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Detection and molecular characterization of the thymus-brain antigen in human brain

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Summary. Anti-human brain sera were found to contain antibodies reacting with determinants present on murine thymocyte plasma membranes. This determinant is borne by the thymocyte-brain antigen of mouse thymocytes.

The non species-specific determinant of the thymocyte-brain system is detectable on the thymocyte-brain antigen of human and mouse brain in comparable amounts. In contrast to these findings, the allogenic Thy-1 and the species-specific determinant of this system were only found on the thymocyte-brain antigen of mouse brain but not on the corresponding antigen of human brain.

The molecular weight, Stokes radius and diffusion coefficient of the thymocyte-brain antigen of human and mouse brain are in good accordance with the data estimated for the corresponding antigen of murine thymocytes.

INTRODUCTION

Antigenic correlations between thymus and brain have been described for different species (Golub 1971; Morris & Williams, 1975a; Reif & Allen, 1964; Thiele, Stark & Keeser, 1972b). However, this phenomenon is less defined in the human (Brown & Greaves, 1974; Greaves & Brown, 1974; Kongshavn, Gold, Shuster, Colquhoun & Fredman, 1974; Takada, Takada, Ito & Minowada, 1974; Thiele, Stark, Keeser & Zimpel, 1973). Preliminary experiments have revealed that human brain and mouse thymocytes have some antigenic structures in common (Golub, 1972; Thiele *et al.*, 1973). The present experiments were designed to investigate further these correlations in serological and biochemical terms.

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MATERIALS AND METHODS

Chemicals

Acetone (p.A. grade), sodium deoxycholate for microbiology, urea p.A. grade and guanidine were purchased from Merck, Darmstadt, Fed, Rep. Germany. Urea and guanidine were further purified by adsorption on charcoal. Nonidet-P40 (polyoxyethylene p-t-octylphenol-NP_{40}) was a gift from Shell A.G., Hamburg.

Reference proteins. Pepsin (twice crystallized), β-lactoglobulin (crystallized, pure, from milk), myoglobulin (purest, from horse) ovalbumin (crystallized five times, from chicken egg), bovine serum IgG (pure) and DNP α-alanine were obtained from Serva, Heidelberg. Bovine serum albumin was purchased from Behringwerke, Marburg. Blue dextran 2000 was obtained from Pharmacia, Uppsala, Sweden.

Column material. Sepharose 6B and cyanogen bromide-activated Sepharose 4B were obtained from Pharmacia; Ultrogel ACA 34 was purchased from LKB, Bromma, Sweden.

Buffers. Phosphate-buffered saline (PBS) (10 mM phosphate buffer pH 7·2, 140 mM NaCl; 0·02% NaN_3) was used in these studies.

Animals and organs

Thymocytes of 6–12-week-old inbred CBA/J/W/Bom mice and outbred Wistar/Han rats were prepared as described previously (Thiele *et al.*, 1972b).

Antisera and complement

Anti-human brain serum (ABS_{Hu}), anti-mouse (CBA) brain serum (ABS_M) and anti-Wistar rat brain serum (ABS_R) were raised in rabbits following the immunization schedule described elsewhere (Thiele *et al.*, 1972b) but without the use of adjuvants. Standard absorptions to remove non-specific antibodies were performed as described (Thiele *et al.*, 1972b). AThy-1.1 was purchased from Searle Laboratories U.K.; AThy-1.2 was kindly provided by Dr Anneliese Schimpl, Würzburg.

The 50% lysis titres for CBA thymocytes were: ABS_{Hu} (1:400); ABS_M (1:700); ABS_R (1:770) and AThy-1.2 (1:500) and for rat thymocytes the 50% lysis titres were: AThy-1.1 (1:140) and ABS_M (1:64).

The globulin-fraction of ABS_M (ABG_M) was prepared by ammonium sulphate precipitation at 50% saturation. Fluorescein-isothiocynate (FITC)-conjugated globulin fractions of goat anti-mouse globulin (FITC/GAMG) and tetra-ethylrhodamine isothiocyanate (TRITC) conjugated goat anti-rabbit globulin (TRITC/GARG) were obtained from Nordic Immunologic Laboratory, Tilburg, The Netherlands.

A commercial guinea-pig serum (Behring Meerschweinchen-Komplement, Behringwerke, Marburg, Fed. Rep. Germany) diluted 1:3, absorbed before use with agar purum (100 mg/3·0 ml complement dilution for 90 min at 0°) served as complement source.

Preparation of brain tissue

Human adult brain was obtained from the Institut for Pathology, University of Hamburg. Ten grams of brain matter were homogenized in 90 ml 0·32 M sucrose at 4° using a Potter Elvejhem homogenizer. The homogenate was filtered through muslin cloth and centrifuged 10 min for 1000 *g*.

The sediment was washed in 0·32 M sucrose and centrifuged in the same way. The combined supernatants were centrifuged for 20 min at 12 000 *g*, the sediment resuspended and dialysed against PBS.

Solubilization

Acetone extraction of human and mouse brain antigens was performed by dropwise addition of 90 ml 90% acetone to 10 ml samples of brain matter (corresponding to 2 g brain, wet weight) at −20°. After 2 h incubation at −20° the preparation was centrifuged at 4000 *g* for 20 min in the cold. The sediment was resuspended in 4 ml PBS and dialysed overnight against this buffer at 4°. Subsequent solubilization in urea-NP_{40} or sodium deoxycholate was performed as described previously (Arndt, Stark, Klein, Müller & Thiele, 1976). Corresponding solubilization experiments were carried out in 6 M guanidine containing 2% NP_{40}.

Analytical chromatography

Determination of mol. wt, Stokes radius and diffusion coefficient ($D_{20,w}$) was performed as described elsewhere in detail (Arndt *et al.*, 1976) by upward-flowing chromatography using: (a) Sepharose 6B Cl (2·5 × 68 cm, flow rate 14 ml/h, 1·75 ml/fraction), equilibrated with 6 M urea–0·2% NP_{40} (v/v), 10 mM Tris buffer (pH 8·0), 1 mM EDTA, 0·02% NaN_3; (b) Sepharose 6B Cl (2·5 × 80 cm, flow rate 32 ml/h, 1·85 ml/fraction), equilibrated with 6 M guanidine 0·2% NP_{40} (v/v), 10 mM Tris buffer (pH 8·0), 1 mM EDTA, 0·02% NaN_3. The proteins used for calibration were reduced with 2-mercaptoethanol and carboxymethylated according to Crestfield (Crestfield, Moore & Stein, 1963); (c) Ultrogel ACA_{34} (2·5 × 65 cm, flow rate 14 ml/h, 1·85 ml/fraction) equilibrated with 10 mM Tris buffer (pH 8·0), 0·2% (w/v) sodium deoxycholate, 0·02% NaN_3.

Affinity chromatography

Affinity chromatography columns were prepared by coupling 100 mg ABG_M to 10 g CNBr-activated Sepharose 4B according to the protocol given by Pharmacia Uppsala/Sweden. The columns (1·5 × 28 cm) were equilibrated with 10 mM phosphate buffer pH 7·2; 100 mM NaCl; 0·2% NP_{40} (column

buffer). Forty-five millilitre samples of the solubilized and chromatographed (Sepharose 6B/6M urea–0·2% NP_{40}) thymocyte-brain antigen (Arndt *et al.*, 1976) (starting material 3 g CBA thymus) were concentrated and dialysed against the column buffer before being applied to the affinity column. The column was first eluted with 100 ml column buffer, followed by 150 ml 0·1 M glycine buffer pH 2·5–0·2% NP_{40}. Both fractions were dialysed first against 6 M urea and then against PBS. After concentration by ultrafiltration (Amicon PM 10 filter) samples were tested for their ability to absorb the cytolytic activity for mouse thymocytes from the ABS_M, ABS_R, ABS_{Hu} and AThy-1·2 sera.

Serological test system

The cytolysis assay system used in these experiments has been described previously in detail (Zimpel, Stark & Thiele, 1973). The estimation of the absorbing capacity of chromatographed antigen fractions was performed as described elsewhere (Arndt *et al.*, 1976). The suitable antiserum dilutions used in the present experiments, i.e. the dilution which lysed approximately 80% of CBA thymocytes as targets were:

$$ABS_M = 1{:}300;$$
$$ABS_{Hu} = 1{:}220.$$

The standard quantitating absorption analyses have been described elsewhere in detail (Thiele, Stark, Keeser & Zimpel, 1974a). The final dilutions used were:

$$ABS_M\ (1{:}64);$$
$$ABS_{Hu}\ (1{:}50).$$

Immunofluorescence-staining procedures

The immunofluorescence technique used in these experiments has been described in detail elsewhere (Thiele & Stark, 1974b). The examinations were performed with the reflected-light-fluorescence technique using a Zeiss Photomicroscope III equipped with a EPI-III RS-condenser. For FITC, blue interference filter KP 500, KP 490, chromatic beam splitter FT 510 and orange filter LP 520 were used. For TRITC, green interference filter combination BP 546, chromatic beam splitter FT 580 and red filter LP 590 were used.

RESULTS

Absorption of ABS_{Hu}, ABS_M and AThy-1 with brain of different species

As has been shown previously, ABS_{Hu}, raised in rabbits is highly cytotoxic to thymocytes of mice and rats although the cytotoxicity to human thymocytes was less pronounced (Thiele *et al.*, 1973). For absorption studies ABS_M and ABS_{Hu} were exhaustively absorbed with mouse, rat and human brain. The remaining cytotoxicity after absorption was estimated for mouse and rat thymocytes as targets as may be seen from Table 1. Absorption of AThy-1·2 and AThy-1·1 with human brain did not reduce cytotoxity to mouse rat thymocytes

Co-capping experiments

Incubation of CBA thymocytes with AThy-1·2 and GAMG/FITC at 0° followed by incubation at 37° and further treatment with ABS_{Hu} and TRITC/

Table 1. Cytotoxicity of absorbed antisera

Antiserum	Target: thymocytes	Per cent cytotoxic antibodies left after absorption with:		
		Mouse brain*	Rat brain*	Human brain*
ABS_M	Mouse	0	~50	~60
	Rat	0	0	0
ABS_{Hu}	Mouse	0	0	0

* The chosen amounts of the different absorbents were found by antecedant experiments to be sufficient for complete, i.e. exhaustive absorptions: 1·0 ml of 1:8 diluted ABS_M+washed (4000 *g*) sediment of 0·5 g (wet weight) homogenized mouse, rat or human brain. 1·0 ml of 1:8 diluted ABS_{Hu}+washed (4000 *g*) sediment of 0·5 g (wet weight) homogenized mouse, rat or human brain.

GARG in the cold led to FITC and TRITC stained caps. Identical results were obtained when the capping procedure was performed with ABS_{Hu} and TRITC/GARG and the counter staining with AThy-1.2 and FITC/GAMG.

Gel filtration of the thymocyte-brain antigen of mouse thymocytes

After solubilization of mouse thymocyte membrane fractions with 6 M urea–2% NP_{40}, the sample was chromatographed on a Sepharose 6B column equilibrated with 6 M urea–0·2% NP_{40}. After extensive dialysis against PBS the single fractions were tested concerning their capacity to absorb cytolytic activity to mouse thymocytes from ABS_M and ABS_{Hu} sera. It was found that the solubilized fractions 75–95 absorbed the cytotoxic activity of both sera, with similar absorption curves (Fig. 1).

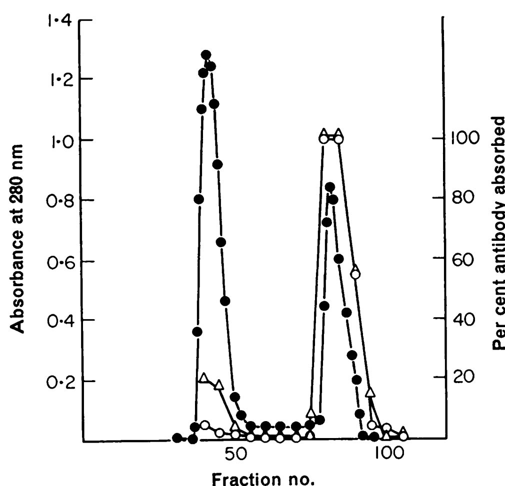

Figure 1. Chromatography of the thymocyte-brain antigen from CBA-thymocytes on Sepharose 6B-Cl in the presence at urea-NP_{40}. Absorbance at 280nm, (●—●); per cent antibody absorbed, (○—○) ABS_M; ABSnm, (△—△).

Affinity chromatography

The thymocyte-brain antigen fraction obtained after gel-filtration was subjected to affinity chromatography with ABG_M linked to Sepharose 4B. The antigen fractions eluted by 0·1 M glycine buffer, pH 2·5 were able to absorb the cytotoxicity of ABS_M (1:120); ABS_R (1:80) and ABS_{Hu} (1:50).

Solubilization and chromatography of the thymocyte-brain antigen of human brain

Ninety per cent acetone extraction of the 12,000 *g* sediment of brain matter after differential centrifugation yielded a 30–40% recovery of the original antigenic activity. The samples with partially reduced lipid content were solubilized with 2% Na deoxycholate; 6 M urea–2% NP_{40}; 6 M guanidine-2% NP_{40}. These procedures caused no further loss of the absorbing capacity of this material as tested with ABS_{Hu} with mouse thymocytes as targets. After chromatography on ACA_{34} in Na deoxycholate, the majority of the antigenic activity was eluted with fractions 90–105 (Fig. 2). A similar result was obtained after solubilization in 6 M urea–2% NP_{40} and subsequent chromatography on Sepharose 6B-Cl: The solubilized antigen eluted in a single peak (fractions 95–110).

In contrast to these findings, after treatment of the antigen with reduced lipid content with 6 M guanidine 2% NP_{40} and chromatography on Sepharose 6B, the bulk of the antigenicity eluted near the void volume.

Analytical studies on LKB ACA_{34} in 0·2% Na deoxycholate yielded an elution volume corresponding to a molecular weight of 55,000 and a Stokes radius of 32·7 Å (Table 2). In 6 M urea–0·2% NP_{40} on Sepharose 6B the mol. wt was calculated to be 35,800 (Table 2).

Solubilization and chromatography of the thymocyte-brain antigen of mouse brain

Corresponding extraction and solubilization procedures and analytical chromatography experiments were performed with the thymocyte-brain antigen of mouse brain. After chromatography on ACA_{34} in Na deoxycholate the solubilized antigen as tested with ABS_M and ABS_{Hu} using mouse thymocytes as targets eluted in one peak (Fig. 3). As may be seen from Table 2 the molecular parameters of this antigen are in good accordance to the data of the thymocyte-brain antigen from human brain.

DISCUSSION

Thymocytes and brain tissue of mice and rats have some particular antigenic structures in common (Golub, 1971; Morris *et al.*, 1975a; Reif *et al.*, 1964; Thiele *et al.*, 1972b). There is now good

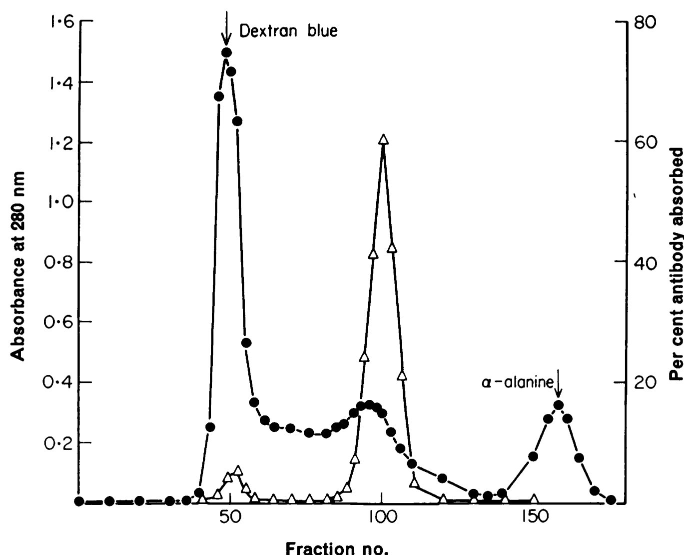

Figure 2. Chromatography of the thymocyte-brain antigen from human brain on LKB ACA_{34} in the presence of sodium deoxycholate. Absorbance at 280nm (●—●); per cent antibody absorbed: (△—△) ABS_{Hu}.

evidence that this antigenic system consists of at least three different antigenic determinants, which are actually borne by the same molecule (Arndt *et al.*, 1976; Morris, Letarte-Muirhead & Williams, 1975b; Thiele *et al.*, 1974b). A similar correlation between thymocytes and brain has been described for the chicken (Feiglová, Pichlikova & Nouza, 1972) although this finding has been challenged (Wick & Schauenstein, 1974). The observation of common antigenic structures of thymocytes and central nervous tissue raises the question as to whether this phenomenon reflects a general biological principle (Thiele, Stark & Földi, 1972a; Thiele *et al.*, 1972b). However, similar experiments performed by several groups with heterologous anti-human brain sera (ABS_{Hu}) using immunofluore-

Table 2. Molecular properties of the thymocyte–brain antigen of human brain

		Thymocyte–brain antigen		
Property	Method	Mouse thymocytes (Arndt *et al.*, 1976)	Mouse brain	Human brain
Mol. wt	Gel filtration LKB-ACA_{34} 0·2% Na-deoxycholate	54,000	54,000	55,000
Mol. wt	Gel filtration Sepharose-6B Cl 6M Urea-0·2% NP_{40}	35,000	34,000	35,800
Stokes radius Å	Gel filtration LKB ACA_{34} 0·2% Na-deoxycholate	31·8	31·5	32·7
$D_{20,w}$ $cm^2\ sec^{-1}$	Gel filtration LKB-ACA_{34} 0·2% Na-deoxycholate	$6{\cdot}28\ 10^{-7}$	$6{\cdot}34\ 10^{-7}$	$6{\cdot}11\ 10^{-7}$

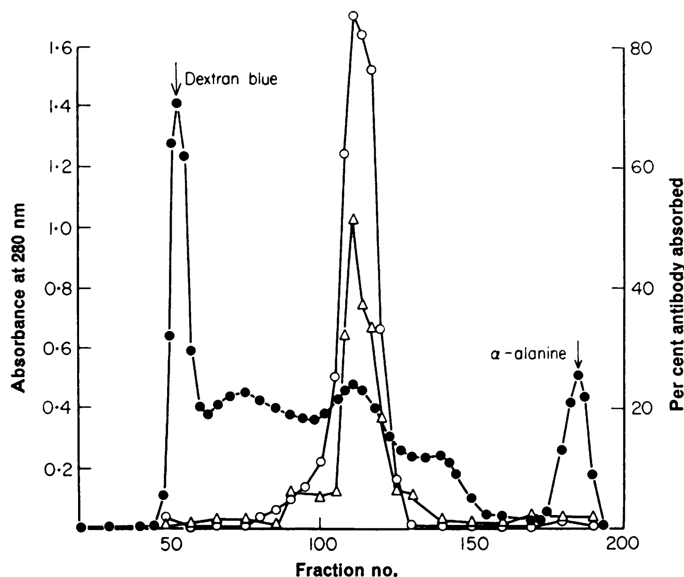

Figure 3. Chromatography of the thymocyte-brain antigen from CBA-mouse brain on LKB ACA_{34} in the presence of sodium deoxycholate. Absorbance at 280 nm (●—●); % antibody absorbed: (○—○) ABS_M; (△—△) ABS_{Hu}.

scence and cytolysis assay are equivocal (Bonet & Toben, 1976; Golub, 1972; Takada *et al.*, 1974; Thiele *et al.*, 1973).

Interestingly, ABS_{Hu} proved to be highly cytotoxic to thymocytes of mice (Doria & Baroni, 1975); Thiele *et al.*, 1973) and rats (Thiele *et al.*, 1973). The co-capping experiments presented here as well as the finding that the solubilized thymocyte-brain antigen prepared from mouse thymocyte membranes after purification by affinity chromatography removed the cytotoxic activity on mouse thymocytes not only out of ABS_M but also of ABS_{Hu}, provide strong evidence that the determinant detected with ABS_{Hu} on mouse thymocytes is part of this antigen molecule.

The observation that the cytotoxic activity of ABS_{Hu} on mouse thymocytes was completely removed by rat brain and the finding that absorption of ABS_M with human brain abolished the cytotoxicity to rat thymocytes but left most of the antibodies reacting with mouse thymocytes, suggests that the non-species specific determinant of the thymocyte-brain antigen detected first in mice and rats (Golub, 1971; Morris *et al.*, 1975b; Thiele *et al.*, 1972b) is also expressed on human brain.

Solubilization experiments performed with human brain led to isolation of a fraction reacting with ABS_M as well as with ABS_{Hu}. This fraction possessed physico-chemical characteristics similar to that of the thymocyte-brain antigen isolated from mouse (Arndt *et al.*, 1976) and rat thymocytes and brain (Barclay. Letarte-Muirhead & Williams, 1975; Letarte-Muirhead, Barclay & Williams, 1975).

The finding that the Thy-1 antigenic determinants are not restricted to mouse strains but are also expressed on rat thymocytes and brain (Douglas, 1972; Micheel, Pasternak & Steuden, 1973) implies phylogenetically its pre-species appearence. The detection of the non-species specific determinant in association with a molecular structure of the human brain resembling that of the murine thymocyte-brain antigen raises the question as to whether the Thy-1 determinant in one of its known allo-antigenic forms is also expressed on the human brain. However, the fact that human brain was completely unable to remove the corresponding antibodies from AThy-1·1 and AThy-1·2 sera makes their expression on human brain unlikely, a conclusion also drawn by Doria *et al.* (Doria *et al.*, 1975).

Recently we tested a rabbit ABS_{Hu} serum which, in contrast to batches of ABS_{Hu} sera used previously in our laboratory (Thiele *et al.*, 1973), had an unequivocal cytotoxicity to human thymocytes even after appropriate absorption with human liver and erythrocytes. In preliminary experiments it was found

that this cytotoxic activity could be removed by absorption with the above mentioned fraction solubilized from human brain. Attempts to solubilize the corresponding antigenic structure from human thymocytes and lymphocytes are now in progress.

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ADDENDUM

After this paper had been submitted for publication we successfully solubilized a structure from human thymus, which is characterized by corresponding physico-chemical data and bears the non-species-specific determinant.

REFERENCES

ARNDT R., STARK R., KLEIN P., MÜLLER A. & THIELE H.-G. (1976) Solubilization and molecular characterization of membrane-bound antigens shared by thymocytes and brain. *Europ. J. Immunol.* **6**, 333.

BARCLAY A.N., LETARTE-MUIRHEAD M. & WILLIAMS A.F. (1975) Purification of the Thy-1 molecule from rat brain. *Biochem. J.* **151**, 699.

BROWN G. & GREAVES M.F. (1974) Cell surface markers for human T and B lymphocytes. *Europ. J. Immunol.* **4**, 302.

CRESTFIELD A.M., MOORE S. & STEIN W.H., (1963) The preparation and enzymatic hydrolysis of reduced and S-carboxymethylated proteins. *J. biol. Chem.* **238**, 622.

BONET J.-C. & TOBEN H., (1976) Characterization of a subpopulation of human T lymphocytes reactive with an heteroantiserum to human brain. *J. Immunol.* **116**, 1041.

DORIA G. & BARONI C.D. (1975) Cross-reactivity between human thymus and mouse lymphoid tissues, as revealed by rabbit antiserum against human brain. *Proc. Soc. exp. Biol. (N.Y.)* **148**, 1126.

DOUGLAS T.C. (1972) Occurrence of a theta-like antigen in rats. *J. exp. Med.* **356**, 1054.

FEIGLOVA E., PICHLIKOVÁ L. & NOUZA K. (1972) Anti-lymphocyte activity of rabbit anti-chicken antibrain sera. *Folia Biologica*, **18**, 25.

GOLUB E.S. (1971) Brain-associated θ antigen: reactivity of rabbit anti-mouse brain with mouse lymphoid cells. *Cell. Immunol.* **2**, 353.

GOLUB E.S. (1972) The distribution of brain-associated θ antigen cross-reactive with mouse in the brain of other species. *J. Immunol.* **109**, 168.

GREAVES M.F. & BROWN G. (1974) Antigenic correlations between brain and thymus. *Lancet*, **i**, 455.

KONGSHAVN P.A.L., GOLD P., SHUSTER J., COLQUHOUN B. & FREDMAN S.O. (1974) Ability of anti-brain hetero-antisera to distinguish thymus-derived lymphocytes in various species. *Clin. Immunol. & Immunopath.* **3**, 1.

LETARTE-MUIRHEAD M., BARCLAY A.N. & WILLIAMS A.F., (1975) Purification of the Thy-1 molecule, a major cell-surface glycoprotein of rat thymocytes. *Biochem. J.* **151**, 685.

MICHEEL B., PASTERNAK G. & STEUDEN J., (1973) Demonstration of θ-AKR differentiation antigen in rat tissue by mouse alloantiserum. *Nature: New Biology*, **241**, 221.

MORRIS R.J. & WILLIAMS A.F., (1975a) Antigens on mouse and rat lymphocytes recognized by rabbit antiserum against rat brain: the quantitative analysis of a xenogeneic antiserum. *Europ. J. Immunol.* **5**, 274.

MORRIS R.J., LETARTE-MUIRHEAD M. & WILLIAMS A.F. (1975b) Analysis in desoxycholate of three antigenic specificities associated with the rat Thy-1 molecule. *Europ. J. Immunol.* **5**, 282.

REIF A.E. & ALLEN I.M. (1964) The AKR thymic antigen and its distribution in leukemias and nervous tissues. *J. exp. Med.* **120**, 413.

TAKADA A., TAKADA Y., ITO U. & MINOWADA J. (1974) Shared antigenic determinants between human brain and human T-cell line. *Clin. exp. Immunol.* **18**, 491.

THIELE H.-G., STARK R. & FÖLDI M. (1972a) Antigenic correlations between brain tissue and thymocytes in rats and mice. *Naturwiss.* **59**, 221.

THIELE H.-G., STARK R. & KEESER D. (1972b) Antigenic correlations between brain and thymus. I. Common antigenic structures in rat and mouse brain tissue and thymocytes. *Europ. J. Immunol.* **2**, 424.

THIELE H.-G., STARK R., KEESER D. & ZIMPEL H. (1973) Lack of antigenic correlations between brain and thymus. *Lancet*, **ii**, 1447.

THIELE H.-G., STARK R., KEESER D. & ZIMPEL H. (1974a) Antigenic correlations between brain and thymus. II. Further evidence for the organ specifity of the thymus-brain system in mice. *Z. Immunol.-Forsch.* **146**, 346.

THIELE H.-G. & STARK R. (1974b) Common antibody-induced redistribution on thymocytes of strain-, species and non-species-specific antigenic determinants shared by brain and thymocytes of mice. *Immunology*, **27**, 807.

WICK G. & SCHAUENSTEIN K. (1974) Lack of antigenic correlation between chicken brain and thymus. *Lancet*, **ii**, 214.

ZIMPEL H., STARK R. & THIELE H.-G. (1973) An approach to avoiding errors in cytotoxic testing. *J. Immunol. Meth.* **3**, 411.