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LYMPHOCYTE PLASMA MEMBRANES

VI. PLASMA MEMBRANE GLYCOPROTEINS OF THYMIC AND SPLENIC LYMPHOCYTES FROM INBRED RATS

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Summary

Plasma membranes of splenic and thymic lymphocytes from ACI rats were analyzed for their protein and glycoprotein components by surface radioiodination with ^{125}I and SDS-polyacrylamide gel electrophoresis. The glycoproteins were extracted with lithium diiodosalicylate, characterized and assayed with antisera to thymic antigen. Plasma membranes of both cell types showed more than 25 proteins of which 10—15 were glycoproteins. Both cells showed five major glycoproteins but their apparent molecular weights or intensities differed. Surface radioiodination showed a 120 000 daltons component, common to both cell types, and a 27 000 daltons thymus-specific component as the most exposed surface glycoproteins. Lithium diiodosalicylate extracts of the plasma membranes contained almost all of the glycoprotein components and comprised 5—6% of the total membrane protein and 40—50% of the total membrane carbohydrate, with sialic acid content in thymus twice that of the spleen cells. About 1% of the total plasma membrane protein and 7% of the total isolated glycoproteins from thymocytes were reactive with rabbit anti-rat thymocyte antiserum and the immune precipitates showed two components with apparent molecular weights of 72 000 and 27 000.

Introduction

The majority of membrane glycoproteins on mammalian cells are on the exterior cell surface [1,2]. In lymphocytes, these surface glycoproteins are of major importance in the maturation and regulation of cell functions, and they

Abbreviation: SDS, sodium dodecyl sulfate.

include surface immunoglobulins, histocompatibility antigens and differentiation antigens [3,4]. Knowledge of the composition and structural orientation of the lymphocyte surface glycoproteins is, therefore, important for understanding their functional properties.

Cell surface glycoproteins have been studied most extensively in erythrocytes [2], but in recent years increasing attention has been directed to lymphocyte membrane glycoproteins [5—8]. There have been a few reports of the isolation of glycoproteins from purified lymphocyte plasma membranes [9,10], but very little is known about their physical, chemical and immunological properties. For immunological studies, it is also important to ascertain whether the specificity of the immunologically important components is preserved in the isolated glycoproteins. In this paper we report the isolation and characterization of glycoproteins from purified splenic and thymic lymphocyte plasma membranes from inbred rats. We have chosen to work with inbred rat strains because of their importance in immunogenetic studies of differentiation and of histocompatibility antigens [11].

Experimental procedures

Materials. Lithium diiodosalicylate, acrylamide, bisacrylamide and *N,N,N′,N′*-tetramethylethylenediamine (TEMED) were purchased from Eastman Kodak Co., Rochester, N.Y. Sodium dodecyl sulfate, 99% pure, was purchased from BDH (Gallard-Schlesinger, Carle Place, N.Y.). Carrier-free ^{125}I was obtained from New England Nuclear, Boston, Mass. Lactoperoxidase (EC 1.11.1.7, 45 units activity/mg), *N*-acetylneuraminic acid (Type VI), dithiothreitol and Triton X-100 were purchased from Sigma Chemical Co., St. Louis, Mo. All other chemicals were reagent grade.

Isolation of lymphocytes and their plasma membranes. The spleens and thymuses from 8—10-week-old ACI inbred rats, obtained from our breeding colony, were used as the source of lymphocytes. The lymphocytes were isolated, purified and their plasma membranes were prepared as described previously [12—14].

Extraction of plasma membrane glycoproteins and their biochemical analysis. The glycoproteins from the purified plasma membranes were isolated by lithium diiodosalicylate extraction [15]. Protein was determined by the method of Lowry et al. [16] with bovine serum albumin as the standard. Carbohydrate was determined by the anthrone reaction [17]. Lipid was extracted by the method of Folch et al. [18]. For sialic acid the samples were heated at 80°C for 1 h in the presence of 50 mM H_2SO_4 and then assayed by the thiobarbituric acid method of Warren [19]. The results were expressed in terms of *N*-acetylneuraminic acid.

SDS-polyacrylamide gel electrophoresis. The samples were dissolved in SDS with a detergent : protein weight ratio of 15 and a final detergent concentration between 2 and 3%, and heated in a boiling water bath for 5 min. For reduction, the samples contained 50 mM dithiothreitol in addition to the detergent. The standard proteins for molecular weight calibration were treated in the same manner. However, when the dimers of beta-galactosidase and tetrameric

forms of phosphorylase A and catalase were needed, these proteins were dissolved in the detergent without heating [20].

The SDS-polyacrylamide gels (0.5 × 5.5 cm or 0.5 × 10.0 cm) contained 7.5% (w/v) acrylamide and 0.5% SDS, and the electrophoresis was done according to the method of Shapiro et al. [21]. The gels were stained for carbohydrate using the periodic acid-Schiff reagent method of Zacharius et al. [22]. The method of Shapiro et al. [21] was used for construction of the molecular weight calibration curve.

Surface radioiodination. The intact lymphocytes were surface-labeled with carrier-free ^{125}I by the method of Cone and Marchalonis [23]. Plasma membranes of the iodinated cells were isolated as described [14], and analyzed by electrophoresis in 10 cm SDS-polyacrylamide gels. The gels were sliced in 1.6 mm fractions, and the radioactivity was counted using a Packard Gamma Scintillation Spectrometer (Model 5975).

Preparation of antisera to thymic antigen. Antisera to thymic antigen were prepared in two ways. (1) New Zealand white rabbits were given 5—7 weekly intramuscular injections of 0.5 ml of rat brain homogenate (equivalent to 250 mg wet weight of brain) emulsified with 0.5 ml Difco complete Freund's adjuvant containing 3 mg/ml additional *M. tuberculosis.* The rabbits were bled 10 days after the last injection. (2) Antiserum to rat thymocytes was prepared by injecting rabbits with a total of 1 ml of rat thymocytes ($2 \cdot 10^8$ cells) emulsified 1 : 1 with Freund's adjuvant, as above, into the hind foot pads. After 2 to 3 weeks, the animals were injected intravenously with $2 \cdot 10^6$ rat thymocytes on 3 successive days. The animals were bled 2 weeks later. The antisera were adsorbed once with 1 vol. of finely minced liver, twice with 1 vol. of packed red cells and once with 1/3 vol. of bone marrow cells, all from ACI rats.

Immunodiffusion of isolated plasma membranes and glycoproteins. Ouchterlony plates were prepared with 1.5% agarose in phosphate-buffered saline. The membranes were dissolved in 1% Triton X-100 (Triton : protein = 10 to 20 : 1, w/w). Immunodiffusion of the samples was carried out against anti-rat brain serum at 22°C for 3 days. The plates were then dialyzed thoroughly against phosphate-buffered saline and stained for protein.

Coprecipitation analysis of thymic antigen. Lithium diiodosalicylate extracted glycoprotein from thymocyte plasma membranes (20 μg), thymocyte plasma membrane (200 μg) and splenic lymphocyte plasma membrane (200 μg) were iodinated with lactoperoxidase. Each sample (0.5 ml) contained 5 μg lactoperoxidase, and 400 μCi ^{125}I in phosphate-buffered saline to which two 10 μl aliquots of 10 mM H_2O_2 were added at 5-min intervals. The samples were then exhaustively dialysed against 10 mM Tris · HCl/0.15 M NaCl, pH 7.5.

The glycoprotein, the thymocyte plasma membranes and the splenic lymphocyte plasma membranes retained 9.3, 5.2 and 8.2%, respectively, of the added iodine. These samples were solubilized in 0.5% Triton X-100 (Triton : protein = 5 : 1, w/w), incubated at 30°C for 1 h and centrifuged at 25 000 × *g* for 1 h. The amounts of glycoprotein, of thymocyte and of splenic lymphocyte plasma membranes solubilized were 91.9, 76.7 and 88.1%, respectively. Rabbit anti-rat brain or anti-rat thymocyte serum (20 μl) was added to each test sample, and normal rabbit serum (20 μl) was added to each control; concentrations of both sera were previously adjusted to equivalence with goat anti-rabbit IgG

serum. The samples were incubated overnight at 4°C. The goat antiserum (20 μl) was then added and the samples were incubated for 24 h at 4°C. The precipitates were washed 6 times in cold phosphate-buffered saline and analysed by SDS-polyacrylamide gel electrophoresis.

Results

Glycoprotein analysis

The yield of protein in the lithium diiodosalicylate extracts was about 6% of the total membrane protein in both splenic and thymic membranes (Table I). The yield of carbohydrate was 38% of the total splenic membrane carbohydrate and 47% of total thymocyte membrane carbohydrate. These findings indicate that the content of carbohydrate in the extracts was enriched 7—11-fold compared to that in plasma membranes, both estimated with respect to protein. In the spleen, the extracted glycoproteins contain 44% carbohydrate (6% sialic acid); in the thymus, the glycoproteins contain 52% carbohydrate (12% sialic acid).

SDS-polyacrylamide gel electrophoresis of isolated plasma membranes and glycoproteins

The molecular weight versus relative mobility calibration curve was a straight line from 10 000 to 200 000. The polyacrylamide gel densitometric tracings for membrane proteins and glycoproteins are shown in Fig. 1. The apparent molecular weights of the proteins and the glycoproteins are given in Table II. There were some differences in the protein patterns of the two membranes: (i) 3—4 minor protein bands in spleen of molecular weight greater than 200 000 were absent from thymus; (ii) an intensely stained splenic protein of 140 000 daltons (band 11) was absent from thymus; (iii) a protein of 27 000 daltons (band 25) was very prominent in thymus but not in spleen; and (iv) protein band 23 (40 000—42 000 daltons) was moderately intense in thymus but weak in spleen. Of the major common proteins, bands 13 (122 000), 16 (96 000) and 20 (66 000) were equally prominent with band 22 (49 000) as the most prominent protein.

TABLE I

PROTEIN AND CARBOHYDRATE CONTENT IN THE LITHIUM DIIODOSALICYLATE-EXTRACTED GLYCOPROTEINS FROM ACI FEMALE RAT LYMPHOCYTE PLASMA MEMBRANES

Each figure represents the average of two observations.

	Speen	Thymus
Protein yield (% of total membrane protein)	6.48	5.57
Carbohydrate yield (% of total membrane carbohydrate)	37.6	47.2
Carbohydrate content in extracted glycoprotein (μg/mg extracted protein)	787	1089
Sialic acid content in extracted glycoprotein (μg/mg extracted protein)	108.1	247.5

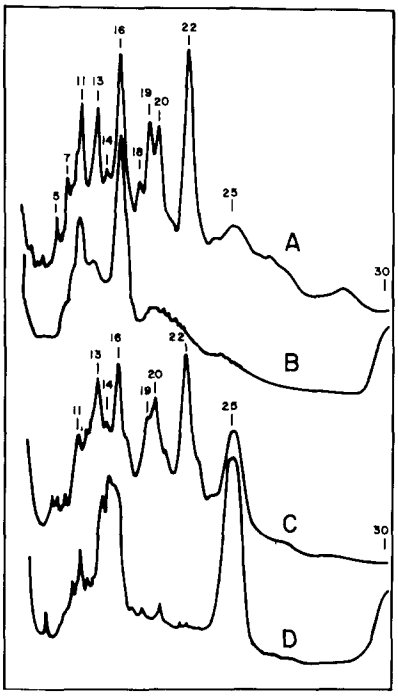

Fig. 1. Densitometric tracings of SDS-polyacrylamide gels stained for protein and carbohydrate. Curves A and C represent Coomassie-blue stained gels from splenic and thymic cell plasma membranes, respectively. Curves B and D show the periodic acid-Schiff positive bands of splenic and thymic cell membranes, respectively. Despite the overall similarity of the protein components, the carbohydrate components of the two membranes are different. The bands are numbered as in Table II.

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Gels B and D in Fig. 1 represent splenic and thymic membranes, respectively, stained with periodic acid-Schiff reagents. The stained components were also stained for protein (gels A and C in the figure). Treatment of the gels with Schiff reagent without prior oxidation by periodic acid did not show any stained component, an indication that the components stained with periodic acid-Schiff reagents represent glycoproteins.

The glycoproteins of the two membranes also differed qualitatively and quantitatively. In the spleen, there were five prominent glycoproteins of molecular weights of 165 000 (band 7), 148 000 (band 9), 140 000 (band 11), 122 000 (band 13) and 96 000 (band 16) and 5—6 minor glycoproteins in the molecular weight range from 250 000 to 25 000; all of the major glycoprotein bands correspond to major protein bands in isolated membranes. Thymocyte

TABLE II

ESTIMATED APPARENT MOLECULAR WEIGHTS OF THE PROTEIN AND GLYCOPROTEIN COMPONENTS OF ACI RAT SPLENIC AND THYMIC LYMPHOCYTE MEMBRANES BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Each result is the average of 5—7 observations.

Component No.	Molecular weight ± standard deviation ($\times 10^{-3}$)			
	Spleen		Thymus	
	Protein components *	Glycoprotein components **	Protein components *	Glycoprotein components **
1	264 ± 5	—	—	—
2	239 ± 3	—	—	—
3	224 ± 6	f	—	—
4	203 ± 5	f	205 ± 5	f
5	190 ± 5	f	189 ± 3	f
6	—	—	179 ± 1	f
7	165 ± 2	p	166 ± 2	p or f
8	—	—	154 ± 3	f
9	148 ± 3	vp	—	—
10	—	—	143 ± 2	f
11	140 ± 2	vp	—	—
12	—	—	135 ± 1	f
13	122 ± 2	p	122 ± 5	p
14	109 ± 2	—	110 ± 4	p
15	104 ± 2	f	105 ± 2	vp
16	96 ± 2	vp	96 ± 3	p
17	89 ± 5	—	90 ± 3	f
18	79 ± 3	—	78 ± 5	f
19	72 ± 3	f	72 ± 3	f
20	66 ± 3	f	66 ± 3	
21	57 ± 3	—	56 ± 3	—
22	49 ± 2	—	50 ± 1	—
23	40 ± 3	—	42 ± 2	—
24	34 ± 3	—	33 ± 1	—
25	27 ± 2	f	27 ± 2	vp
26	24 ± 1	—	—	—
28	18 ± 1	—	17 ± 0.3	—
29	10 ± 1	—	—	—
30	≈8 ***	p ***	≈8 ***	p ***

* Underlined protein components are major bands. A dash indicates no component in this position.

** The symbols are: f, faint periodic acid-Schiff (PAS)-positive bands; p, prominent PAS-positive bands; vp, very prominent PAS-positive bands.

*** Glycolipids only.

membranes showed four prominent glycoproteins of molecular weights 122 000 (band 13), 119 000 (band 14), 105 000 (band 15) and 96 000 (band 16), and 9—10 minor glycoproteins in the molecular weight range from 200 000 to 50 000. In addition, the thymocyte membranes alone contained an intense glycoprotein of 27 000 daltons (band 25). When the two membranes were compared, the relative intensities of some of the carbohydrate bands did not correspond to the intensities of the respective protein bands (examples: thymic bands 15 and 16). These results suggest that the carbohydrate composition of membrane glycoproteins vary considerably in different lymphocytes.

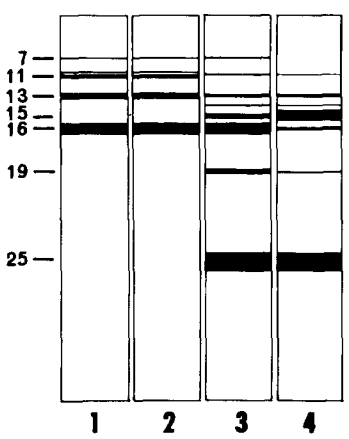
In both membranes, there was an intense periodic acid-Schiff-positive band of 8000 daltons (band 30, Fig. 1, gels B and D) which did not stain for protein but did stain with Sudan black. In addition, lipid extracts of the plasma membranes showed only this band when stained with periodic acid-Schiff stain and Sudan black. These findings suggest that this band contains glycolipids.

The gel patterns of the extracted glycoproteins are shown in Fig. 2. All observed bands were stained for carbohydrate and protein indicating that the extracts consisted only of glycoproteins. When compared with the glycoproteins in the whole plasma membranes, the results showed that all observed glycoprotiens were extracted by lithium diiodosalicylate.

Surface components of rat splenic and thymic lymphocytes

Since glycoproteins in plasma membranes are surface components, we attempted to label them by surface radioiodination of intact lymphocytes. Fig. 3 shows the results of iodine incorporation in the surface of splenic or thymic lymphocytes. The thymocytes required more H_2O_2 than splenic lymphocytes for maximal iodine uptake, and this probably indicates that the rate of iodination is slower for thymocytes than for splenic lymphocytes. The maximal uptake of iodine by splenic lymphocytes was almost twice that by thymocytes.

Gels of the radioiodinated membrane proteins (Fig. 4) showed that the most intensely labeled component in splenic cells had a molecular weight of 120 000 (band 13, Fig. 1). These cells also showed a component of 210 000 daltons together with a few other less intense components. The ends of the gels con-



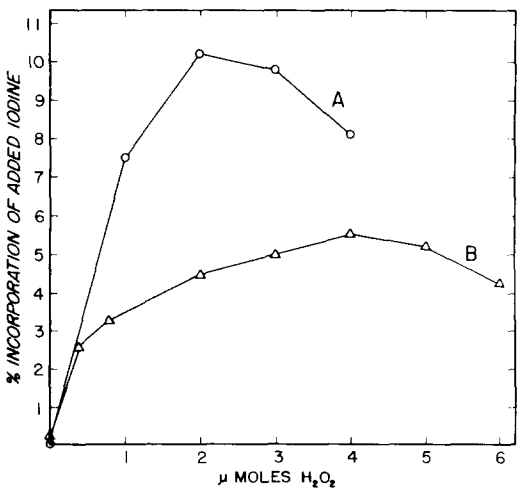


Fig. 2. SDS-polyacrylamide gels of membrane glycoproteins extracted by lithium diiodosalicylate. The splenic glycoproteins stained for protein and carbohydrate are gels 1 and 2, respectively. The thymic glycoproteins stained for protein and carbohydrate are gels 3 and 4, respectively.

Fig. 3. Incorporation of ^{125}I into the surface proteins of rat splenic lymphocytes and thymocytes (10^7 cells each). Curve A shows the iodination of splenic cells, and curve B, the same for thymocytes. Maximal iodination of thymocytes required a longer time and a higher total amount of H_2O_2. The maximal incorporation of iodine into splenic lymphocytes was nearly twice that into thymocytes.

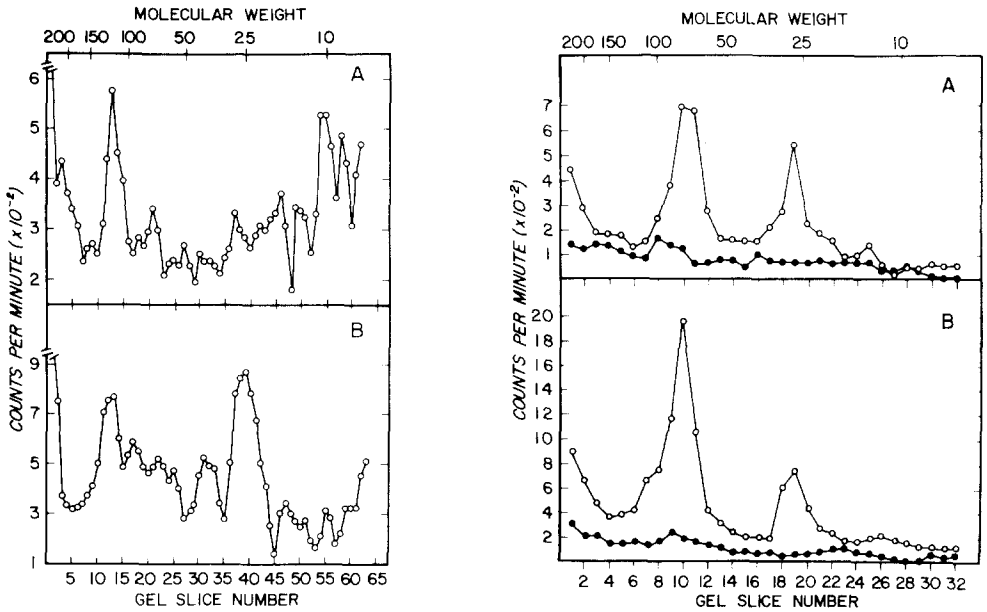


Fig. 4. SDS-polyacrylamide gels of ^{125}I-labeled splenic membranes (A) and thymocyte membranes (B). Membrane samples equivalent to $2 \cdot 10^7$ splenic lymphocytes and $8 \cdot 10^7$ thymocytes were separated by electrophoresis and the gels were sliced into 1.6 mm fractions and counted in a gamma scintillation counter.

Fig. 5. The Triton X-100 extract of radioiodinated thymocyte membranes (A) and radioiodinated lithium diidosalicylate-extracted thymocyte membrane glycoproteins (B) were precipitated with normal rabbit plus goat ant-rabbit serum (control: •) and with rabbit anti-rat thymocyte serum plus goat anti-rabbit serum (test: ○). The precipitates were analyzed by SDS-polyacrylamide gel electrophoresis.

tained some labeling, presumably due to uptake of iodine by the glycolipid components. Thymic cells showed two major labeled components: one in the position of the major labeled peak in the spleen (band 13) and the other in the position of the prominent thymus-specific glycoprotein (band 25, 27 000). In both cell types, the relative intensities of the labeled components did not correspond to those of the glycoproteins.

Characterization of the thymus-specific antigen

Immunodiffusion of thymocyte plasma membranes or extracted glycoproteins against anti-rat brain serum showed one major and one minor precipitin lines. The results showed that the extracted glycoproteins contained the antigen in much higher concentration than the plasma membranes and that lithium diiodosalicylate extraction did not destroy the reactivity of the antigen. Extracted glycoproteins from splenic cells did not show any visible precipitin line.

The results of coprecipitation of the plasma membranes or the glycoproteins with antithymocyte serum are given in Table III. The plasma membrane and the glycoprotein extract from thymocytes both yielded specifically precipitable protein, and its amount increased proportionately with the amount of sample used. Approximately 7% of the extracted glycoprotein and about 1% of

TABLE III

IMMUNOPRECIPITATION OF THYMIC ANTIGENS FROM LYMPHOCYTE PLASMA MEMBRANE GLYCOPROTEIN EXTRACTS AND TRITON X-100 SOLUBILIZED PLASMA MEMBRANE PROTEINS

Extracted glycoprotein from thymocyte plasma membrane and plasma membranes from thymocytes and splenic lymphocytes were iodinated with ^{125}I, dissolved in Triton X-100 and coprecipitated with normal rabbit serum plus goat anti-rabbit IgG serum (control) or with rabbit anti-rat thymocyte serum plus goat anti-rabbit IgG serum (test). The resulting precipitates were washed 6 times with phosphate-buffered saline and the protein in the precipitates was estimated from counts. Each figure represents the mean of duplicate samples.

Sample	Amount of iodinated protein added (*c*)	Amount of iodinated protein precipitated			Percent of total iodinated protein specifically precipitated (*a*-*b*) × 100/*c*)
		Test (*a*) (ng)	Control (*b*) (ng)	Specific (*a*-*b*) (ng)	
Thymocyte glycoprotein	2.16	254	105	149	6.91
	5.40	594	208	386	7.15
Thymocyte plasma membrane	12.50	254	130	124	0.99
	25.00	563	300	263	1.05
	50.00	1065	510	555	—
Splenic lymphocyte plasma membrane	14.68	205	253	—	—
	29.36	273	327	—	—

the total plasma membrane protein precipitated specifically with the antiserum. Hence, the thymic antigen content of the glycoprotein extract is enriched 7-fold compared to the plasma membrane. The splenic lymphocyte plasma membrane did not yield any specific precipitation with the antisera.

The precipitates, analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 5) showed that both the glycoprotein extract and the plasma membrane contained two components in identical positions: the smaller component is the same as the thymocyte-specific 27 000-dalton component, and the larger component has an apparent molecular weight of 72 000 which coincides with the minor glycoprotein band 19 (Fig. 1). The radioactive count density of the lighter component was lower than the other component, especially in the glycoprotein extract. Reduction of the precipitates with 50 mM dithiothreitol gave identical results, an indication that these two components are non-covalently linked polypeptides.

Discussion

Characteristics of protein and glycoprotein components

The two membranes of rat lymphocytes contained a large number of protein components with some differences. These results are similar to those reported by other investigators for different lymphocytes [24—29]. The 27 000 daltons glycoprotein was absent from spleen, although about 50% of the splenic lymphocytes share T-cell properties [30,31]. This indicates that the thymocytes and the splenic T-cells differ in their surface glycoproteins. Similar results were reported by Gahmberg et al. [5] for murine thymocytes and peripheral

T-cells. This difference may correlate with the difference in the functional properties of the two cell types [32].

Glycoproteins

The significant difference between the two membranes was in the sialic acid content. Allan and Crumpton [33] and Snary et al. [8] found that 18—20% of the total carbohydrate in pig lymphocyte membranes was sialic acid. In rat we obtained comparable results, but the sialic acid content in thymus was almost double that in spleen. Such difference between lymphocytes from different organs was previously reported for chicken and mouse [34,35]. Since sialyl residues carry net negative charge, the higher sialic acid content in thymocytes probably explains their higher electrophoretic mobility [36].

Surface components by lactoperoxidase-catalyzed iodination

Both membranes contained 5—6 prominent glycoproteins, but surface radioiodination labeled only one major glycoprotein in spleen and two in thymus. This finding corroborates the observation by others that iodination by lactoperoxidase-catalysis does not label all cell surface glycoproteins to the same extent [37]. Recently Gow and Wardlow [38] also reported differential iodination for some soluble proteins by this method where labeling did not correlate with their tyrosine content. Therefore, differential and limited labeling of lymphocyte surface proteins by this technique may be due to differences in their conformation or availability for lactoperoxidase catalysis.

Molecular weight and chemical nature of thymic antigens

Immunodiffusion and immunoprecipitation results showed that the extracted glycoproteins were enriched in thymic antigen content. It appears that lithium diiodosalicylate does not affect the reactivity of the thymic antigens, in agreement with the observation of Bustin et al. [39]. The immune precipitates contained two components which corresponded to two membrane glycoproteins. We conclude that rat thymic antigens are glycoprotein in nature.

Our immunofluorescence results with anti-rat brain serum showed weak staining of about 50% of the splenic cells and intense staining of all thymocytes [30,31]. Membranes of the splenic cells, however, did not show any significant reactivity with anti-thymocyte or anti-brain sera. These results support the observation by others that peripheral T-cells contain much less thymic antigen than thymocytes [32,40].

There are conflicting reports regarding the physical chemical properties of thymic antigens. Several investigators reported that murine thymic antigens are proteins [23,41—43] of 60 000 daltons [23,41] or of 35 000—40 000 daltons [42] or they are glycoproteins of 25 000 daltons [44]. Others found that the antigens are glycolipids or gangliosides [45,46] of 35 000 daltons [45]. In recent studies, Stein-Douglas et al. [47] disputed the ganglioside nature of the antigens, and Arndt et al. [48] reported that murine brain-associated thymic antigens are lipoproteins.

Rat thymic antigens are less well described, but their properties are probably similar since the murine thymic antigen Thy 1.2 is common to both species. Bustin et al. [39] reported that these antigens are proteins of 60 000—

70 000 daltons but their reactivity is dependent on carbohydrate and phospholipid. Williams and co-workers reported that the antigens are glycoproteins of 25 000—27 000 daltons [49—52].

We find that rat thymic antigens are glycoproteins comprising two components. Interestingly, the larger component has a molecular weight of approx. 72 000 which corresponds to the value reported by several investigators [23,39,41] and the smaller component has a molecular weight of 27 000, a value reported by others [44,49—52]. In our earlier studies, we initially reported the 27 000-dalton glycoprotein being unique to rat thymocyte plasma membranes [53] and we subsequently suggested that it may be the thymus-specific antigen [54]. Our present results indicate that both components are present in these antigens and suggest that the conflicting results from different laboratories may be due to the existence of two components of different molecular size. The two alternative hypotheses are either that there are two independent thymus-specific antigens which are operationally defined by the antisera used by different investigators, or that the thymic antigen consists of two non-covalently linked components.

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