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LYMPHOCYTE PLASMA MEMBRANES

I. THYMIC AND SPLENIC MEMBRANES FROM INBRED RATS

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SUMMARY

Thymic and splenic lymphocyte plasma membranes were isolated from inbred ACI and F344 rats. Chemical and enzymic analyses indicated a highly purified plasma membrane preparation with a high cholesterol to phospholipid ratio and over 12-fold enrichment of the membrane enzyme 5'-nucleotidase. Polyacrylamide-gel electrophoresis of externally radioiodinated membranes showed a major 117 000 dalton glycoprotein in both thymic and splenic lymphocytes. Thymocytes differ from splenic lymphocytes by having an unique external glycoprotein of 27 000 daltons and by the relative lack of membrane components above 200 000 daltons

INTRODUCTION

Genetic studies in inbred rats have shown that the antibody response to the synthetic polypeptide antigen poly($Glu^{52}Lys^{33}Tyr^{15}$) is under polygenic control [1]. The interaction of the antigen with a specific receptor on the plasma membrane of the immunocompetent cell probably initiates the response [2], and this receptor is likely to be an immunoglobulin or immunoglobulin-like molecule [3]. In addition to our studies on the mechanism of action of antigen, we are pursuing a detailed serological and chemical investigation of the genetically controlled differences among lymphocyte plasma membranes. As the initial step in approaching both of these problems, we have purified and analyzed thymic and splenic lymphocyte plasma membranes from normal high responder (ACI) and low responder (F344) strains of inbred rats

MATERIALS AND METHODS

The spleens and thymuses of 15–20 female rats (8–10 weeks old) were removed and minced in 15–20 ml of 10 mM Tris buffer (pH 7.4)–0 15 M saline at 4 °C. The cell suspensions were overlaid on 1·2 solution of 39 % (v/v) Hypaque and 9 % (w/v) Ficoll ($\rho = 1.16$ g/cm^3) and centrifuged at $1000 \times g_{av}$ min [4]. The interface consisting of over 95 % lymphocytes was aspirated, washed once and suspended in

5–10 ml of 10 mM Tris–0 15 M saline The cell suspension was diluted with 4 vol of 10 mM Tris buffer (pH 7 4) and kept at 0 C for 5 min Sufficient Tris-buffered 1 5 M NaCl was added to restore the molarity to 0 15 M, and the cell suspension was homogenized in a Potter–Elvejhem homogenizer The process was monitored by the trypan blue dye exclusion test, and homogenization was continued until more than 85 % of the cells were disrupted The homogenate was centrifuged first at $3000 \times g_{av}$ min to remove nuclei The supernatant was centrifuged at 250 000 g_{av} min to remove mitochondria and then at $600\,000 \cdot g_{av}$ min to pellet the microsomal fraction This pellet was suspended in 20 ml of 10 mM Tris–HCl buffer (pH 7 4), overlaid with a discontinuous 20–50 % (w/v) sucrose gradient and then centrifuged at $75\,000\,000 \times g_{av}$ min at 4 °C to isolate the microsomal fraction The various subcellular fractions were identified by electron microscopy

The total protein was determined by the method of Lowry et al [5] RNA and DNA were extracted by the procedure of Munro and Fleck [6] RNA concentration was estimated spectrophotometrically by assuming an absorbance of 1 00 at 260 nm for 32 μg/ml RNA, and DNA was determined by the diphenylamine method [7] using calf thymus DNA as the standard Carbohydrate was estimated by the anthrone reaction [8] Lipid was extracted by the method of Folch et al. [9], and the total lipid was determined gravimetrically as well as colorimetrically [10] Cholesterol was determined by the method of Bowman and Wolf [11] Lipid phosphorus was determined by the method of Ames [12], and the phospholipid content was estimated assuming 25 μg phospholipid per μg phosphorus The 5′-nucleotidase (EC 3 1 3 5) was assayed by the method of Michell and Hawthorne [13] Glucose 6-phosphatase (EC 3 1 3 9) and acid phosphatase (EC 3 1 3 2) activities were estimated by the KF–EDTA inhibition method suggested by Hubscher and West [14] The inorganic phosphorus released in these enzyme reactions was determined by the method of Ames [12] Succinic dehydrogenase (EC 1 3.99 1) and NADH dehydrogenase (EC 1 6 99 3) were assayed by the method of Earl and Kornei [15], and Wallach and Kamat [16], respectively

The isolated plasma membranes were solubilized with 2 % sodium dodecylsulphate at 100 °C for 5 min using 10 mg/mg protein Complete solubilization was indicated by the absence of any precipitate after centrifugation at $6\,000\,000 \times g_{av}$ min The membrane proteins were separated by electrophoresis in 7 5 % polyacrylamide gels (50 mm × 6 mm) containing 0 5 % sodium dodecylsulphate, and the gels were calibrated with 10 proteins of known molecular weight in the range of 300 000–10 000 Experiments using radioiodinated membranes showed that less than 3 % of the membrane proteins failed to enter the separating gel The gels were fixed in 10 % acetic acid and stained with Coomassie Brilliant Blue for protein or with the periodic acid–Schiff reagent for carbohydrate Analyses for lipids were done either by prestaining solubilized membrane with Sudan black or by staining polyacrylamide gels with Sudan black after fixation and dehydration through a graded series of ethyleneglycol (10–50 %) concentrations

Since the antigen-immunocompetent cell interaction involves external membrane components, experiments were carried out to identify these components in thymic and splenic lymphocytes A purified suspension of 1 10^7 viable lymphocytes was surface-labeled with radioiodine by the lactoperoxidase method [17] In these experiments, over 97 % of the iodinated proteins were recovered in the membrane frac-

tion After labeling the intact cells, the membranes were isolated, solubilized in sodium dodecylsulphate and analyzed on 7 5 % polyacrylamide gels (100 mm × 6 mm) containing 0.5 % sodium dodecylsulphate. The gels were sliced into 64 fractions, each of which was approx. 1.5-mm thick, and assayed for radioactivity.

In order to identify membrane-bound immunoglobulins, we iodinated thymic and splenic lymphocytes ($1 \cdot 10^8$ cells), solubilized them in 1 % Triton X-100 and incubated them with rabbit anti-rat immunoglobulin G (IgG) antiserum at 4 °C for 16–18 h. The precipitates were washed with 0.15 M phosphate-buffered NaCl, dissolved in sodium dodecylsulphate, separated on polyacrylamide gels and assayed as described above.

RESULTS

The preparation of the various cell fractions was monitored electron microscopically and assayed chemically The results of the studies on chemical composition and enzymatic activity are summarized in Table I. The 30–35 % and 35–40 % interfaces of the sucrose gradient consisted entirely of smooth membrane vesicles and contained the highest specific activity of 5'-nucleotidase, which is considered to be a specific marker for plasma membrane [18]. On the basis of the electron microscopic findings, the high cholesterol content [19], the 12–15-fold enrichment of nucleotidase activity, the low level of contamination by nucleic acids and a variety of other enzyme activities, these two fractions were combined and subsequently used as the plasma membrane fraction The analytical results are in general agreement with those reported in studies of plasma membranes from lymphocytes of the pig [20, 21], human [22–24] and calf [25]

A comparison of the composition and enzyme activities in the plasma membranes from splenic and thymic lymphocytes is summarized in Table II. There are no significant differences ($P \leqq 0.01$) between spleen and thymus The succinate dehydrogenase activity in purified plasma membrane preparations was very low and 50–100 times lower than in the mitochondrial fraction. The total activity of NADH dehydrogenase, which is considered to be a relatively reliable marker for endoplasmic reticulum [16], was 3 1 and 2 5 % for the membrane fractions of splenic and thymic lymphocytes, respectively. These results are consistent with the data for purified plasma membrane fractions of L-cells [26], Ehrlich ascites cells [16] and calf lymphocytes [25], and they provide further evidence that we have separated highly purified plasma membrane from the smooth endoplasmic reticulum

The membrane protein patterns of both splenic and thymic lymphocytes showed about 25 protein bands, of which 15 were relatively sharp Densitometric analyses of Coomassie blue-stained gels are shown in Fig. 1 (Curves 1 and 2), and there is an overall similarity between the band patterns of the two membranes However, there are some significant differences (1) three minor proteins greater than 200 000 daltons are always present in the spleen but absent from the thymus, (2) a band of 145 000 daltons is very prominent in the spleen (Band c) but very faint or absent from the thymus, and (3) a major band of about 27 000 daltons stains prominently for protein in the thymus but not in the spleen (Band h).

The densitometric analyses of periodic acid–Schiff-stained gels (Fig 1, Curves 3 and 4) showed that all the proteins above 100 000 daltons in thymic and splenic

membranes stained with the reagent indicating that they were glycoproteins The major glycoprotein of 145 000 daltons (Band c) was much more prominent in splenic than in thymic membranes Although this finding suggests that this component

TABLE I

CHEMICAL AND ENZYME COMPOSITION OF THE DIFFERENT FRACTIONS OF RAT THYMOCYTES

The experiments were performed with material obtained from 20 thymuses yielding about 10^{10} cells, and the results represent the average of two typical experiments Absolute yields from homogenates were 342 mg protein, 30 mg DNA, 16 9 mg RNA, 11 3 mg cholesterol and 36 6 mg phospholipid Total activity of enzymes in homogenates, expressed as μmoles of product liberated per h, were 5'-nucleotidase, 202, succinate dehydrogenase, 87, glucose 6-phosphatase, 79 acid phosphatase 380, NADH dehydrogenase, 3680 Specific activities represent μmoles of product liberated/h per mg protein

	Nuclear pellet	Mitochondrial pellet	20 000 × *g* Supernatant	Sucrose bottom	Plasma membrane
Protein					
recovery (%)	22 8	15 5	32 8	4 6	1 3
DNA					
recovery	76 7	8 7	4 0	0 2	0 1
μg/mg protein	297	49	10.8	3 6	5 0
RNA					
recovery (%)	17 8	18 9	55 0	6 2	0.7
μg/mg protein	39	61	83	66	24 9
Cholesterol					
recovery (%)	18 6	18 7	16 5	6 9	8 7
μg/mg protein	27	40	16 7	49	222
Phospholipid					
recovery (%)	21 9	36 3	15 8	9 6	6 0
μg/mg protein	103	250	52	221	491
Cholesterol/phospholipid (molar ratio)	0 52	0 32	0 64	0 44	0 91
5'-Nucleotidase activity					
recovery (%)	9 7	24 7	14 4	19 2	18 0
spec act	0 25	0 94	0 26	2 44	8 24
Succinate dehydrogenase activity					
recovery (%)	9 7	46 2	15 2	0 9	0 1
spec act	0 108	0 758	0 118	0 049	0 015
Glucose-6-phosphatase activity					
recovery (%)	12 2	14 8	52 0	7 3	0 9
spec act	0 123	0 220	0 367	0 367	0 171
Acid phosphatase activity					
recovery (%)	6 4	39 0	30 5	4 8	1 0
spec act	0 31	2 83	1 04	1 14	0 83
NADH dehydrogenase activity					
recovery (%)	7 6	44 7	16 8	3 8	0 6
spec act	3 56	31 04	5 51	8 82	5 29

TABLE II

COMPARISON OF THE CHEMICAL AND ENZYME COMPOSITION OF THE ISOLATED PLASMA MEMBRANES FROM THYMOCYTES AND SPLENIC LYMPHOCYTES

10^9 purified lymphoid cells were used which yielded 30–40 mg of homogenate protein. The phospholipid molecular weight was assumed to be 775 The total enzymic activities are expressed as μmoles of product liberated per h. Specific activities represent μmoles of product/h per mg protein The number of experiments is given in parentheses, and the means are given ±S E

	Spleen	Thymus
Protein (% of homogenate protein)	0 90±0 11 (8)	1 36±0 29 (6)
DNA (μg/mg protein)	6 48±1 68 (8)	7 97±1 61 (8)
RNA (μg/mg protein)	36 8±4 81 (8)	33 6±7 07 (8)
Carbohydrate (μg/mg protein)	93 3±9 78 (9)	82 1±13 24 (9)
Lipid (μg/mg protein)	678±39.7 (8)	807±50 8 (9)
Cholesterol (μg/mg protein)	168±19 4 (9)	235±31 9 (7)
Phospholipid (μg/mg protein)	381±32 2 (7)	481±24 3 (7)
Cholesterol/phospholipid (molar ratio)	0 88	0 98
5′-Nucleotidase	10 19±1 46 (12)	6 84±1 89 (12)
Glucose 6-phosphatase	0 131±0 033 (6)	0 237±0 035 (5)
Acid phosphatase	1 18±0 18 (5)	0 79±0 08 (5)
Succinate dehydrogenase	0.024±0 004 (10)	0 029±0 006 (12)
NADH dehydrogenase	13 30±3 14 (10)	11 43±2 20 (10)

may be an immunoglobulin, purified rat serum IgG immunoglobulin, analysed repeatedly under identical conditions, had a mobility equivalent to 160 000 daltons. The glycoproteins of 127 000 (Band d) and 100 000 daltons (Band e) were equally prominent in both membranes. In both thymus and spleen, there were two major membrane proteins of 65 000 (Band f) and 50 000 daltons (Band g) which stained very weakly with the periodic acid–Schiff reagent. The most striking difference betweeen thymus and spleen was the intensely periodic acid–Schiff-positive 27 000 dalton glycoprotein (Band h) in the thymocyte plasma membrane. In both thymic and splenic membranes, there was a broad band of about 8000 daltons (Band i) which only stained with the periodic acid–Schiff reagent or with Sudan black. In addition, polyacrylamide gel analysis of a chloroform–methanol extract of isolated membranes showed only this band. These findings suggest that the band consists of membrane glycolipids [22] or unsaturated lipids (Wallach, D. F H., personal communication).

Typical electrophoretic profiles for surface-labeled thymic and splenic lymphocytes are shown in Fig 2. The major externally labeled components were the 117 000-dalton glycoprotein in both thymus and spleen and the prominent 27 000-dalton thymic membrane glycoprotein. The gel fractions in the region of 200 000 daltons were labeled to a significantly greater extent in splenic than in thymic membranes. These studies indicate that the 117 000-dalton component of thymic and splenic lymphocytes and the 27 000-dalton component of the thymus are the major external membrane glycoproteins and that the 200 000-dalton component of splenic lymphocyte membranes is a minor external component

The immunoprecipitation studies with anti-rat IgG antiserum showed that there were two labeled peaks of 117 000 and 200 000 daltons in the precipitates from

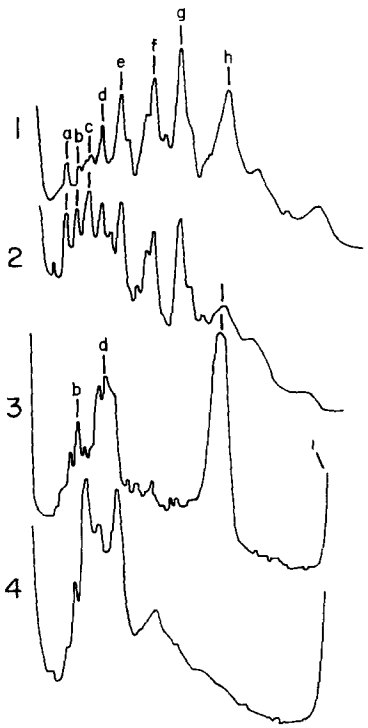

Fig 1 Densitometric tracings of the polyacrylamide-gel electrophoresis patterns of solubilized plasma membranes The membrane preparations were analyzed in 7 5 % polyacrylamide gels containing 0 5 % sodium dodecylsulphate Curves 1 and 2 are tracings of Coomassie blue-stained gels from thymus and spleen and Curves 3 and 4 are tracings of periodic acid–Schiff-stained gels of thymus and spleen, respectively There are quantitatively less Components b and c (175 000 and 145 000 daltons) in thymic than in splenic membranes Component h (27 000 daltons) is a major, unique glycoprotein in thymic lymphocytes (Curves 1 and 3) and is a minor component in splenic lymphocytes (Curve 2) Component i probably represents membrane glycolipids or unsaturated lipids (about 8000 daltons), since it is stained only with the periodic acid–Schiff reagent (Curves 3 and 4) and with Sudan black

splenic and thymic membranes, although there was less precipitate from thymic membranes This finding does not necessarily mean that the surface immunoglobulins are related, since the antiserum contained light chain specificity and different classes of immunoglobulin may have been precipitated by reaction of the anti-IgG antibody with their light chains

DISCUSSION

The major differences between thymic and splenic lymphocyte membranes lie in the components above 200 000 daltons, the immunoglobulins and the 27 000-dalton glycoprotein (Figs 1 and 2) There were no significant differences in either the chemical composition or in the enzymatic activities (Table II).

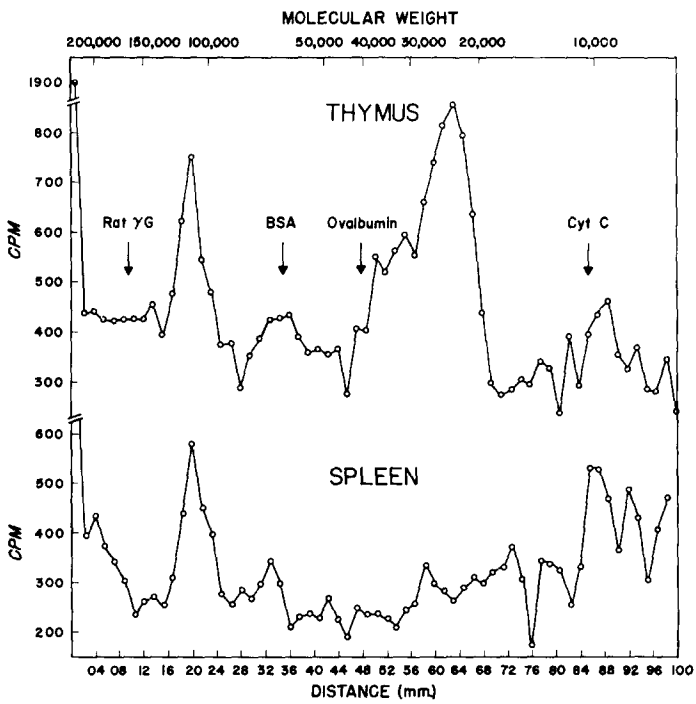

Fig 2. The pattern of labeled external plasma membrane proteins from surface iodinated thymocytes and splenic lymphocytes after electrophoresis in 7 5 % polyacrylamide gels containing 0 5 % sodium dodecylsulphate Each point represents the counts in one gel slice and is the average of several experiments The molecular weight scale at the top is derived experimentally by calibration with 10 proteins of known molecular weight, and the relative mobilities of four of these standards are indicated by arrows The distance of migration from the origin is indicated on the scale at the bottom

Membrane-bound immunoglobulins radioiodinated by the lactoperoxidase method [17, 27] have been studied in the lymphocytes of human and mouse thymuses [28], mouse spleen [28, 29] and cultured human lymphocytes [30]. Both IgM and IgG have been isolated from spleen cells, and there is still some disagreement about the presence of immunoglobulins on thymus. Our studies to date indicate that there are immunoglobulins on the splenic membrane and probably also on the thymic membrane The 200 000-dalton component on both membranes may be monomeric IgM similar to that obtained from mouse lymphocyte membranes The 117 000-dalton component is a lower weight than that observed for serum immunoglobulin (160 000 daltons), and it may represent an unique molecular species of membrane-bound immunoglobulin.

These studies have established a basic framework for subsequent investigation into the control of the immune response at the level of the membrane interaction with antigen and for the investigation of genetic, maturational and functional differences among lymphocytes.

ADDENDUM

When unreduced rat myeloma IgM was analyzed by electrophoresis in sodium dodecylsulphate, as described, we observed one species having an apparent molecular weight of 115 000. After reduction with dithiothreitol, only two subunits were found with apparent molecular weights of 95 000 and 27 000 These correspond closely to the expected molecular weights of heavy and light chains, respectively Therefore, the molecular species of unreduced IgM having an apparent molecular weight of 115 000 probably represents a half-molecule consisting of one heavy and one light chain It is possible that the lymphocyte membrane immunoglobulin component having apparent molecular weight of 117 000 represents a half-molecule of membrane-bound IgM

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