Preliminary Characterization of Two Thymus-Dependent Xenoantigens from Mouse Lymphocytes

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Preliminary characterization of two mouse thymus-dependent (T) lymphocyte xenoantigens, T25 and T200, which are selectively labelled by lactoperoxidase-catalysed iodination of T-cells, is described. Both molecules are membrane-bound glycoproteins. Fractionation of membrane vesicles prepared from BW5147 lymphoma cells by sedimentation through sucrose density gradients show that antigens T25 and T200 are in fractions enriched with plasma membrane. Moreover antigen T200 is partially degraded when viable cells are treated briefly with low concentrations of trypsin. Both molecules are efficiently solubilized in buffers containing sodium deoxycholate or Nonidet P-40, as measured by failure to sediment at 100000g for 60 min. However, gel filtration on Sepharose 6B showed the presence of aggregated material in Nonidet P-40 extracts which was not found in deoxycholate-solubilized membranes. After solubilization in detergent, antigens T25 and T200 bind to, and may be specifically eluted from, columns of pea lectin–Sepharose or concanavalin A–Sepharose. Both molecules are heterogeneous when examined by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. As judged by its binding to columns of pea lectin, at least part of the heterogeneity of mouse thymocyte antigen T25 resides in its carbohydrate moiety.

The cell surface is believed to be the site of many important functions, including cell recognition and the regulation of cell growth (Edelman, 1976; Holley, 1975). Moreover, tissue-specific surface molecules probably play a role in the differentiated functions of that tissue. With the possible exception of the erythrocyte, the surface molecules of mammalian cells have not been extensively characterized. Until recently, most progress in the study of mammalian cell surfaces has been the result of immunogenetic analysis of differentiation antigens (Boyse & Old, 1969). Because of the availability of monospecific alloantisera and for other technical reasons, the surface antigens of mouse lymphoid cells have been more extensively characterized by serological methods than any other differentiated mammalian cells. For this reason a molecular study of the surface antigens of mouse lymphoid cells is not only of intrinsic interest, but also provides a favourable model with which to develop methods to attack more difficult structural questions about the surface molecules of normal and malignant cells.

We have been studying the surface molecules of mouse lymphoid cells by using xenoantisera against whole cells and lactoperoxidase-catalysed iodination to label selectively surface molecules. We detected two labelled species (Trowbridge *et al.*, 1975a,b) which, after reduction with 2-mercaptoethanol,

migrate on SDS*/polyacrylamide-gel electrophoresis as molecules with approximate apparent mol.wts. of 200000 (antigen T200) and 25000 (antigen T25). The immunological properties of these molecules have been investigated; antigen T25 carries Thy-1 alloantigenic determinants and antigen T200 is a lymphocyte-specific antigen (Trowbridge *et al.*, 1975b; Trowbridge & Hyman, 1975; Trowbridge & Mazauskas, 1976).

Selective labelling of these molecules by lactoperoxidase-catalysed iodination and the fact that they are the major species recognized by antisera raised against thymus-derived lymphocytes enable these molecules to be defined by their electrophoretic mobility on polyacrylamide gels and by immunological criteria. Consequently, some properties of these molecules, before their isolation in amounts necessary for classical biochemical characterization, can be determined by using radioactively labelled material. One important advantage of this is that sufficient information to select methods of purification can be obtained with material from relatively small numbers of cells.

Here we show that antigens T200 and T25 are membrane-bound glycoproteins which may be efficiently extracted from cell membranes by deter-

* Abbreviations: SDS, sodium dodecyl sulphate.

gent and subsequently fractionated by gel filtration and affinity chromatography on lectin affinity columns.

Experimental

Cells

Thymocytes were prepared from Balb/c mice, bred at The Salk Institute, by teasing thymus glands apart in balanced-salt solution, removing erythrocytes by treatment with Tris-buffered iso-osmotic NH_4Cl and isolating viable cells on gradients of bovine serum albumin (Trowbridge *et al.*, 1975a). BW5147 lymphoma cells were grown in the modified Eagle's medium of Vogt & Dulbecco (1963) supplemented with 10% (v/v) foetal calf serum and 2mM-glutamine.

Methods

Radioactive labelling. Cells were labelled by the glucose oxidase modification of the lactoperoxidase technique (Hubbard & Cohn, 1972) as described earlier (Trowbridge *et al.*, 1975a; Trowbridge & Hyman, 1975).

BW5147 cells were metabolically labelled with 50 μCi of D-[2- ^3H]mannose/ml (sp. radioactivity 2 Ci/mmol; Amersham/Searle, Arlington Heights, IL 60005, U.S.A.), 50 μCi of D-[G- ^3H]galactose/ml (sp. radioactivity 1.6 Ci/mmol) or 50 μCi of D-[6- ^3H]glucosamine/ml (sp. radioactivity 7 Ci/mmol) for 16h at a cell concentration of 1×10^6 cells/ml of Vogt & Dulbecco's (1963) modified Eagle's medium containing 370mg of glucose/litre and supplemented with 2mM-glutamine, 1mM-pyruvate, $1 \times$ Eagle's non-essential amino acids and 10% (v/v) foetal calf serum. The foetal calf serum was passed through a column of Sephadex G-50 to remove low-molecular-weight components (Piez *et al.*, 1960).

The chemical nature of the species into which the radioactivity from the sugars was incorporated was determined by hydrolysing cell proteins for 90min in 2M-trifluoroacetic acid. Trifluoroacetic acid was removed by drying *in vacuo*, the dried hydrolysates were dissolved in 0.1M-HCl, samples applied to Whatman's no. 1 paper, and descending chromatography was carried out for 40h with butan-1-ol/pyridine/water (6:4:3, by vol.) as solvent. More than 90% of D-[2- ^3H]mannose, 85% of D-[6- ^3H]glucosamine and 80% of D-[G- ^3H]galactose was incorporated into glycoproteins as the species originally added to the cells.

Cells were also labelled with 100 μCi of L-[4,5- ^3H]leucine (60 Ci/mmol, New England Nuclear, Boston, MA 02118, U.S.A.) for 4h at 1×10^6 cells/ml in leucine-free Vogt & Dulbecco's (1963) modified Eagle's medium.

Subcellular fractionation. Homogenization of cells by nitrogen cavitation, sucrose-density-gradient

centrifugation and measurement of enzyme activities were essentially as described by Watson *et al.* (1976). Enzyme activities were measured in gradients containing postnuclear supernatants of unlabelled cells run in parallel with gradients loaded with iodinated material.

Solubilization of membranes in detergent. Iodinated cells (4×10^7 cells) were homogenized by nitrogen cavitation, and nuclei and unbroken cells removed as described above. A crude membrane pellet was prepared by centrifuging the postnuclear supernatant at 100000g for 60min at 4°C (42000 rev./min, Beckman type 50 fixed-angle rotor). Membrane pellet from 10^7 cells was resuspended in 2ml of 0.01M-Tris/HCl buffer, pH 8.0, which, in some cases, contained either 1% (w/v) sodium deoxycholate or 1% (v/v) Nonidet P-40. The samples were incubated at 4°C for 60min, centrifuged again at 100000g for 60min and total and trichloroacetic acid-insoluble radioactivity that remained in the supernatant was determined by counting in a Nuclear-Chicago γ -radiation counter. The solubilization of antigens T25 and T200 was measured by separating the iodinated proteins by SDS/polyacrylamide-gel electrophoresis as described below and locating the position of antigens T25 and T200 in the dried gel by radioautography. Areas of the gel containing the two proteins were cut out and their radioactivity was measured as described above. Antigens T25 and T200 originally present in the membrane were similarly measured.

Gel filtration. A downward-flowing column (1.5cm $\times$ 110cm) of Sepharose 6B was equilibrated and eluted with 0.01M-Tris/HCl buffer, pH 8.0, containing 0.2% NaN_3 and either 1% sodium deoxycholate or 1% Nonidet P-40 and 0.15M-NaCl. The column was eluted at a flow rate of 3.2ml/h in the cold (6°C), and 1.25ml fractions were collected. Solubilized membrane, equivalent to 1×10^7 iodinated thymocytes, was applied to the column in 1.2ml of the Tris/HCl buffer containing the appropriate detergent and 5mg each of ovalbumin (Sigma, St. Louis, MO 63178, U.S.A.) and rabbit immunoglobulin G (Miles Laboratories, Elkhart, IN 46514, U.S.A.), 3mg of horse heart cytochrome *c* (Calbiochem, San Diego, CA 92037, U.S.A.) and 2mg of ferritin (6 $\times$ crystallized, Miles Laboratories). The total radioactivity in each eluted fraction was determined, and the A_{280} and A_{412} were measured. Selected fractions were then mixed with an equal volume of ice-cold 20% (w/v) trichloroacetic acid and the radioactivity was measured as described above in the insoluble pellet that was obtained after centrifugation at 100g for 30min at 4°C. The precipitates were washed twice at room temperature (21°C) with acetone and the radioactivity was measured again. Then the precipitates were dissolved in 0.1ml of electrophoresis sample buffer, and

analysed by SDS/polyacrylamide-gel electrophoresis to determine the radioactivity associated with antigens T200 and T25.

For the marker proteins, Stokes radius was plotted against the cube root of the partition coefficient (Siegel & Monty, 1966). A linear relationship was obtained and from this the Stokes radii for antigens T25 and T200 were calculated from the experimentally determined partition coefficients for the antigens.

Affinity chromatography on lectin columns. Pea lectin (Trowbridge, 1974) and concanavalin A (Miles Laboratories, 3 $\times$ crystallized) were coupled to Sepharose 4B that had been activated with 150 mg of CNBr/ml of washed beads (Cuatrecasas, 1970). The coupling reaction was for 18 h at 4°C in 0.1 M-NaHCO₃ buffer, pH 8.5, containing 50 mM-methyl α -D-glucoside, 0.5 M-NaCl and 10 mg of protein/ml of activated beads. After 18 h, 0.1 ml of 2-amino-ethanol was added per 100 ml of reaction mixture and the reaction continued for another 60 min. The coupled beads were stored at 4°C in 0.02% NaN₃. Lectin columns (6.0 cm $\times$ 0.5 cm) were washed just before use with 0.5 M-NaCl and then 0.15 M-NaCl/0.02% NaN₃/0.01 M-Tris/HCl buffer (pH 8.0) containing 0.5% Nonidet P-40 and 1 M-methyl α -D-mannoside. In most experiments, Nonidet P-40 lysates of iodinated cells were prepared as for immunoprecipitation studies and solubilized proteins from 2 $\times$ 10⁷ cells applied to each column equilibrated with the Tris/HCl buffer containing 0.5% Nonidet P-40. The column was washed until no further radioactivity was eluted and then the above buffer containing 0.5% Nonidet P-40 and 1 M-methyl α -D-mannoside was used to elute the column. Fractions containing unbound and specifically eluted radioactive proteins were separately pooled and analysed by polyacrylamide-gel electrophoresis in the presence of SDS.

Immunological procedures and polyacrylamide-gel electrophoresis. Antisera, immunoprecipitation procedures and electrophoresis on 7.5–15% linear gradients of polyacrylamide in the presence of SDS have been described (Trowbridge *et al.*, 1975a; Trowbridge & Mazauskas, 1976). Species labelled with ³H were detected by fluorography (Laskey & Mills, 1975). Samples were concentrated before electrophoresis by precipitation with ice-cold 10% (w/v) trichloroacetic acid. Precipitates thus obtained were then washed with acetone at room temperature. From measurement of total radioactivity and examination of labelled species in samples directly applied to the gel, almost quantitative recovery (more than 90%) of all radioactive species resolved on the gels was obtained after these procedures.

Identification of the iodinated species. Identification of the chemical nature of the ¹²⁵I incorporated into antigen T25 and T200 was determined by the

method of Izzo *et al.* (1972), as described by Hubbard & Cohn (1972) by using Pronase instead of crude trypsin for proteolysis.

Proteolysis experiments. Pronase (Calbiochem: predigested by incubation at 37°C for 2 h in 0.01 M-Tris/HCl buffer, pH 8.0, containing 1 mM-CaCl₂), trypsin (Worthington Biochemicals, Freehold, NJ 07728, U.S.A.; 1-chloro-4-phenyl-3-L-tosyl-*p*-sulphonamidobutan-2-one-treated) and chymotrypsin (Sigma, 3 $\times$ crystallized) were used.

Three types of experiments were performed. Firstly, BW5147 cells (2 $\times$ 10⁷ cells/ml) were treated with 20 μ g of trypsin/ml at 23°C for various times in 1.0 ml of 0.15 M-NaCl/0.01 M-sodium phosphate buffer, pH 7.2, just before iodination of the cells by the lactoperoxidase technique. The reaction was terminated by the addition of 2 M-di-isopropyl phosphorofluoridate in anhydrous propan-2-ol to a final concentration of 0.01 M. Two control incubations were performed; in one, di-isopropyl phosphorofluoridate was added just before trypsin; in the other, cells were incubated for 20 min without the addition of trypsin. Immunoprecipitates were prepared from some samples as described above.

Secondly, samples (0.05 ml) of Nonidet P-40 lysates of iodinated thymocytes were incubated with 50 μ g of trypsin, chymotrypsin or Pronase for 4 h at 37°C. The reaction was terminated by the addition of 0.2 ml of 0.15 M-NaCl/0.01 M-sodium phosphate buffer pH 7.2, containing 0.2% Nonidet P-40, 500 Kallikrein inactivator units of Trasylol (Calbiochem) and 0.02 M-di-isopropyl phosphorofluoridate and then immunoprecipitates were prepared as described below.

Lastly, immunoprecipitates were prepared from Nonidet P-40 lysates of iodinated thymocytes with rabbit anti-(mouse thymocyte) serum and treated with 10% (w/v) trichloroacetic acid followed by washing with acetone. The denatured precipitates were incubated with 50 μ g of each proteinase for 18 h at 37°C in electrophoresis sample buffer (Maizel, 1971) (0.1 ml) from which 2-mercaptoethanol and SDS were omitted. The reaction was terminated by boiling the samples after the addition of 1 μ l of 2-mercaptoethanol and 10 μ l of 10% (w/v) SDS.

Results

Subcellular distribution of antigens T200 and T25

Since antigens T200 and T25 were labelled by lactoperoxidase-catalysed iodination of cells, it was probable that both molecules were cell-surface components. However, because a small proportion of damaged cells (usually less than 5% of the total) are always present, some intracellular components could also be labelled. For this reason, independent evidence was sought to define the subcellular distribution of antigens T200 and T25.

Firstly, we investigated the subcellular distribution of antigens T200 and T25 by fractionation of postnuclear supernatants from iodinated BW5147 cells on sucrose gradients. After cell disruption by nitrogen cavitation, followed by removal of nuclei and unbroken cells, about 70% of the trichloroacetic acid-insoluble radioactivity was recovered in the postnuclear supernatant. A similar recovery of antigens T200 and T25 in the postnuclear supernatant

Fig. 1. Distribution of labelled species from iodinated BW5147 cells in sucrose-density-gradient fractions

The postnuclear supernatant from unlabelled or iodinated BW5147 cells was fractionated by sucrose-density-gradient centrifugation as described in the Experimental section. Shown are sucrose density (—), total protein (○), ^{125}I -labelled protein (□), 5'-nucleotidase activity expressed as pmol/h per ml of gradient fraction (■) in each gradient fraction. Also shown is the distribution of antigen T25 (open columns) and antigen T200 (closed columns) in pooled gradient fractions expressed as the radioactivity in gel slices containing each labelled species after polyacrylamide-gel electrophoresis. The same proportion of each pooled gradient fraction was loaded on the gel.

was estimated from an analysis of individual labelled species by SDS/polyacrylamide-gel electrophoresis. The results of sucrose-density-gradient fractionation of the postnuclear supernatant are shown in Fig. 1. In contrast with free ^{125}I , which was found predominantly at the top of the gradient, most of the trichloroacetic acid-insoluble radioactivity was distributed between two peaks of membrane fragments. Examination of the individual iodinated proteins by gel electrophoresis of pooled fractions from the gradients showed that both of the membrane peaks contained all detectable iodinated species including antigens T25 and T200. The distribution of enzymes with known subcellular locations in the gradient fractions was similar to that reported for splenic lymphocytes (see Figs. 5 and 6 of Watson *et al.*, 1976), showing that mitochondria and intact lysosomes were sedimented in the membrane fraction with a peak at 40–42% (w/w) sucrose. As Fig. 1 shows, the plasma-membrane marker enzyme 5'-nucleotidase (EC 3.1.3.5) was found in both membrane peaks and its distribution was quantitatively similar to that of the total iodinated proteins and antigens T200 and T25 individually.

Secondly, we determined whether antigen T200 or T25, both known to be glycoproteins (see below), were degraded by trypsin treatment of viable cells. Under the conditions used, cell viability was greater than 95%, as judged by Trypan Blue exclusion, and no degradation of the major cell proteins was detected by inspection of gels stained with Coomassie Blue. Nevertheless, a decrease in antigen T200 and the appearance of a new radioactive band (band 1, apparent mol.wt. 100000) was seen on the radioautograph of iodinated cell proteins as early as 1 min after the addition of trypsin (Plate 1a). This is shown more clearly on the radioautograph of precipitates prepared from the cells with rabbit anti-thymocyte serum (Plate 1b). We conclude that band 1 is derived from antigen T200, since the latter molecule is the only labelled species with a higher molecular weight than band 1 that is precipitated by this serum (see also Plate 2). Although incubation of the cells with higher concentrations of trypsin completely degraded antigen T200, significant losses of viable cells occurred under these conditions. Partial loss of another iodinated species (band 2) and the appearance of a second degradation product (band 3) was seen, but there was no detectable degradation of antigen T25.

Solubilization of antigens T200 and T25

Conditions were determined to solubilize antigens T25 and T200 from the membrane. On the basis of the experience of others (Helenius & Simons, 1975; Allan & Crumpton, 1971; Letarte-Muirhead *et al.*, 1975) extraction with the non-ionic detergent Nonidet P-40 and the weakly ionic detergent

EXPLANATION OF PLATE I

Effects of trypsin treatment on the iodinated proteins of BW5147 cells

Radioautographs of the total iodinated proteins (a), and iodinated proteins precipitated by rabbit anti-(mouse thymocyte) serum (b) from labelled BW5147 cells treated for the indicated time (min) with trypsin as detailed in the Experimental section. The gel tracks marked C are of proteins from control cells that were incubated for 20min in the absence of trypsin. Equal amounts of cell protein were loaded on each gel track.

EXPLANATION OF PLATE 2

Proteolysis of antigens T200 and T25

The radioautograph is of iodinated proteins precipitated by rabbit anti-thymocyte serum from Nonidet P-40 lysates of iodinated thymocytes untreated (*a*) or treated with Pronase (*b*), trypsin (*c*), chymotrypsin (*d*), or with a mixture of trypsin and chymotrypsin (*e*) before immunoprecipitation. Equal amounts of precipitates were loaded on each gel track. Additional experimental details are given in the Experimental section.

EXPLANATION OF PLATE 3

Affinity chromatography of iodinated membrane proteins from BW5147 cells and thymocytes on concanavalin A-Sepharose and pea lectin-Sepharose

Iodinated proteins from BW5147 cells (a); unbound (A) and bound fractions (B) from a pea-lectin column. Unbound (C) and bound fractions (D) from a concanavalin A column, and an unfractionated sample (E). Iodinated proteins from thymocytes (b); unbound (F) and bound (G) fractions from a pea-lectin column. Unbound (H) and bound fractions (I) from a concanavalin A column and an unfractionated sample (J). Fractions were concentrated by precipitation with trichloroacetic acid as described in the Experimental section, allowance being made in the final volume of the chromatographed fractions for incomplete recovery of radioactivity. Equal volumes of samples were then loaded on each gel track.

sodium deoxycholate was tested. Between 75 and 90% of the iodinated proteins in a crude membrane pellet from either BW5147 cells or thymocytes remained in the supernatant after resuspension in buffer, containing either detergent, and centrifugation at 100000g for 60 min. Less than 5% of the labelled proteins remained in the supernatant after centrifugation in buffer alone. Recovery of antigens T200 and T25 in the supernatant of detergent solutions was almost quantitative, as judged by gel electrophoresis of the original pellet and detergent extracts.

Solubilization was also tested by chromatography of the detergent extracts of the iodinated proteins on Sepharose 6B. All the iodinated proteins solubilized and eluted in deoxycholate were included in the Sepharose 6B column (Fig. 2), and recovery of applied labelled protein was greater than 90%. Antigens T200 and T25 were well-separated as discrete peaks. The Stokes radius of the antigen T25-detergent complex was determined as 2.9 nm (29 Å) in two separate experiments; that of antigen T200 was too large to determine accurately but was greater than 7.0 nm (70 Å). In addition to iodinated proteins and a trichloroacetic acid-soluble peak of ^{125}I , a discrete peak of radioactivity eluted in the position of cytochrome *c* was found. The radioactivity in these fractions was precipitated by trichloroacetic acid, but was solubilized when the

precipitate was washed with acetone. This fraction, which we presume to be iodinated lipid as no loss of the radioactive bands resolved on the gels was observed, accounted for 5–28% of the total trichloroacetic acid-insoluble radioactivity in different experiments.

Only 30–40% of the applied iodinated proteins were recovered in the eluate of the Sepharose 6B column run in Nonidet P-40, moreover, a peak of radioactivity containing mainly aggregated antigen T200 was eluted in the void volume. Higher recovery of radioactivity and less aggregation was noted if membranes solubilized with Nonidet P-40 were applied directly to the Sepharose column without storage. A variable fraction of antigens T25 and T200 was eluted from the column run in Nonidet P-40 in positions similar to those found for extracts run in deoxycholate.

Chemical nature of antigens T200 and T25

Under the appropriate conditions, antigen T200 and T25 are degraded by proteinases. Incubation of Nonidet P-40 extracts of iodinated thymocytes with Pronase totally degraded antigens T200 and T25; treatment of the extract with chymotrypsin or trypsin alone, or as a mixture, resulted in the cleavage of antigen T200 to give a species of apparent mol.wt. 100000 (Plate 2). Antigen T25 was relatively resistant to digestion by trypsin or chymotrypsin under these conditions, although the methods used are not sufficiently quantitative to exclude partial degradation of antigen T25. Antigens T200 and T25 were both completely degraded by Pronase, trypsin or chymotrypsin after denaturation with trichloroacetic acid, and about 70% of the ^{125}I incorporated into either molecule was recovered as 3-iodotyrosine after Pronase digestion.

Antigens T200 and T25 could also be metabolically labelled with [^3H]leucine, but only 0.1% of the trichloroacetic acid-insoluble radioactivity incorporated by BW5147 cells was in antigen T200 and less than 0.01% was recovered in antigen T25, which could only be identified in immunoprecipitates prepared from glycoproteins isolated by lectin affinity chromatography (see below). Both molecules were metabolically labelled by mannose, galactose and glucosamine and in this case accounted for a much higher fraction of the incorporated radioactivity (3–6% and 0.5–1% of mannose, galactose and glucosamine being incorporated into antigens T200 and T25 respectively).

Affinity chromatography on lectin-Sepharose columns

Solubilized iodinated proteins of BW5147 cells and thymocytes were passed through a column of either concanavalin A-Sepharose or pea lectin-Sepharose to investigate the usefulness of this procedure in the purification of antigens T200 and

Fig. 2. Gel filtration on Sepharose 6B of deoxycholate-solubilized iodinated membrane proteins from thymocytes. Total radioactivity (—), trichloroacetic acid-insoluble radioactivity after washing with acetone (●), antigen T25 (○) and antigen T200 (□) in each column fraction. Details of procedures are given in the Experimental section. The elution positions of standard proteins (IgG, immunoglobulin G; Cyt *c*, cytochrome *c*; Ova, ovalbumin) co-chromatographed with membrane proteins are shown. The void volume (59 ml) of the column was determined separately with Blue Dextran 2000 (Pharmacia, Piscataway, NJ 08854, U.S.A.).

T25. In parallel experiments the recovery of trichloroacetic acid-soluble radioactivity was always higher from pea-lectin columns (62–85%) than from concanavalin A columns (51–74%). Antigen T200 from either cells was bound by both lectin columns and could be specifically eluted with buffer containing methyl α -D-mannoside (Plate 3). Antigen T25 from thymocytes had different properties from that of BW5147 cells; whereas antigen T25 from BW5147 cells was bound quantitatively to both lectin columns, thymocyte antigen T25 was only partially bound by pea lectin–Sepharose. The bound fraction migrated with a faster mobility than the unbound fraction on the gels (Plate 3b).

Similar results were obtained with membrane fractions from BW5147 cells or thymocytes solubilized and chromatographed in sodium deoxycholate-containing buffers.

Heterogeneity of antigens T200 and T25

Antigens T200 and T25 are heterogeneous, as judged by SDS/polyacrylamide-gel electrophoresis. Antigen T200 from BW5147 cells consists of three components and thymocyte antigen T200 of two components. These components cover a span of about 20000 mol.wts. and they vary from 210000 on 7% (w/v) polyacrylamide gels with *N,N'*-diallyltartardiamide as cross-linking reagent (Anker, 1970) to 175000 on 14% gels.

Antigen T25 from thymocytes migrates as a broad band within which individual components are unresolved. On 7% polyacrylamide gels the components range from 23000 to 36000 mol.wt., and on 14% and 18% gels there is less trailing and the molecular-weight range is from 23000 to 27000. In contrast, antigen T25 from BW5147 cells is more homogeneous, giving a discrete band of material with an apparent mol.wt. of 23000 on an 18% gel, 24000 on a 14% gel and 26000 on a 10% gel. Thus antigen T25 from BW5147 cells may more closely resemble the faster-migrating components of thymocyte antigen T25.

Preparation of unreduced samples in buffer containing 50mM-3-iodoacetamide did not change the electrophoretic mobility of either antigen T200 or T25.

Discussion

Subcellular distribution of antigens T200 and T25

Antigens T200 and T25 are among the species selectively labelled by lactoperoxidase-catalysed iodination of viable cells, suggesting that both molecules are on the cell surface. The results of subcellular-fractionation studies indicate that both molecules are membrane-bound and that their distribution on sucrose gradients is similar to that of most of the other iodinated species and to that of 5'-

nucleotidase, an enzyme known to be located on the plasma membrane of lymphocytes (Misra *et al.*, 1974, 1975). Moreover, antigen T200 was partially degraded by brief treatment of viable cells with trypsin. These results, taken together, strongly suggest that both molecules are plasma-membrane components and are located on the cell surface. Further, Gahmberg *et al.* (1976) have shown that a high-molecular-weight mouse thymocyte glycoprotein, which is almost certainly antigen T200, is labelled by the galactose oxidase technique, which also selectively labels cell-surface glycoproteins.

Properties of antigens T200 and T25

Sensitivity to proteinases, biosynthetic labelling experiments and lectin affinity chromatography establish that antigens T25 and T200 are glycoproteins. Both molecules are heterogeneous, and for thymocyte antigen T25 heterogeneity in the carbohydrate portion of the molecule was suggested from the results of lectin affinity chromatography. From a comparison of the value of the Stokes radius of antigen T25 in deoxycholate and its apparent molecular weight from SDS/polyacrylamide-gel electrophoresis, this molecule is probably monomeric in solution in deoxycholate.

The properties of antigen T25 are in good agreement with those reported for the rat Thy-1 molecule, the equivalent surface glycoprotein on rat thymocytes (Letarte-Muirhead *et al.*, 1975). In particular, the Thy-1 glycoprotein from thymocytes of both species show only partial binding to lentil or pea lectin [lectins with similar carbohydrate-binding specificity (Allen *et al.*, 1976)].

More detailed characterization will be necessary to establish the molecular structure of antigen T200 in its native form; it is eluted from Sepharose 6B run in deoxycholate earlier than expected for a globular protein with a mol.wt. of 200000, but many factors could account for this (Tanford *et al.*, 1974). Antigen T200 does not contain disulphide-bonded subunits.

Solubilization of antigens T200 and T25

Antigens T200 and T25 are effectively solubilized by deoxycholate. As judged by sedimentation studies, Nonidet P-40 also solubilized both molecules, but gel filtration showed the presence of aggregated protein which included antigen T200 and to a lesser extent antigen T25. This observation contrasts with the result of immunoprecipitation studies in Nonidet P-40, which show that antigens T200 and T25 can be precipitated independently from each other and from other labelled molecules (see, e.g., Trowbridge *et al.*, 1975b). One explanation is that aggregation occurs after solubilization of the membrane proteins and is a relatively slow process which is favoured by freezing of the Nonidet P-40 extracts. It seems less likely that solubilization is incomplete, since iodinated lipid is

eluted on gel filtration in Nonidet P-40 as a discrete peak of radioactivity well-separated from most iodinated proteins. The presence of aggregated membrane antigens has been noted after solubilization with other non-ionic detergents (Letarte-Muirhead *et al.*, 1974).

On the basis of these preliminary results it is probable that solubilization of lymphoma membrane pellets in deoxycholate followed by lectin affinity chromatography and gel filtration will be useful in the purification of these molecules on a preparative scale.

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References

- Allan, D. & Crumpton, M. J. (1971) *Biochem. J.* **123**, 967-975
- Allen, A. K., Desai, N. N. & Neuberger, A. (1976) *Biochem. J.* **155**, 127-135
- Anker, H. S. (1970) *FEBS Lett.* **7**, 293-294
- Boyse, E. A. & Old, L. J. (1969) *Annu. Rev. Genet.* **3**, 269-290
- Cuatrecasas, P. (1970) *J. Biol. Chem.* **245**, 3059-3065
- Edelman, G. M. (1976) *Science* **192**, 218-226
- Gahmberg, C. G., Hayry, P. & Andersson, L. C. (1976) *J. Cell Biol.* **68**, 642-653
- Helenius, A. & Simons, K. (1975) *Biochim. Biophys. Acta* **415**, 29-79
- Holley, R. W. (1975) *Nature (London)* **258**, 487-490
- Hubbard, A. L. & Cohn, Z. A. (1972) *J. Cell Biol.* **55**, 390-405
- Izzo, J. L., Roncone, A., Izzo, M. J., Foley, R. & Bartlett, J. W. (1972) *J. Biol. Chem.* **247**, 1219-1226
- Laskey, R. A. & Mills, A. D. (1975) *Eur. J. Biochem.* **56**, 335-341
- Letarte-Muirhead, M., Acton, R. T. & Williams, A. F. (1974) *Biochem. J.* **143**, 51-61
- Letarte-Muirhead, M., Barclay, A. N. & Williams, A. F. (1975) *Biochem. J.* **151**, 685-697
- Maizel, J. V. (1971) *Methods Virol.* **5**, 179-246
- Misra, D. N., Gill, T. J. & Estes, L. W. (1974) *Biochim. Biophys. Acta* **352**, 455-461
- Misra, D. N., Ladoulis, C. T., Estes, L. W. & Gill, T. J. (1975) *Biochemistry* **14**, 3014-3024
- Piez, K. A., Oyama, V. I., Levintow, L. & Eagle, H. (1960) *Nature (London)* **188**, 59-61
- Siegel, L. M. & Monty, K. J. (1966) *Biochim. Biophys. Acta* **112**, 346-362
- Tanford, C., Nozaki, Y., Reynolds, J. A. & Makino, S. (1974) *Biochemistry* **13**, 2369-2376
- Trowbridge, I. S. (1974) *J. Biol. Chem.* **249**, 6004-6012
- Trowbridge, I. S. & Hyman, R. (1975) *Cell* **6**, 279-287
- Trowbridge, I. S. & Mazauskas, C. (1976) *Eur. J. Immunol.* **6**, 557-562
- Trowbridge, I. S., Ralph, P. & Bevan, M. J. (1975a) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 157-161
- Trowbridge, I. S., Weissman, I. L. & Bevan, M. J. (1975b) *Nature (London)* **256**, 652-654
- Vogt, M. & Dulbecco, R. (1963) *Proc. Natl. Acad. Sci. U.S.A.* **49**, 171-179
- Watson, J., Nilsen-Hamilton, M. & Hamilton, R. T. (1976) *Biochemistry* **15**, 1527-1534