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Isolation and Characterization of the Thymus–Brain Antigen (Analogous to Thy-1 Antigen) from Human Brain

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1. The human thymus–brain antigen, which corresponds to the murine (mouse or rat) Thy-1 antigen complex, was isolated from brain after solubilization in deoxycholate by gel-permeation chromatography, wheat-germ-lectin affinity chromatography and ion-exchange chromatography. 2. The isolated antigen is a glycoprotein displaying an apparent molecular weight of 26000–29000 in sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. 3. No antigen activity was found with the lipid fraction from human brain. 4. The protein has a tendency for spontaneous self-association (dimerization), leading to aggregates resistant to dissociating and reducing agents on prolonged storage. 5. The antigen is microheterogeneous with respect to size, charge (approximate isoelectric points of the monomer 7.7, 7.0 and 6.5) and to lectin-binding affinity. 6. The antigen can be reconstituted to protein–lipid vesicles. The antigen activity of solubilized antigen is strongly increased by reconstitution and that of membranes decreased by solubilization with detergent.

By using appropriate alloantisera, Reif & Allen (1964) discovered an antigen in mice that is mainly displayed by thymic lymphocytes and brain. This antigen, which exists in two allelic forms, now designated Thy-1.1 and Thy-1.2, was found to reside on a glycoprotein with an apparent molecular weight of about 25000 in SDS/polyacrylamide-gel electrophoresis (Trowbridge *et al.*, 1975; Letarte & Meghji, 1978). Previously we have found (Thiele *et al.*, 1972) that this molecule bears at least two further determinants, one of them being species-specific (mouse or rat), the other one shared by several species, including man (Thiele *et al.*, 1973; Arndt *et al.*, 1977, 1978; Dalchau & Fabre, 1979). This complex antigen was provisionally called thymus–brain antigen (Thy-Br antigen) (Arndt *et al.*, 1977). More recently it became apparent that this structure is also expressed on the surface membranes of some other cell types (Scheidt *et al.*, 1972; Raedler *et al.*, 1978; Lennon *et al.*, 1978), although its expression on distinct lymphocyte populations differs in mice, rats and man (Thiele *et al.*, 1972; Acton *et al.*, 1974; Douglas & Dowsett, 1975; Williams, 1976; Arndt *et al.*, 1978; Goldschneider *et al.*, 1978).

In the human the Thy-Br antigen is displayed on brain and thymic epithelium (Raedler *et al.*, 1979), but in contrast with the mouse it is not expressed on thymic and peripheral lymphocytes as well (Arndt *et al.*, 1978). Chromatographic analyses of the molecule solubilized from human brain and thymus revealed a close resemblance to the Thy-Br-antigen glycoprotein isolated from mouse and rat thymic lymphocytes and brain (Arndt *et al.*, 1977, 1978).

The aim of the present study was the isolation of the Thy-Br antigen from human brain and its further characterization. The structural analysis of this protein seems to be of particular interest because of its remarkable phylogenetic stability. Moreover, this could contribute to an elucidation of its as yet unknown biological function.

Abbreviations used: Thy-Br_{Hu} antigen, human thymus–brain antigen; SDS, sodium dodecyl sulphate.

Materials and Methods

Chemicals

Acrylamide, bisacrylamide, *NN*-diallyltartardiamide and SDS were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Ampholytes (Ampholine and Servalyte) were from LKB Produkter (Bromma, Sweden) and Serva (Heidelberg, Germany) respectively. Chymotrypsinogen, β-lactoglobulin and cytochrome *c* were purchased from Serva, aldolase was from Boehringer (Mannheim, Germany), and bovine serum albumin and *Vibrio cholerae* neuraminidase [EC 3.2.1.18; activity 0.2 unit (μmol of sialic acid cleaved from human

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α_1-acid glycoprotein / min at 37°C) / ml] were from Behring (Marburg, Germany). Triton X-100 (scintillation grade), hen's-egg phosphatidylcholine and absorber resin Amberlite XAD-2 were from Serva. Cholate and n-octyl glucoside were from Sigma (München, Germany), and sodium deoxycholate (for microbiology) was from Merck (Darmstadt, Germany). DEAE-Sepharose CL-6B and CNBr-activated Sepharose were from Pharmacia (Uppsala, Sweden), and AcA 34 gel was from LKB Produkter. Wheat-germ agglutinin coupled to Sepharose was obtained from Pharmacia or prepared by coupling of 2–4 mg of lectin/ml of CNBr-activated Sepharose in the presence of 20 mM-*N*-acetylglucosamine. The lectin was purified from wheat germ by the method of Bloch & Burger (1974). Alkaline phosphatase coupled to pig immunoglobulin having antibody specificity for rabbit immunoglobulin G was purchased from Orion Diagnostics (Helsinki, Finland).

Buffers

Tris-buffered saline contained 20 mM-Tris/HCl, 130 mM-NaCl and 0.04% NaN_3, pH 8.0. Phosphate-buffered saline contained 10 mM-sodium phosphate, 140 mM-NaCl and 0.02% NaN_3, pH 7.2. Sucrose medium contained 0.34 M-sucrose, 10 mM-Tris/HCl and 1 mM-phenylmethanesulphonyl fluoride as proteinase inhibitor (added from 100 mM stock solution in ethanol before use), pH 7.4.

Cells, tissues and antisera

Post-mortem human brain taken 1–2 days after death was provided by the Department of Pathology of the University Hospital. Thymic lymphocytes from CBA/J/Han mice were prepared as described by Thiele *et al.* (1972).

Anti-(human brain) serum was raised in rabbits as described by Thiele *et al.* (1972).

Antigen detection

Antigen activity was assayed by absorption of appropriately diluted rabbit anti-(human brain) serum with the sample containing antigen and subsequent determination of remaining free antibodies in either a cytotoxicity assay or a solid-phase enzyme-linked immunosorbent assay. The cytotoxicity assay was performed as described by Zimpel *et al.* (1973) with CBA-mouse thymocytes as target cells and guinea-pig serum as complement source. For the enzyme-linked immunosorbent assay microtitre plates were coated with deoxycholate-solubilized CBA-mouse thymocyte membranes (1 mg of protein/ml, diluted 1:400 with Tris/HCl-buffered saline) and residual sites on the plastic surface saturated with 1% bovine serum albumin. A neat antiserum dilution or antiserum previously reacted with antigen was put into the antigen-coated wells for 3 h. After a washing, antibody bound to the wells was detected by incubation with alkaline phosphatase-linked anti-(rabbit immunoglobulin G) antibody and photometric determination of substrate (4-nitrophenyl phosphate) hydrolysed by the bound enzyme.

Interfering detergent contained within the samples was neutralized by addition of bovine serum albumin (Springer *et al.*, 1977) after reaction with the antiserum. Quantitative comparisons of antigen activity of single samples with the enzyme-linked immunosorbent assay were done by using a calibration curve of dilutions of a standard preparation of the antigen (lectin-purified antigen in Tris-buffered saline containing 0.2% sodium deoxycholate). Values of absorbed antibody determined by the enzyme-linked immunosorbent assay method were based on a calibration curve of antiserum dilutions. Protein content was determined with a modified Lowry procedure (Bensadoun & Weinstein, 1976), with bovine serum albumin as standard.

Purification procedure

For the preparation of a crude membrane fraction, slices of human brain were dissected to obtain mainly grey matter. The tissue pieces were stored at −70°C after being frozen in liquid N_2. The brain matter (150 g) was homogenized in 10 vol. (v/w) of sucrose medium by 15 strokes in a Potter–Elvejhem homogenizer and centrifuged at 700 ***g*** for 10 min. After the sediment had been rewashed, the supernatants of both centrifugation steps were combined and the sucrose concentration was lowered to 0.32 M by addition of water. The samples were centrifuged at 12000 ***g*** for 30 min, and the sediment containing the membranes was washed once with 1.5 litres of buffer of higher ionic strength (60 mM-Tris/HCl/390 mM-NaCl/0.12% NaN_3, pH 8.0, containing phenylmethanesulphonyl fluoride), to remove as much non-integral membrane protein as possible. The sediment was then suspended in 1 litre of 10 mM-Tris/HCl buffer, pH 8.0, containing 1 mM-EDTA and 0.02% NaN_3. All procedures were performed at 4°C. In some experiments the membranes were freeze-dried and washed with acetone at −10°C in order to remove part of the lipid. After addition of 2% sodium deoxycholate, the membranes were solubilized for 1 h at 37°C and centrifuged at 100000 ***g*** for 1 h. The sediments were extracted once again with 2% sodium deoxycholate, and the supernatants were combined and dialysed against Tris-buffered saline, pH 8.0, containing 0.2% sodium deoxycholate and 1 mM-EDTA. The extracts were chromatographed on a column (5 cm × 100 cm) of AcA 34 gel either directly or after concentration by freeze-drying and dialysis. Frozen or freeze-dried antigen samples were routinely resolubilized after thawing by short warming up to 37°C. Fractions containing antigen were combined and further

purified by affinity chromatography on a column (5cm x 20cm) of Sepharose-coupled wheat-germ lectin. Elution of the antigen was performed with 0.2M-*N*-acetylglucosamine in Tris-buffered saline containing 0.2% sodium deoxycholate. Specifically eluted material was dialysed against 20mM-Tris/HCl buffer, pH8.0, containing 0.02% NaN_3 and absorber resin Amberlite XAD-2 to remove all deoxycholate. The sample was then adjusted to 0.1% Triton X-100. Occasionally precipitated material was resolubilized by short warming up to 37°C. Alternatively, deoxycholate was removed by ethanol precipitation of the antigen (Barclay *et al.*, 1976).

For final purification the antigen material was passed through a DEAE-Sephadex column (1cm x 4cm) equilibrated with 20mM-Tris/HCl buffer, pH8.0, containing 0.1% Triton X-100. The purified antigen is found in the unretarded fractions and was concentrated by freeze-drying.

Electrophoresis and two-dimensional separation

Analytical SDS/polyacrylamide-gel electrophoresis was performed in slab gels (11% acrylamide) as described by Maizel (1971). For preparative SDS/polyacrylamide-gel disc electrophoresis the same gel system was used in rods. After electrophoresis the gel was sectioned with a Gilson gel fractionator and eluted at room temperature for 1 day in 0.2–0.5ml of phosphate-buffered saline. SDS was removed by dialysis (1 day against phosphate-buffered saline and another 1 day against phosphate-buffered saline containing absorber resin XAD-2).

Two-dimensional electrophoresis with isoelectric focusing in the first dimension and SDS/polyacrylamide-gel electrophoresis in the second was carried out essentially as described elsewhere (O'Farrell, 1975; Hamann & Drzeniek, 1978) but without urea in the focusing gel. Gel rods (3mm diam.; 5% acrylamide with 15% diallyltartardiamide as cross-linker) (Baumann & Chrambach, 1976), 1% ampholytes pH3.5–10 (LKB) or 2–11 (Serva) and 1% pH7–9 (LKB), purified on activated charcoal (Hamann & Drzeniek, 1978), and 0.1% Triton X-100 were used for the first-dimension gel. Deoxycholate has to be replaced by non-ionic detergent within the sample since it causes precipitation at lower pH.

Reconstitution

Lectin-purified material was reconstituted to protein–lipid vesicles by the method described by Engelhard *et al.* (1978), with antigen solution in 0.2% sodium deoxycholate and egg phosphatidylcholine mixed with cholate [phosphatidylcholine/cholate ratio 1:5 (w/w)]. Protein and lipid were mixed in a ratio of 2:1 and dialysed against Tris-buffered saline, pH8.0, containing 10% sucrose and 0.1mM-EDTA.

Reconstituted vesicles were analysed by a 0–50% sucrose gradient (Engelhard *et al.*, 1978).

Radiolabelling procedures

Antigen (in Tris-buffered saline containing 0.2% sodium deoxycholate or detergent-free) was labelled with ^{125}I by using the chloramine-T method (Jensenius & Williams, 1974). By this method, only a few per cent of radioactive iodine were incorporated into the protein; a substantial part of the iodine label was non-covalently bound to the protein (partially associated with lipid or detergent traces, as found after SDS/polyacrylamide-gel electrophoresis or t.l.c.).

Thin-layer chromatography

Lipid of purified antigen preparations was analysed by t.l.c. on silica-gel 60 (Merck) plates with chloroform/methanol/water (12:7:1, by vol.) as solvent. Lipid bands were located by 40% (v/v) H_2SO_4 spray followed by 10min heating at 110°C.

Neuraminidase treatment

For neuraminidase treatment purified antigen in medium containing Triton X-100 was dialysed against 50mM-sodium acetate buffer (pH6.0)/150mM-NaCl containing 1mg of $CaCl_2$/ml and incubated with 0.04 unit of neuraminidase/ml for 24h at 37°C. The neuraminidase had been coupled to CNBr-activated Sepharose (0.2 unit of enzyme/ml of gel). Control incubations were performed with glycine-conjugated (blocked) Sepharose.

Periodate treatment

Lectin-purified antigen was freed of detergent and dialysed against 50mM-sodium acetate buffer, pH4.5. Then 20mM-sodium periodate was added and the mixture was incubated at 4°C for 6h in the dark. The reaction was terminated by addition of 2% (v/v) glycerol, and the solution was dialysed against Tris-buffered saline and finally against Tris-buffered saline containing 0.2% sodium deoxycholate.

Lipid extraction

For the extraction of total lipid, brain was homogenized in 30vol. (v/w) of chloroform/methanol (2:1, v/v) in an Ultra-Turrax homogenizer. The homogenate was filtered and the residue was washed with the same volume of chloroform/methanol (1:2, v/v). Approximate lipid content was determined by weighing the dried residue. Lipid vesicles were prepared by addition of the detergent octyl glucoside (lipid/detergent ratio 1:5) to the extract, drying and dialysis of the detergent/lipid mixture dissolved in phosphate-buffered saline against deter-

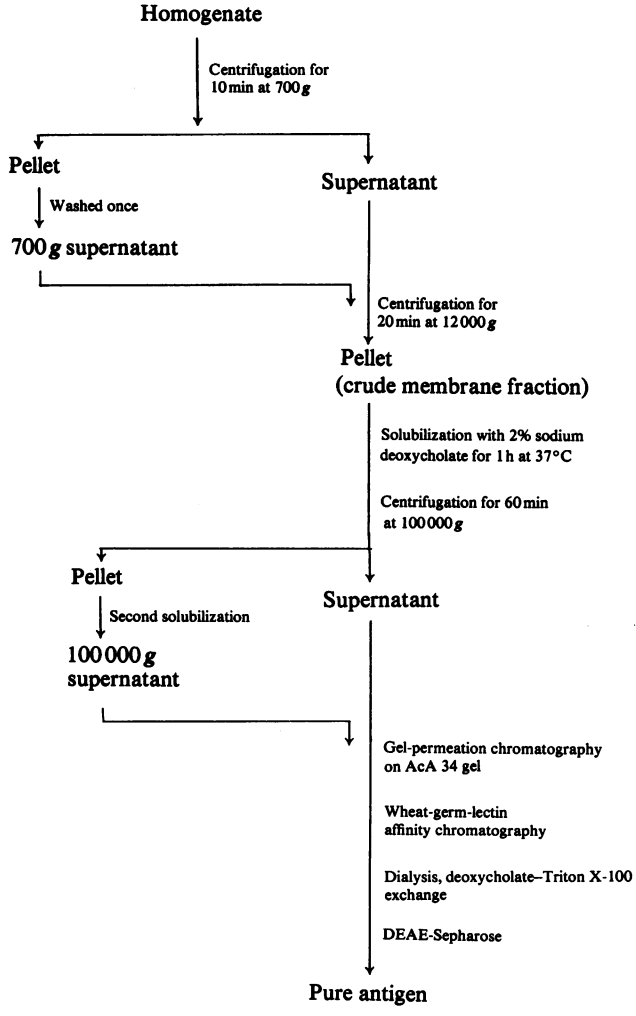
gent-free phosphate-buffered saline (Engelhard *et al.*, 1978).

Results

Purification procedure

Starting material for the isolation of human thymus-brain antigen was grey matter of post-mortem brain, which, as quantitative absorption analysis had revealed, contains 3–5-fold more Thy-Br antigen than does white matter. A similar distribution pattern was found for Thy-1 antigen in mouse brain (Stohl & Gonatas, 1977). The purification procedure and the yields obtained after the individual purification steps are summarized in Scheme 1 and Table 1 respectively.

The antigen is solubilized from membranes by deoxycholate. Maximal yield is obtained by two successive extractions at 37°C. A crude pre-purification by gel-permeation chromatography on AcA 34 gel removes part of lipid and especially some wheat-germ-lectin-binding proteins of high molecular weight. The subsequent lectin affinity chromatography on wheat-germ agglutinin leads to a reasonable enrichment of the antigen. It has to be mentioned that part of the antigenic material (about 40%) is not bound by the lectin column (Fig. 1). This material is therefore not contained within the antigen preparation finally isolated. Elution of the bound antigen with an *N*-acetylglucosamine gradient reveals a heterogeneity of the antigen with respect to binding affinity (Fig. 1). Two-dimensional analysis (isoelectric focusing followed by SDS/polyacryl-



Scheme 1. *Procedure for the purification of Thy-Br$_{Hu}$ antigen from human brain*
For full experimental details see the text.

Table 1. *Purification of Thy-Br antigen from human brain*

Protein content and antigen activity after the individual purification steps are shown. For details of the purification procedure see the Materials and Methods section and Scheme 1. Antigen activity is expressed as equivalents of purified Thy-Br antigen. It must be mentioned that antigen activity does not reflect the real content of antigen, since solubilization and removal of lipids lowers apparent antigen activity (see the Discussion section).

Preparation	Protein content (mg from 150g of brain)	Antigen activity (units* from 150g of brain)	Apparent specific activity (units/mg of protein)
Homogenate	10200	440	0.043
700*g* supernatant	7000	310	0.044
12000*g* sediment	3100	280	0.090
Deoxycholate extract, 100000*g* supernatant	1400	54	0.039
Gel-permeation chromatography, antigen-containing fractions	260	34	0.13
Wheat-germ-lectin eluate	24	14	0.58
DEAE-Sepharose unbound fraction	7	7	1

* 1 unit is equivalent to 1 mg of purified antigen.

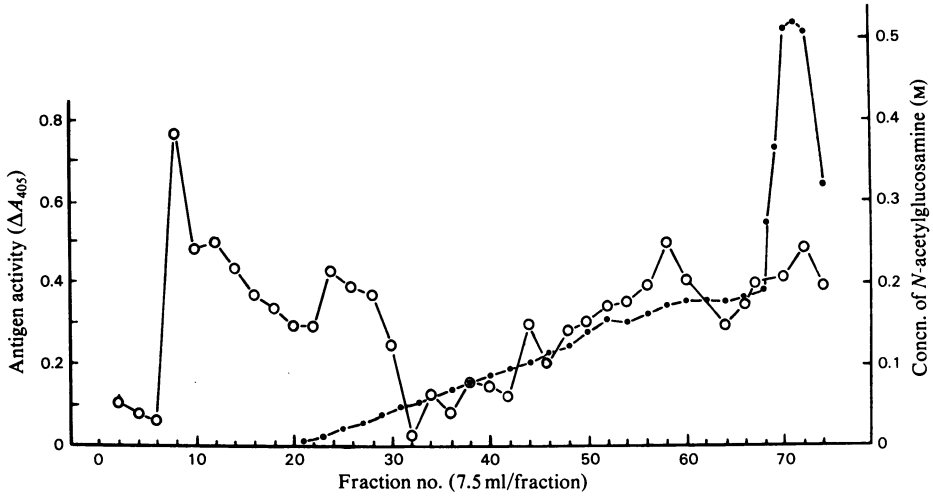


Fig. 1. *Elution of Thy-Br$_{Hu}$ antigen from a wheat-germ-lectin–Sepharose column with an N-acetylglucosamine gradient*

A 20ml portion of gel-permeation-chromatographically purified membrane extract was applied to the column, washed with 1 column volume of Tris-buffered saline containing 0.2% sodium deoxycholate and eluted with a linear gradient (300ml) of 0–0.2M-*N*-acetylglucosamine in Tris-buffered saline containing 0.2% sodium deoxycholate, followed by 30ml of 0.5M-*N*-acetylglucosamine. For full experimental details see the text. O, Antigen activity, assayed by the enzyme-linked immunosorbent assay method; ●, *N*-acetylglucosamine concentration. The unbound fraction (fractions 1–20) contained 40% and the bound one (fractions 21–74) 60% of the total eluted activity.

amide-gel electrophoresis) of the lectin-purified antigen material revealed that accompanying proteins and also the dimerized antigen (see below) have lower isoelectric points than the antigen monomer (Fig. 3). These differences in charge allow final purification of the antigen by ion-exchange chromatography. At an ionic strength of 20mM-Tris/HCl and pH8.0, the impurities, including the dimer, are bound to the ion-exchanger, whereas the antigen (monomer) is found in the unretarded fraction. Before ion-exchange chromatography, deoxycholate has to be removed by dialysis and to be replaced by the non-ionic detergent Triton X-100, since in deoxycholate most of the antigen is eluted at 100–150mM-Tris/HCl buffer together with accompanying proteins.

Characterization of the pure antigen

The purified Thy-Br_{Hu} antigen displays in SDS/polyacrylamide-gel electrophoresis one single band with an apparent molecular weight of 26000–29000 (Fig. 2). The protein band is rather broad, indicating a microheterogeneity with respect to size, as is often found for heavily glycosylated proteins. Microheterogeneity is also found with regard to charge. After isoelectric focusing (first dimension, Fig. 3) the antigen appears in three regularly spaced spots. The influence of different treatments on the antigen activity of lectin-purified antigen is shown in Table 2.

Dimerization

Although several separation methods were tested, it was not possible to obtain the antigen in a homogeneous single fraction in SDS/polyacrylamide-gel electrophoresis. In fact, it turned out that the antigen could exist in two forms, differing by molecular weight and charge. If crude membrane or purified preparation were subjected to preparative SDS/polyacrylamide-gel electrophoresis in rods, in addition to the 26000–29000-mol.wt. peak a second peak having antigen activity was detected at an apparent mol.wt. of 50000–60000 (Fig. 4). If the 26000–29000-mol.wt. material was isolated by

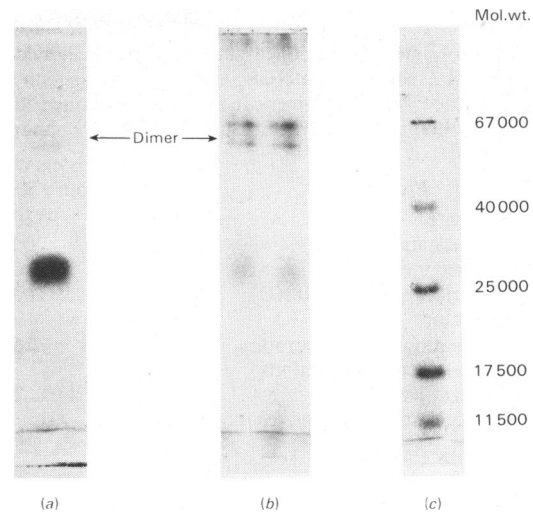


Fig. 2. *SDS/polyacrylamide-gel-electrophoresis of purified Thy-Br_{Hu} antigen*

(*a*) Antigen electrophoresed immediately after purification; (*b*) dimer has formed in a stored sample of pure Thy-Br_{Hu} antigen; (*c*) molecular-weight reference markers. An 11% acrylamide gel was used. Sample in (*a*) was unreduced, and those in (*b*) and (*c*) were reduced. For full experimental details see the text.

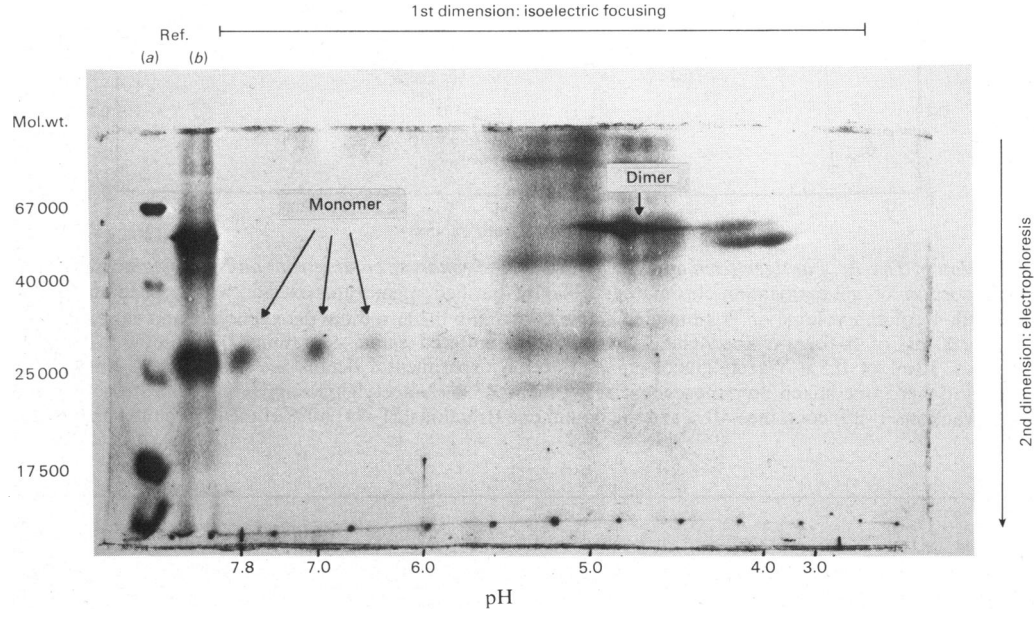


Fig. 3. *Coomassie Blue-stained gel of a two-dimensional separation of Thy-Br_{Hu} antigen as obtained after the wheat-germ-lectin purification step*

For full experimental details see the text. First dimension: isoelectric focusing in polyacrylamide-gel rod containing Triton X-100. Second dimension: SDS/polyacrylamide-gel (11% acrylamide) electrophoresis. Ref.: reference samples [molecular-weight markers (*a*) and lectin-purified material (*b*)] of the second dimension. Some diffuse staining in the pH 5 region is caused by residual tightly bound deoxycholate, which is precipitated at acid pH.

Table 2. *Influence of SDS, reducing agents, carbohydrate reagents and heating on the antigen activity*

Antigen (lectin-purified material in Tris-buffered saline containing 0.2% sodium deoxycholate) was: (*a*) diluted in the same buffer (control); (*b*) diluted (100-fold) in Tris-buffered saline containing 0.1% SDS; (*c*) and (*d*) incubated for 1h with cysteine or dithioerythritol and then dialysed for 24h against deoxycholate buffer; (*e*) heated to 95°C (before dilution); (*f*) and (*g*) treated with periodate or neuraminidase as described in detail in the Materials and Methods section. Antigen activity in the samples was determined by the enzyme-linked immunosorbent assay.

Treatment	Antigen activity (% of control)
(*a*) Antigen in 0.2% deoxycholate (control)	100
(*b*) Antigen in 0.1% SDS	50
(*c*) Antigen + 10mM-cysteine	31
(*d*) Antigen + 5mM-dithioerythritol	10
(*e*) Antigen, 10min at 95°C	78
(*f*) Periodate, 20mM, 6h	22
(*g*) Neuraminidase, 0.04 unit/ml, 24h at 37°C	105

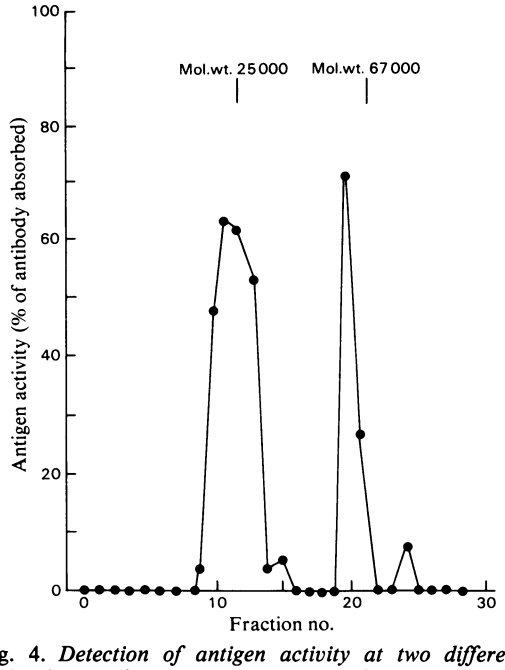


Fig. 4. *Detection of antigen activity at two different molecular-weight ranges, representing the monomer and the dimer, after SDS/polyacrylamide-gel electrophoresis*

Gel-permeation-chromatographically purified membrane extracts were subjected to preparative SDS/polyacrylamide-gel electrophoresis and fractionated, and antigen activity was assayed with the cytotoxicity assay after elution and removal of SDS. For full experimental details see the text.

preparative electrophoresis, concentrated and immediately subjected to a second SDS/polyacrylamide-gel electrophoresis, the antigen was found again in a single band at 26000–29000 mol.wt. On storage the higher-molecular-weight derivative (mol.wt. 56000, unreduced) reappeared in this material, indicating that this fraction is a dimer formed by self-association of the 26000–29000 mol.wt. protein (Fig. 2). The dimer cannot be cleaved by the usual agents known to break non-covalent and disulphide bonds (50mM-dithioerythritol, 1% SDS, 6M-urea, EDTA, 100°C). Reduction, however, splits the 56000-mol.wt. band into two or three bands with slightly differing molecular weights (58000–64000). We suggest that cleavage of intramolecular disulphide bonds leads to an unfolding and therewith to an increase in apparent molecular weight, but that this process is prevented in a part of the molecules by cross-linking. Reduction of the monomer does not lead to a detectable change in apparent molecular weight, but antigen activity is destroyed even by mild reductive agents such as cysteine.

Reconstitution

As was shown by Helenius *et al.* (1978) membrane proteins can be reconstituted by a simple procedure to lipid–protein vesicles with an arrangement of the protein resembling that of the intact membrane and partly connected with an increase in

Table 3. *Influence of solubilization and reconstitution on antigen activity*

(*a*) Crude membrane fraction was incubated for 1h at 37°C in the presence or absence of 2% sodium deoxycholate in 10mM-Tris/HCl buffer, pH8.0. (*b*) Antigen (lectin-purified material) dissolved in Tris-buffered saline containing 0.2% sodium deoxycholate was compared with the same amount of antigen reconstituted to protein–lipid vesicles (see the Materials and Methods section). Values of four different reconstitution experiments, performed under the same conditions, are given. Antigen activity was determined by the enzyme-linked immunosorbent assay.

Material	Antigen activity (%)
(*a*) Crude membrane fraction (1h at 37°C)	100
Membrane, 1h at 37°C in 2% deoxycholate	61
(*b*) Antigen in 0.2% deoxycholate	100
Antigen, reconstituted in phospholipid vesicles	
Expt. 1	2000
Expt. 2	400
Expt. 3	250
Expt. 4	650

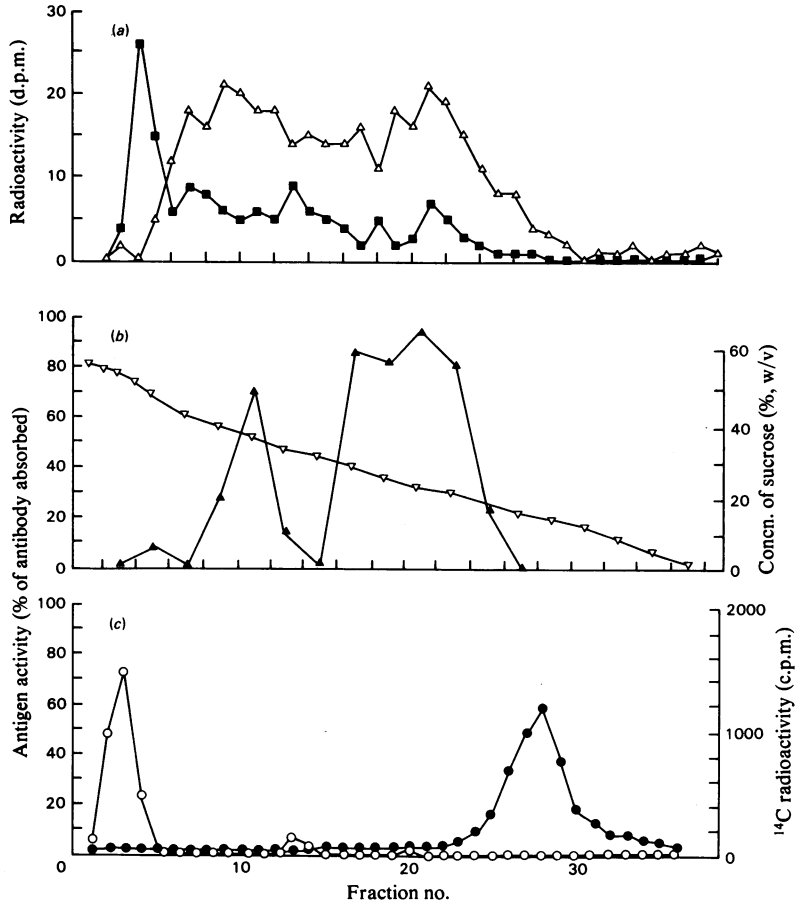


Fig. 5. *Density-gradient profiles of reconstituted phospholipid–Thy-Br$_{Hu}$-antigen vesicles*

Lectin-purified antigen was reconstituted and analysed as described in the text. (*a*) Distribution of radioactivity; ■, ^{125}I-labelled Thy-Br$_{Hu}$ antigen; △, ^{14}C-labelled phospholipid. (*b*) ▲, Antigen activity; ▽, sucrose concentration. (*c*) Controls: ○, antigen activity of lectin-purified antigen alone (lipid traces were removed from the material by ethanol precipitation before dialysis against sucrose); ●, ^{14}C-labelled phospholipid without protein (separate runs).

immunological and biological reactivity. If lectin-purified Thy-Br$_{Hu}$ antigen is reconstituted with egg phosphatidylcholine, almost all antigenic activity is found with reconstituted vesicles having a density between that of pure protein and lipid (Fig. 5). If ^{125}I-labelled Thy-Br$_{Hu}$ antigen is used, some of the ^{125}I label is retained at the start.

This probably does not represent unreconstituted protein but rather free iodine not entirely removed from the protein (see the Materials and Methods section). Two populations of vesicles with different densities are found, which may correspond to classes of vesicles differing in size and structure. The antigen activity of reconstituted material is increased to a variable extent (Table 3). It is assumed that these differences are due to variances in the arrangement of the antigen within the vesicle bilayers, as may occur in different experiments or alternatively to aggregation of the vesicles after reconstitution. It has indeed been reported that by using this method some membrane proteins yield vesicles that rapidly undergo aggregation (Engelhard *et al.*, 1978).

In control experiments phospholipid vesicles without incorporation of antigen did not absorb significant amounts of antibody out of the antiserum.

Furthermore, no antigenic activity was found with vesicles (in a lipid concentration of 0.1 μg–1 mg/ml) prepared from an extract of total lipid from human brain. Presence of a glycolipid with side chains cross-reacting with Thy-Br$_{Hu}$ antigen seems very unlikely therefore.

Discussion

The molecular properties of Thy-Br_{Hu} antigen much resemble those of murine Thy-1 antigen, indicating a close relationship and stability of the structure during phylogenetic development. The molecular weight of 26000–29000 in SDS/polyacrylamide-gel electrophoresis is very similar to those found for Thy-Br antigens from rat brain (24000; Barclay *et al.*, 1975), rat thymocytes (25000–27000; Letarte-Muirhead *et al.*, 1975) and mouse brain (25000; Letarte & Meghji, 1978). By ultracentrifugation a molecular weight of 17500 was established for the Thy-1.1 glycoprotein from rat brain. Thereof 12500 is accounted for by the peptide moiety as revealed by amino acid and carbohydrate analyses (Barclay *et al.*, 1976; Kuchel *et al.*, 1978). Remarkable differences between molecular-weight values as estimated by SDS/polyacrylamide-gel electrophoresis and the more reliable values obtained by ultracentrifugation and amino acid analyses are common to glycoproteins with high carbohydrate content, owing to diminished binding of SDS.

Unexpectedly we observed a dimerization tendency of the solubilized Thy-Br_{Hu} antigen. It may be speculated that Cone & Marchalonis (1974), who reported a molecular weight of 60000 for Thy-1 antigen from mouse thymocytes in SDS/polyacrylamide-gel electrophoresis after immunoprecipitation, in fact detected with their experimental system the dimer of Thy-1 antigen.

As far as we know, a dimerization process leading to a bond that cannot be cleaved by the usual dissociating agents known to break non-covalent bonds and by thiol reagents has not been reported before for membrane proteins. One explanation for self-association could be cross-linking of the protein by tightly bound autoxidizing lipids (Roubal & Tappel, 1966). This may also explain the occurrence of deoxycholate-insoluble precipitates during some purification steps. Hitherto it has not been clear whether the dimerization is a process occurring only under experimental conditions *in vitro* or whether it represents a specific property of the protein correlated to a biological function *in vivo*.

Besides its size, Thy-Br_{Hu} antigen resembles the mouse and rat analogues in some other aspects too. Thus it is a glycoprotein, as revealed by its binding to wheat-germ lectin. Like Thy-1 antigen from rat thymocytes (Letarte-Muirhead *et al.*, 1975), Thy-Br_{Hu} antigen displays a heterogeneity in the carbohydrate moiety, since not all of the antigen is bound by lectin. Additionally it was found that the bound antigen consists of several fractions differing in binding affinity to the lectin.

Microheterogeneity is also found with respect to charge, as demonstrable by isoelectric focusing. The antigen (monomer) appears in three fractions with slightly differing isoelectric points, which may be caused by differences in neuraminic acid content (Fig. 3). In some earlier studies Thy-1 antigen was claimed to be a glycolipid (Vitetta *et al.*, 1973; Esselman & Miller, 1974). Wang *et al.* (1978) found a structure with Thy-1 activity displaying t.l.c. behaviour only little deviating from that of the ganglioside G_{M1}. The discrepancy with the results obtained by other authors, who found the antigenic activity to be associated with the isolated glycoprotein (Trowbridge *et al.*, 1975; Barclay *et al.*, 1976; Arndt *et al.*, 1976), was explained by the suggestion that a carbohydrate structure common to protein and glycolipid could be the antigenic determinant (Wang *et al.*, 1978). The results obtained for the non-species-specific determinant of Thy-Br_{Hu} antigen allow no clear decision whether the determinant is a carbohydrate or a peptide structure. On the one hand antigenic activity was found to be greatly diminished by periodate treatment, but on the other hand antigenic activity is decreased by SDS and almost completely destroyed by reducing agents, which cleave intramolecular disulphide bonds of the peptide chain.

These conflicting results could be interpreted by a model suggesting a complex carbohydrate structure as antigenic site, composed of several carbohydrate chains fixed at different sites of the peptide backbone and held in a specific spatial arrangement by the disulphide-bond-stabilized tertiary structure of the protein.

Practically no antigen activity could be found with lipid. Vesicles prepared from lipid extract from human brain did not absorb significant amounts of antibodies out of the antiserum, and after SDS/polyacrylamide-gel electrophoresis of solubilized material the antigen activity was associated with the protein peak at the molecular weight of 26000–29000 (and 60000 for the dimer). Under these conditions all glycolipid should have been liberated from a possible carrier protein and run in the front. This finding is important, since absence of lipid even after some purification steps in detergent-containing buffers is not self-evident. Even in severalfold-dialysed preparations, purified by gel-permeation chromatography and lectin affinity chromatography, some lipid is present, as became obvious by t.l.c. Also, extractions of membranes with cold acetone, which diminishes antigen activity of the preparation, did not yield lipid-free protein. Bound lipid is probably the reason for strikingly different elution behaviour of the antigen in ion-exchange chromatography. It was found that the antigen pre-purified by gel-permeation chromatography is eluted from a DEAE-Sepharose column in sodium deoxycholate-containing buffer at 0.7M-NaCl and after a further purification by lectin affinity chromatography at 0.15M-NaCl. Though the antigen

content after the different purification steps was assayed routinely, we do not give values for the quantitative yield of antigen along the purification pathway, since values obtained by the assay techniques used apparently did not give true reflection of antigen molecule content. Thus quantitative comparisons between solubilized antigen preparations and particle-bound antigen assayed by using bivalent antibody can lead to wrong results under certain conditions (Reynolds, 1979; Mason & Williams, 1980). Further, the accessibility and conformation of a membrane antigen may be influenced by its molecular environment (lipid matrix or detergent). This is documented by the significantly lowered absorbing capacity of a given amount of antigen after solubilization (Table 3) and by the strong increase of antigen activity after the purified antigen is reconstituted to protein–lipid vesicles. Integration in lipid bilayers is an effective way to arrange purified membrane proteins in a conformation much resembling that presented by native membranes (Helenius *et al.*, 1978). Differences in the affinity even of univalent Fab antibody to Thy-1 antigen either in the membrane or when solubilized were also described by Mason & Williams (1980).

Beyond the relevance for comparative phylogenetic considerations, the purification of Thy-Br_{Hu} antigen is a prerequisite for analysis of the primary structure. Information on the amino acid sequence may also contribute to the hitherto poor knowledge of structural relationships among membrane proteins.

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