonidet P-40 and Thy-1.1 antigen.

The ratio of the 200000g extract/buffer activity measures the solubilization of antigen. Only Lubrol-PX effectively released Thy-1.1 from whole thymocytes, whereas Lubrol-PX, Nonidet P-40, Triton X-67, and Triton X-100 were all completely effective in solubilizing Ag-B(4) antigens. Tween 40 and Brij 35 gave relatively poor solubilization of both Ag-B(4) and Thy-1.1 antigens. The release of Thy-1.1 from whole thymocytes by Lubrol-PX occurred in the presence or absence of 15% (w/v) sucrose, and in the latter case at least the antigen would not be simply floating on the extracting solution (see below). In many detergents, more antigen was found than could be assayed with the original cells. This may be due to the detergent revealing antigen previously unavailable to antibody.

Table 1. *Solubilization of Ag-B(4) and Thy-1.1 antigens from DA thymocytes by various detergents*

C, Whole cells; M, membrane. Detergent/buffer, activity in the presence of detergent divided by activity in buffer only. Soluble/buffer, activity in 200000g supernatant divided by activity in buffer only. Detergent concentration was usually 2% for extraction, with cells at 2×10^8 /ml and membrane at 2×10^8 cell equivalents/ml. Experiments with different detergent or cell concentrations were also done with similar results to those below.

Detergent	Fraction	Ag-B(4) activity (%)		Thy-1.1 activity (%)	
		Detergent/buffer	Soluble/buffer	Detergent/buffer	Soluble/buffer
Triton X-100	C	240	170	93	4
	M	—	120	60	13
Nonidet P-40	C	170	170	47	17
	M	—	80	56	17
Triton X-67	C	150	110	160	27
	M	—	200	100	20
Lubrol-PX	C	116	158	114	99
	M	—	200	85	92
Tween-40	C	74	27	180	13
	M	—	34	120	6
Brij 35	C	100	38	—	26
Deoxycholate	M	58	70	78	80

Solubilization of Ag-B(4) and Thy-1.1 antigens from DA membrane fractions

The crude membranes prepared from DA thymocytes contained 40% of the original 5'-nucleotidase activity, and 3–4% of the protein. Thus the yield of membrane was about 40% at tenfold purification.

The solubilization of antigen from membranes gave much the same results as obtained with whole cells (Table 1). In addition deoxycholate solubilized Ag-B(4) and Thy-1.1 antigens from membrane preparations. Deoxycholate could not be used with whole cells since unlike the non-ionic detergents it solubilized the cell nuclei releasing DNA.

The presence of deoxycholate in the inhibition assays raised the background both in the Ag-B(4) and Thy-1.1 assay systems. Satisfactory assays were possible, however, by using the PVG/c anti(DA) and Balb/c anti(rat brain) antisera.

In contrast with this deoxycholate interfered with the inhibition of the binding assay with C₃H anti(AKR thymocyte) serum, without affecting the binding itself. Assays of fractions from sucrose gradients to check the Balb/c anti(rat brain) results were possible, but the C₃H anti(AKR thymocyte) serum was not used as a routine for assays in deoxycholate.

Solubilization of Thy-1.1 from PVG/c leukaemia cells and brain homogenate

Brain and leukaemia cells are other rat tissues rich in Thy-1.1 antigen (Acton *et al.*, 1974).

Solubilization of leukaemia cells was carried out as for thymocytes, but satisfactory results were not obtained with any of the detergents. The results were unusual in that the Thy-1.1 activity seemed to be

masked in the presence of a number of detergents. For this reason the use of the PVG/c rat leukaemia cells was abandoned.

With brain homogenate suspended in BBSS buffer the effects of Lubrol-PX, Triton X-67, Nonidet P-40, and Triton X-100 were investigated. Although none of these detergents masked the Thy-1.1 activity when added to the homogenate, they were not effective in releasing activity into a 200000g supernatant, less than 1% of the activity was solubilized by Lubrol-PX or Triton X-67, and the best results were with Triton X-100, which released about 25% of the activity.

Sucrose-density-gradient centrifugation of deoxycholate and Lubrol-PX-solubilized thymocyte membranes

The properties of Ag-B(4) and Thy-1.1 antigens solubilized in deoxycholate and Lubrol-PX were examined by sucrose gradient centrifugation.

Fig. 4 shows sedimentation of Thy-1.1 and Ag-B(4) antigens in 5–20% (w/v) sucrose gradients in water (Fig. 4a) and in ²H₂O (Fig. 4b). Sedimentation in ²H₂O gradients, in which the measured density was from 1.128–1.174 g/ml compared with 1.027–1.083 g/ml for water gradients, was carried out to indicate whether antigens were associated with low-density material. If this were the case, the relation between marker proteins and antigen would differ on water and ²H₂O gradients (Meunier *et al.*, 1972). Thy-1.1 antigen sedimented a little faster than lysozyme on both gradients, and Ag-B(4) antigen activity ran slightly behind rat serum albumin. By measuring the distances moved in both gradients, and comparing

Fig. 4. Sucrose gradient centrifugation of deoxycholate extracts from thymocyte membranes

(a) Distribution of Ag-B(4) (Δ) and Thy-1.1 ($\circ$) activity after centrifugation for 40h at 44000 rev./min of deoxycholate extract from DA thymocyte membrane on 5–20% (w/v) sucrose gradients in water and 0.2% deoxycholate (see under 'Methods'). Activity is plotted as the percentage of total antigen in the original extract. The position of marker proteins sedimenting on the same gradient are shown: RSA, rat serum albumin; Ov, ovalbumin; Lys, lysozyme. (b) Activities after centrifugation as above but with gradients and extracts prepared in $^2\text{H}_2\text{O}$.

Fig. 5. Sucrose gradient centrifugation of Lubrol-PX extracts from thymocyte membranes

Details are as for Fig. 4. (Δ), distribution of Ag-B(4) activity and ($\circ$) of Thy-1.1 activity of extracts prepared in Lubrol-PX and run on 5–20% (w/v) sucrose gradients in water (a) and $^2\text{H}_2\text{O}$ (b).

these with the sedimentation of the marker proteins $\bar{v}$ values were calculated as described by Meunier *et al.* (1972). For Thy-1.1 antigen a value of 0.70 ml/g was obtained and for Ag-B(4) antigen the $\bar{v}$ was 0.75 ml/g.

Apparent $s_{20,w}$ values were calculated by comparison with marker proteins (Martin & Ames, 1961) and similar results were obtained after sedimentation on water or $^2\text{H}_2\text{O}$ gradients for 20 or 40h. From a total of nine gradients the mean $s_{20,w} \pm \text{S.E.}$ was 2.4 ± 0.04 for Thy-1.1 antigen and 4.4 ± 0.10 for Ag-B(4) antigen.

In contrast with these results, those obtained after centrifugation of Lubrol-PX extracts on sucrose gradients containing 0.2% Lubrol-PX were anomalous. Distribution of antigen activity after centrifuga-

tion for 40h on 5–20% (w/v) sucrose gradients in water and in $^2\text{H}_2\text{O}$ is shown in Fig. 5. Thy-1.1 antigen activity sedimented more slowly than lysozyme in the water gradient (apparent $s_{20,w}$ of 1.1), and did not enter the $^2\text{H}_2\text{O}$ gradient at all. The distribution of Ag-B(4) antigen activity was more like that seen in the deoxycholate gradient, but the peak was much broader. The marker proteins ran in the same positions on the gradients in Lubrol-PX as they had in the equivalent in deoxycholate. Thus in Lubrol extract, the Thy-1.1 activity behaved as if associated with low-density material, and this was confirmed by the finding that it did not enter a 10–30% (w/v) sucrose gradient in water after 40h centrifugation.

All the above results were obtained by using the binding assay with Balb/c anti(rat brain) serum. Assays with C_3H anti(AKR thymocyte) serum were also carried out for the 5–20% (w/v) sucrose gradients in water and in $^2\text{H}_2\text{O}$. In Lubrol-PX the assays were entirely comparable with those with the Balb/c anti-serum and activity on the sucrose gradients was in the same fractions as shown in Fig. 5. In deoxycholate

Fig. 6. Gel filtration of solubilized thymocyte membranes on Sepharose 6B columns

A deoxycholate extract (0.5 ml) (a) or a Lubrol-PX extract (0.5 ml) (b) of thymocyte membrane was chromatographed on a column (57 cm $\times$ 0.9 cm) of Sepharose 6B in the presence of deoxycholate or Lubrol-PX respectively (see under 'Methods'). Fractions (0.5 ml) were collected and assayed for Ag-B(4) (Δ) and Thy-1.1 activity ($\circ$). Results are plotted as percentage of the activity in the starting extract. Positions of markers co-chromatographed with the extracts are shown (BD, Blue Dextran 2000; Ov, ovalbumin).

inhibitions were weak, but the activity assayed was in the same fractions as shown in Fig. 4.

Gel filtration of solubilized thymocyte membrane on columns of Sepharose 6B and Sephadex G-200

Gel filtration could be carried out in both deoxycholate and Lubrol-PX. Marker proteins ran similarly in both detergents, except that the elution volume

was greater for all markers in Lubrol-PX than in deoxycholate. This was observed in gel filtration on both Sepharose 6B and Sephadex G-200 columns.

The distribution of antigen activity after chromatography on Sepharose 6B in deoxycholate is shown in Fig. 6(a). Ag-B(4) antigen eluted in one peak between IgA and IgG, and the Thy-1.1 antigen activity was eluted between IgG and ovalbumin. Chromatography on Lubrol-PX columns gave different results (Fig. 6b). Some Ag-B(4) activity eluted at about the same place as IgA, but a considerable amount of activity was also found at the column front. With Thy-1.1 antigen two discreet peaks were obtained, one at the column front (75% of the activity in this experiment and 66% in another run), the other at approximately the same elution volume as IgG. The latter peak is presumably comparable with that found for gel filtration in deoxycholate.

These results were confirmed by gel filtration on Sephadex G-200 (Fig. 7). In deoxycholate (Fig. 7a) Ag-B(4) antigen eluted ahead of the IgG marker, and Thy-1.1 antigen eluted slightly ahead of ovalbumin. In Lubrol-PX (Fig. 7b) most of the activity of both Ag-B(4) and Thy-1.1 antigens was close to the gel front, with a small peak of Thy-1.1 antigen eluting between rat serum albumin and IgG. In both detergents, the results were very similar to those obtained with Sepharose-6B gel filtration.

From the chromatography on Sephadex G-200 in deoxycholate, estimates were made of the Stokes radii for Ag-B(4) and Thy-1.1 antigens. For the marker proteins, IgG, rat serum albumin, ovalbumin and myoglobin, Stokes radius was plotted against the cube root of the partition coefficient (Siegel & Monty, 1966). A linear relation was obtained and from this Stokes radii for Ag-B(4) and Thy-1.1 antigens were calculated by using the experimentally determined partition coefficients for the antigens. The values obtained in two experiments were 3.1 and 3.1 nm for Thy-1.1 and 5.1 and 5.4 nm for Ag-B(4). Thus both antigens were considerably larger than the deoxycholate micelle which would be expected to have a molecular weight of about 2000 with the conditions used (Small, 1968).

Calculation of apparent molecular weight of Ag-B(4) and Thy-1.1

Taking the values for $s_{20,w}$, $\bar{v}$ and Stokes radius determined above, the apparent molecular weight for the antigens in deoxycholate was calculated with the equation used by Siegel & Monty (1966):

$$M = \frac{6\pi\eta NaS}{1 - \bar{v}\rho}$$

where symbols are as follows: M, molecular weight; η , viscosity; N, Avogadro's number; a, Stokes

Fig. 7. Gel filtration of solubilized thymocyte membranes on Sephadex G-200 columns

A deoxycholate extract (5ml) (a) or a Lubrol-PX extract (5ml) (b) from thymocyte membrane was chromatographed on a column (87 cm × 2.6 cm) of Sephadex G-200 in the presence of deoxycholate or Lubrol-PX respectively (see under 'Methods'). Fractions (5ml) were collected and assayed for Ag-B(4) (Δ) and Thy-1.1 activity (○). Results are plotted as percentage of the activity in the starting extract. Positions of markers co-chromatographed with the extracts are as described in the legends to Figs. 4 and 6.

radius; $S_{20,w}$; $\bar{v}$, partial specific volume; ρ , density of medium.

From this the molecular weight of Ag-B(4) antigen was estimated to be 100000 and that of Thy-1.1 28000. The frictional ratio f/f_0 was calculated with the equation:

$$f/f_0 = a \left(\frac{4\pi N}{3\bar{v}M} \right)^{1/3}$$

and values of 1.71 and 1.57 were obtained for Ag-B(4) and Thy-1.1 antigens respectively.

Discussion

Solubilization of Thy-1.1 and Ag-B(4) antigens

The solubilization of Thy-1.1 antigen in detergents was very unusual in that only deoxycholate and Lubrol-PX released the antigen into a 200000g supernatant. Even the Lubrol-PX was apparently not giving true solubilization since the antigen was found in a low-density complex by sucrose gradient centrifugation and gel filtration.

Vitetta *et al.* (1973) studied the solubilization of Thy-1.1 antigen from mouse thymocytes by precipitation with specific anti(Thy-1.1) antiserum of membrane protein labelled with ^{125}I by the lactoperoxidase method. They reported that Nonidet P-40 does not solubilize the antigen. However, they also claimed that the antigen activity was destroyed by detergents, and this was clearly not supported by our results. We did find, however, that Nonidet P-40 was the one detergent which led to interference with the antigen-antibody binding reaction in the Thy-1.1 system and this may explain their results. However, it is very difficult to evaluate studies using ^{125}I labelling with lactoperoxidase, since there is no clear evidence that the Thy-1.1 antigen is being labelled, and further the amount of antigen solubilized cannot be calculated.

In contrast with the results for Thy-1.1 antigen, solubilization of Ag-B(4) antigen was achieved by several detergents. Lubrol-PX, Nonidet P-40, Triton X-67 and Triton X-100 were all effective in solubilizing Ag-B(4) from thymocytes and their membranes, and deoxycholate gave relatively good solubilization of antigen from membranes. Other workers have shown that H-2 histocompatibility antigen can be solubilized in Nonidet P-40 (Schwartz & Nathenson, 1971) and that H-LA can be solubilized in deoxycholate (Snary *et al.*, 1974).

In terms of yielding antigenic activity in discrete molecular forms deoxycholate was better than Lubrol-PX for the solubilization of Ag-B(4) antigen. Activity in large aggregates was observed in Lubrol-PX as well as in fractions of a size corresponding to that found in deoxycholate. Other non-ionic detergents may give better results; for example Schwartz *et al.* (1973) found all of the H-2 activity in one peak after solubilization in Nonidet P-40 and chromatography on Sepharose 6B.

Estimates of molecular weight and asymmetry of the antigen-detergent complexes

As a result of the studies with sucrose gradient centrifugation and gel filtration in deoxycholate, some hydrodynamic parameters of Ag-B(4) and Thy-1.1 antigen activities were determined. These apply to antigen plus bound detergent and thus should be interpreted with caution. The molecular sizes of the antigens are almost certainly over-

estimated since they are derived by comparison with marker proteins which probably bind much less deoxycholate than the membrane molecules (Helenius & Simons, 1972). Also the $\bar{v}$ of the antigens may be altered by the binding of deoxycholate which has a $\bar{v}$ of 0.76 (Small, 1968). Despite this the values obtained give some indication of the likely properties of the antigens and can be compared with the results of other workers. Thus the molecular weight of 28000 calculated for Thy-1.1 in deoxycholate is consistent with a value of 35000 reported by Vitetta *et al.* (1973), but not compatible with that of 60000 obtained by Atwell *et al.* (1973). Both of the latter values were determined by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate of ^{125}I -labelled material immunoprecipitated from membrane extracts with anti-(Thy-1) antiserum. Vitetta *et al.* (1973) attribute the lack of agreement to differences in the specificity of the antiserum.

For histocompatibility antigens more data are available, and the values for Ag-B(4) can be compared with those for H-LA and H-2. The $s_{20,w}$ values for Ag-B(4) antigen in deoxycholate are very similar to those found for H-LA, i.e. 5.15S and 4.65S for LA and Ag-B(4) determinants respectively (Snary *et al.*, 1974). In contrast the molecular-weight estimate of 100000 for Ag-B(4) antigen is lower than the value of 380000 suggested by Schwartz *et al.* (1973) for H-2 antigen solubilized in Nonidet P-40. However, the latter value may overestimate the size of H-2 in Nonidet P-40 since it is clear from the data on Ag-B(4) antigen that histocompatibility antigen behaves as if of higher molecular weight in gel filtration than on sucrose gradients. It is likely that the 88000 dimer identified by Schwartz *et al.* (1973) after polyacrylamide-gel electrophoresis in sodium dodecyl sulphate of immunoprecipitated H-2 antigen is the same entity as that extrapolated to 380000 molecular weight after Sepharose 6B gel filtration. The fourfold discrepancy could be due to the binding of large amounts of non-ionic detergent as suggested by Snary *et al.* (1974), in addition to asymmetry of the molecule as suggested by the results with Ag-B(4) antigen.

The apparent asymmetry of both Thy-1.1 and Ag-B(4) antigens was clearly established by the behaviour of the antigens in sucrose gradient centrifugation compared with gel filtration, and is expressed in the high values for frictional coefficient (f/f_0) calculated of 1.57 for Thy-1.1 and 1.71 for Ag-B(4).

Many other membrane proteins also appear to be asymmetric including the acetylcholine receptor (Meunier *et al.*, 1972), insulin receptor (Cuatrecasas, 1972) and gonadotropin receptor (Dufau *et al.*, 1973), as well as viral membrane proteins (Simons *et al.*, 1973). Such proteins may be integrated into the cell membrane by a small hydrophobic tail (Marchesi *et al.*, 1972) and it may also be this part of the molecule that results in asymmetry in detergent extracts.

Chemical nature of Ag-B and Thy-1 antigens

The results in the present paper give only indirect information as to the chemical nature of Ag-B(4) or Thy-1.1 antigens. However, the $\bar{v}$ of 0.75 estimated for Ag-B(4) in deoxycholate would be consistent with the antigen being a glycoprotein as suggested for H-LA (Snary *et al.*, 1974) and H-2 (Schwartz *et al.*, 1973).

The chemical nature of Thy-1 antigen is obscure, and it has variously been suggested that it is a protein (Atwell *et al.*, 1973), a glycolipid (Vitetta *et al.*, 1973) or a ganglioside (Esselman & Miller, 1974). The $\bar{v}$ value of 0.70 estimated for Thy-1.1 in deoxycholate in this study would be more compatible with a protein or carbohydrate molecule than a lipid.

Any clear analysis of the nature of Thy-1 antigen will require purified preparations.

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