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# LYMPHOCYTE PLASMA MEMBRANES

# III. COMPOSITION OF LYMPHOCYTE PLASMA MEMBRANES FROM NORMAL AND IMMUNIZED RATS

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## SUMMARY

Isolated plasma membranes of thymic and splenic lymphocytes from unimmunized and immunized rats of the inbred ACI and F344 strains were analyzed for chemical and enzymatic composition, for membrane protein patterns by polyacrylamide gel electrophoresis and for membrane-associated immunoglobulins. After immunization, the thymic and splenic lymphocyte membranes from F344 rat contained less carbohydrate and higher phospholipid contents than control animals. In both ACI and F344 inbred rat strains the membrane phospholipid to cholesterol weight ratio increased significantly after immunization. The electrophoretic patterns of solubilized membrane proteins and of iodinated external membrane proteins were similar in unimmunized and immunized animals.

When thymic and splenic lymphocytes of normal or immunized animals were surface radioiodinated, solubilized in Triton X-100, NP-40 or 10 M urea in 1.5 M acetic acid and analyzed by immunoprecipitation, labeled IgM immunoglobulin was recovered from thymic lymphocytes but both labeled IgG and IgM were recovered from splenic lymphocytes. However, when unlabeled isolated plasma membranes were solubilized in 1 % Triton X-100 and analyzed by immunodiffusion in agarose gels, both IgG and IgM were identified in thymic and splenic cells.

## INTRODUCTION

The initiating event in the antibody response is believed to be the interaction of antigen with a specific receptor on the plasma membrane of an immunocompetent cell (1). The system in which we have chosen to investigate this process is the response to poly ($Glu^{52}Lys^{33}Tyr^{15}$) in the highly responding ACI and the poorly responding F344 rat strains. Our overall approach is to study lymphocytes from different organs of these animals and to determine whether there are any genetically determined differences in membrane structure which correlate with their different abilities to respond to this antigen.

Various methods have so far been used for studying the plasma membrane of lymphocytes which include isolation of the plasma membrane followed by chemical and enzymatic analyses [2, 3], sodium dodecylsulfate-polyacrylamide gel electrophoresis [2, 4], identification of surface components by immunological methods [5], ultrastructural studies [6] and surface iodination [7, 8]. These studies have been reviewed recently [9]. Some controversy exists about the nature and identity of immunoglobulins on the surface of lymphocytes from the spleen and the thymus, and much of this has been attributed to the different detergents used, since each may solubilize the membrane in a different way [7].

This paper presents studies in three areas. First, the chemical and enzymatic composition and the patterns of membrane components in polyacrylamide gel electrophoresis were determined before and after immunization in the highly responding ACI and the poorly responding F344 strains of rats. Secondly, the effects of various detergents in solubilizing the plasma membranes of splenic and thymic lymphocytes were examined. Finally, the immunoglobulins on the lymphocytes from the spleen and thymus of unimmunized and immunized animals were studied by surface radioiodination of whole cells and by immunoprecipitation or immunodiffusion of isolated membranes.

## MATERIALS AND METHODS

ACI and F344 inbred rats(Microbiological Associates, Walkersville, Md.) 10–12 weeks old were immunized subcutaneously with 0.75 mg poly ($Glu^{52}Lys^{33}$-$Tyr^{15}$) dissolved in 0.25 ml of distilled water and emulsified with an equal volume of Freund's complete adjuvant containing additional tubercle bacilli (3 mg/ml final concentration). A second injection of 0.75 mg in 1.0 ml water was administered intraperitoneally 6 days later, and the animals were sacrificed at the peak of the immune response 5 days later.

The spleens and thymuses were collected at 4 °C in basal Eagle's tissue culture medium (Flow Laboratories, Rockville, Md.) adjusted to pH 7.4 with 10 mM Tris buffer. Unimmunized littermates were sacrificed simultaneously to provide normal cells for comparative study. Spleens and thymuses were minced and washed through a coarse mesh screen. The cells were suspended in culture medium at 4 °C, layered over a 2:1 mixture of 9 % Ficoll (Pharmacia Fine Chemicals, Uppsala, Sweden) and 29.9 % Hypaque (Winthrop Laboratories, New York, New York) and centrifuged for 30 min at $300 \times g$. The cells at the interface were collected, resuspended at 4 °C in 0.15 M NaCl/10 mM Tris buffer, pH 7.4, and washed twice. Lymphocyte yields averaged $4 \cdot 10^7$ cells per spleen and $8 \cdot 10^7$ cells per thymus. The average viability as determined by trypan blue dye exclusion was 97–98 %.

Anti-brain serum cross-reacts with thymic lymphocytes in several species [10] and this can be used as a criterion for T-lymphocytes [11]. When indirect immunofluorescence was performed with rabbit anti-rat brain serum, results showed that 100 % of thymic lymphocytes were intensely positive, whereas splenic lymphocytes comprised only 50 % positive cells (Fig. 1). Enumeration was based routinely on counts of at least 200 lymphocytes in each sample preparation. This finding is consistent with those in other species [12, 13]. Histological examination of the thymuses also confirmed that there was no contamination with perithymic lymph nodes. Isolation of lymphocyte

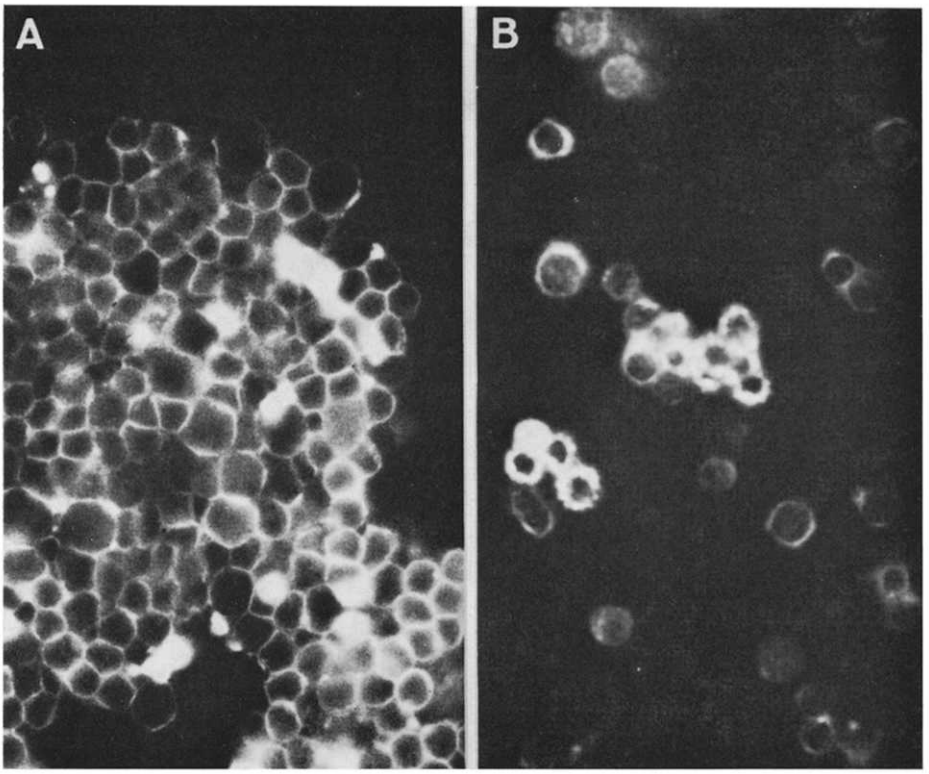


Fig. 1. Indirect immunofluorescence photomicrographs of purified unfixed lymphocyte suspensions from rat thymus (panel A) and rat spleen (panel B). Lymphocytes were incubated for 20 min with rabbit anti-rat brain serum, washed and then incubated with fluorescein-labeled goat anti-rabbit IgG for 20 min. All incubation was carried out at 4 °C. All thymic lymphocytes were uniformly stained over the entire surface, whereas only 50 % of spleen lymphocytes were positive and these cells showed only discontinuous or patchy fluorescence. Negative spleen lymphocytes are visible in the periphery of the field (panel B) surrounding the positive cells in the center of the field.

plasma membranes and biochemical and enzymatic determinations were performed as described previously [2].

The cells suspended in 10 mM Tris/0.15 M NaCl were 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 NaCl, and the cell suspension was homogenized in a Potter-Elvejhem homogenizer. The homogenate was centrifuged at $300 \times g_{av}$ for 10 min to pellet the nuclei. The supernatant was centrifuged at $4000 \times g_{av}$ for 20 min to pellet down the mitochondria. The resulting supernatant was centrifuged at $20\,000 \times g_{av}$ for 1 h to pellet the microsomal fraction. The latter was dissolved in 10 mM Tris · HCl (pH 7.4), mixed with sucrose solution to make the resulting sucrose concentration 30 % and put on a 40 % sucrose solution. The sample was centrifuged at $90\,000 \times g_{av}$ for 15 h. The 30–40 % sucrose interphase was pooled, washed 3 times with 10 mM Tris · HCl buffer (pH 7.4). This fraction was found to be the plasma membranes as judged by morphology and chemical and enzymatic criteria as described in the Results section.

The intact lymphocytes were surface labeled with carrier-free $^{125}$I-iodide (New England Nuclear, Boston, Mass.) by the lactoperoxidase method under conditions similar to those described by Cone and Marchalonis [7]. Aliquots of $1 \cdot 10^8$ cells were suspended in 1.85 ml 0.15 M NaCl, 10 mM phosphate buffer, pH 7.4, containing 100 $\mu$g lactoperoxidase (EC 1.11.1.7) (Sigma Chemical Company, St. Louis, Mo., 45 units activity/mg), 0.5 M KI and 1 mCi $^{125}$I-iodide which was adjusted to pH 7.0 prior to use. The mixture was warmed to 30 °C, and the pH was maintained at 7.2–7.4. Three 50 $\mu$l aliquots of 10 mM $H_2O_2$ were added at 5 min intervals with constant shaking of the cell suspension.The reaction was stopped by the addition of 35 ml phosphate buffered saline at 4 °C. The cells were washed four times in phosphate buffered saline, and 95–96 % remained viable as assayed by the trypan blue dye exclusion test.

Three methods of solubilization of cell surface components were employed. In the first method, intact radioiodinated cells ($1 \cdot 10^8$ in 0.5 ml) were shaken with 0.5 ml of 1 % (v/v) Nonidet P-40 (NP-40, Shell Chemical Co., New York, N. Y.) or 1 % (v/v) Triton X-100 (Sigma Chemical Co.) for 10 min at room temperature. The suspension was then centrifuged at $1300 \times g$ for 30 min at 4 °C, and the supernatant was retained. In the second method, $1 \cdot 10^8$ labeled cells were incubated with 5 ml 10 M urea/ 1.5 M acetic acid for 1–2 h at 37 °C with periodic shaking. The mixture was centrifuged at $1300 \times g$ for 30 min, and the supernatants were extensively dialyzed at 4 °C against 0.5 M Tris · HCl/0.15 M NaCl, pH 8.0, for 24 h with three changes of the buffer. The dialyzed material was centrifuged at $15\,000 \times g$, and the supernatant was retained. In the third method, the cells were radiolabeled and the plasma membranes were isolated, as described previously [2]. The membranes from $2 \cdot 10^8$–$5 \cdot 10^8$ cells were suspended in 1 ml of buffer containing 2 % sodium dodecylsulfate and heated at 70 °C for 1 h to effect solubilization.

All these samples were resolved on 10 cm 7.5 % polyacrylamide gels containing 0.19 % bisacrylamide and 0.5 % sodium dodecylsulfate, using the method previously described [2]. The gels were then sliced into 60–64 fractions with a Canalco slicer (Canalco, Inc., Rockville, Md.), and the slices were counted in glass tubes using a Packard Gamma Scintillation Spectrometer (Model 5975). Mobilities were calculated relative to that of a cytochrome *c* marker which was labeled with $^{125}$I and added to the samples or run simultaneously in a separate gel. The calibration curve for molecular weight was determined using proteins of known molecular weights.

Rat IgG was prepared from an 18 % sodium sulfate precipitate of normal rat serum. The precipitate was redissolved and chromatographed on a Sephadex G-200 column ($2.5 \times 30.0$ cm) using 0.15 M sodium borate, pH 8.0, as the eluant. The second peak was lyophilized, redissolved in a minimal volume of distilled water, dialyzed against 0.15 M sodium borate buffer, pH 8.0, and rechromatographed on Sephadex G-200; the second peak was again retained. The sample was demonstrated to be pure $IgG_2$ by immunoelectrophoretic analysis using rabbit anti-rat antiserum. A 1.0 mg sample of rat IgG was emulsified in complete Freund's adjuvant and injected intradermally into a rabbit. A second intradermal injection of 1.0 mg of rat IgG in water was given two weeks later, and the rabbit was bled 10 days thereafter.

In order to prepare $\gamma$-chains, purified IgG was reduced with 2-mercaptoethanol, alkylated with iodoacetamide and chromatographed on a Sephadex G-100 column, ($1.0 \times 25.0$) in 0.5 M propionic acid [14]. Polyacrylamide gel electrophoresis of the

material in the second peak gave a single band with a molecular weight of approx. 60 000. Double diffusion in gel of this sample against rabbit anti-rat IgG demonstrated its partial antigenic identity with IgG, which indicated that the material was pure γ-chain. Antiserum to rat γ-chain was produced by injection of a rabbit following the schedule described above. The anti-rat γ-chain antiserum was further treated by absorption two times with IgM coupled to Affi-Gel 10 beads (Bio-Rad Laboratories, Richmond, Ca.) and then three times with purified rat light chains in order to assure its specificity. This serum was monospecific for rat IgG when tested against whole rat serum by immunoelectrophoresis.

Rat IgM monoclonal protein was purified from the serum of LOU/Wsl rats transplanted with ileocecal immunocytomas (IR202) [15]. A rabbit was immunized with purified rat myeloma IgM by the schedule described above, except that 0.5 mg of protein was used for each injection. The serum was absorbed with chromatographically purified rat light chains and then with a rat IgG immunoadsorbant [16]. Its specificity was demonstrated by immunoelectrophoresis and by double diffusion in gel. The concentrations of the rabbit antibody to the IgG and IgM were each determined by quantitative precipitin reactions.

Aliquots (0.1–0.2 ml) of solubilized radioiodinated cell surface components were coprecipitated with specific antiserum and carrier IgG or IgM following a method similar to that of Cone and Marchalonis [7] and Vitetta et al. [8]. Rat IgM (25μg) and/or IgG (25 μg) was added as the carrier to a radiolabeled sample of membrane in a glass tube coated with 1 % normal rabbit serum, and then 100 μl of the appropriate antiserum, diluted to provide a 25–50 % antibody excess, was added. The mixture was incubated for 1 h at 22 °C and for two days at 4 °C with daily shaking. A membrane preparation to which methylated bovine serum albumin and rat antiserum to methylated bovine serum albumin were added served as the control. The precipitates were washed with phosphate buffered saline at least 5 times; the counts in the control precipitate were generally twice the background level. The washed precipitates were then resuspended in 0.1 ml phosphate buffered saline and incubated for 1 h with 2 % sodium dodecylsulfate at 70 °C. The components were separated by polyacrylamide gel electrophoresis, and the gel was sliced and counted as described above.

The membrane preparations were analyzed for IgM and IgG alternatively by double diffusion in gel. Isolated membrane from splenic lymphocytes (300 μg protein) or from thymic lymphocytes (800 μg protein) was solubilized in 2 % Triton X-100. Monospecific rabbit anti-rat IgM, anti-rat IgG and anti-rat γ-chain antisera, prepared as described above, were used. Membrane samples and monospecific antisera were placed in wells in 1.5 % agar plates, and the plates were incubated at room temperature for 72 h, washed with 3 changes of 1 % NaCl for 24 h, fixed in 10 % acetic acid for 2 h and stained with Coomassie blue.

## RESULTS

### *Chemical and enzymatic analysis*

The purity of the plasma membrane fractions was demonstrated by the lowest DNA (5–11 μg/mg protein) and RNA (17–32 μg/mg protein) content and the highest cholesterol to phospholipid molar ratio (0.74–0.91) of all fractions, as well as by enzymatic criteria. Table I shows the enzyme composition of the different fractions of

TABLE I

ENZYME COMPOSITION OF THE DIFFERENT FRACTIONS OF RAT SPLEEN LYMPHOCYTES

The results represent the average values from two typical experiments. Specific activities represent μmol of product liberated/h per mg protein in the case of 5′-nucleotidase, glucose-6-phosphatase and acid phosphatase, and μmol of product liberated/min per mg protein in the case of NADH-dehydrogenase. In the case of succinate dehydrogenase the specific activity is expressed as change of absorbance/min per mg protein.

| Enzyme | Homogenate | Nuclear pellet | Mitochondrial pellet | 20 000 × *g* Supernatant | Sucrose bottom | Plasma membrane | Total recovery as percent of homogenate content |
|---|---|---|---|---|---|---|---|
| 5′-Nucleotidase | | | | | | | |
| recovery (%) | | 2.42[a] | 24.1 | 34.0 | 19.0 | 18.5 | 98.1 |
| specific activity | 0.62 | 0.53 | 0.76 | 0.64 | 1.43 | 7.55 | |
| Succinate dehydrogenase | | | | | | | |
| recovery (%) | | 3.93 | 48.0 | 34.8 | 13.2 | 0.56 | 100.5 |
| specific activity | 0.023 | 0.036 | 0.859 | 0.012 | 0.105 | 0.019 | |
| Acid phosphatase | | | | | | | |
| recovery (%) | | 3.11 | 27.4 | 45.4 | 13.9 | 0.60 | 90.4 |
| specific activity | 1.65 | 0.80 | 3.10 | 1.10 | 2.20 | 1.06 | |
| Glucose-6-phosphatase | | | | | | | |
| recovery (%) | | 4.50 | 7.70 | 58.6 | 1.64 | 0.53 | 73.0 |
| specific activity | 0.38 | 0.34 | 0.40 | 0.36 | 0.29 | 0.23 | |
| NADH dehydrogenase | | | | | | | |
| recovery (%) | | 11.4 | 28.0 | 39.0 | 16.4 | 3.1 | 97.7 |
| specific activity | 0.08 | 0.14 | 0.30 | 0.04 | 0.44 | 0.23 | |

[a] Recovery expressed as percent of homogenate.

TABLE II

THE CHEMICAL AND ENZYMATIC COMPOSITION OF ISOLATED PLASMA MEMBRANES FROM THE HIGHLY RESPONDING ACI STRAIN OF INBRED RATS BEFORE AND AFTER IMMUNIZATION WITH POLY ($Glu^{52}Lys^{33}Tyr^{15}$)

The results are the averages from 3–7 determinations using cells from male rats.

| Component | Spleen | | | | Thymus | | | |
|---|---|---|---|---|---|---|---|---|
| | Unimmunized | | Immunized | | Unimmunized | | Immunized | |
| | Mean | ±S.D. | Mean | ±S.D. | Mean | ±S.D. | Mean | ±S.D. |
| Carbohydrate ($\mu$g/mg protein) | 96.6 | 10.8 | 82.5 | 16 | 70.2 | 7.1 | 70.6 | 14.4 |
| Lipid ($\mu$g/mg protein) | 793 | 40 | 727 | 58 | 975 | 60 | 819 | 55 |
| Cholesterol ($\mu$g/mg protein) | 234* | 3 | 182 | 6 | 223 | 6 | 182 | 11 |
| Phospholipid ($\mu$g/mg protein) | 533 | 6 | 495 | 43 | 493 | 4 | 542 | 78 |
| 5′-Nucleotidase ($\mu$mol product/h/mg protein) | 10.23 | 2.35 | 9.15 | 0.23 | 5·81 | 1.88 | 7.10 | 0.29 |

* Difference between unimmunized and immunized animals is significant at $P \leq 0.01$

TABLE III

THE CHEMICAL AND ENZYMATIC COMPOSITION OF ISOLATED PLASMA MEMBRANES FROM THE POORLY RESPONDING F344 STRAIN OF INBRED RATS BEFORE AND AFTER IMMUNIZATION WITH POLY ($Glu^{52}Lys^{33}Tyr^{15}$)

The results are the averages from 3–4 determinations using cells from male rats.

| Component | Spleen | | | | Thymus | | | |
|---|---|---|---|---|---|---|---|---|
| | Unimmunized | | Immunized | | Unimmunized | | Immunized | |
| | Mean | ±S.D. | Mean | ±S.D. | Mean | ±S.D. | Mean | ±S.D. |
| Carbohydrate (μg/mg protein) | 101.1* | 6.7 | 80.1 | 9.1 | 82.7* | 2.7 | 67.7 | 3.2 |
| Lipid (μg/mg protein) | 772* | 36 | 856 | 30 | 825 | 91 | 880 | 41.3 |
| Cholesterol (μg/mg protein) | 228 | 6 | 258 | 20 | 232 | 15 | 227 | 9 |
| Phospholipid (μg/mg protein) | 500* | 39 | 587 | 34 | 534* | 18 | 611 | 14 |
| 5′-Nucleotidase (μmol product/h/mg protein) | 8.06* | 1.13 | 6.0 | 0.4 | 11.4 | 2.4 | 8.9 | 0.9 |

* Difference between unimmunized and immunized animals is significant at $P \leq 0.01$.

rat spleen lymphocytes. The specific activity of 5'-nucleotidase, which we have shown to be a plasma membrane marker for rat lymphocytes [17] was consistently 10–15 times higher than in the homogenates. The specific activity of succinate dehydrogenase (EC 1.3.99.1), the well-known marker for mitochondria, is about 50 times less compared to that in the mitochondrial pellet. The lysozomal enzyme, acid phosphatase (EC 3.1.3.2) has lower specific activity in the plasma membrane than in the mitochondrial and sucrose bottom pellet. Only less than 1 percent of the total homogenate activity of this enzyme is in the membrane fraction. Glucose-6-phosphatase (EC 3.1.

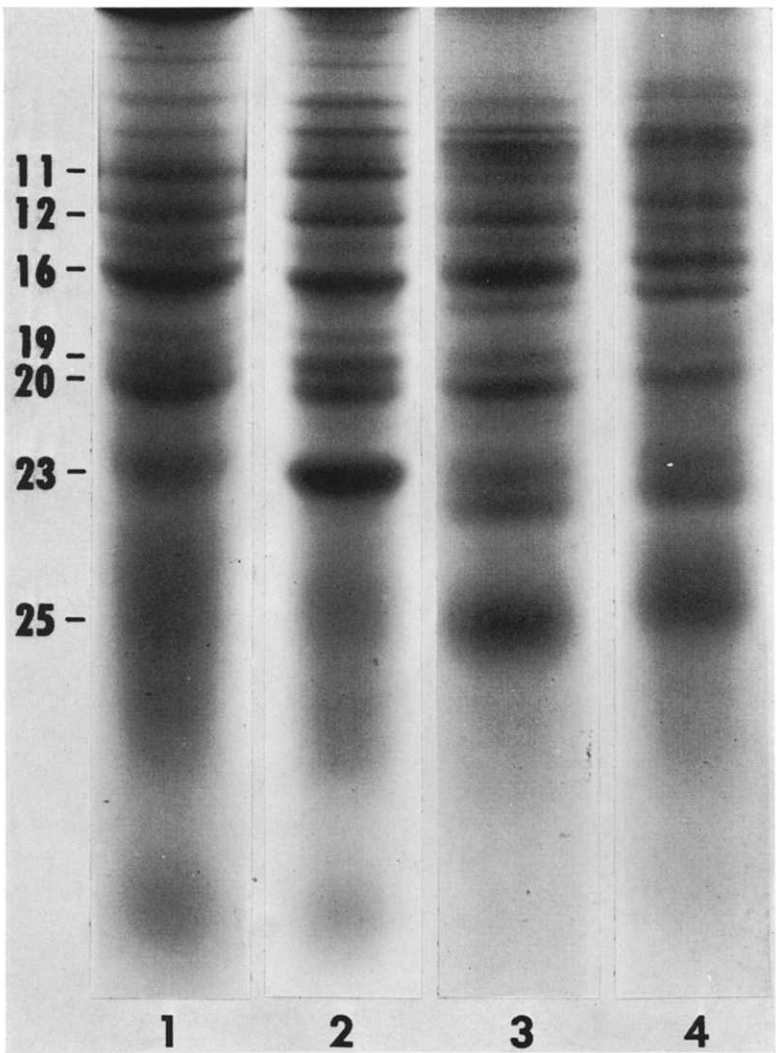


Fig. 2. Sodium dodecylsulfate-polyacrylamide gel electrophoresis patterns of rat lymphocyte plasma membranes. Gels 1 and 2 show the patterns for splenic cell membranes from unimmunized and immunized animals, respectively. Gels 3 and 4 are the patterns for thymocyte membranes from unimmunized and immunized animals, respectively. Several major protein bands are numbered corresponding to the bands in the densitometric tracings in Fig. 3.

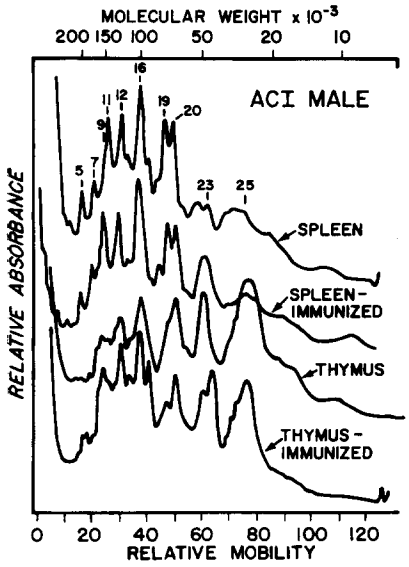


Fig. 3. Densitometric tracings of Coomassie blue-stained sodium dodecylsulfate-polyacrylamide gels (7.5 %) following electrophoresis of isolated plasma membranes from unimmunized and from immunized rats.

3.9) and NADH dehydrogenase (EC 1.6.99.3), the marker enzymes for endoplasmic reticulum, show more or less even distribution of their specific activities in all the fractions. However, the content of these enzymes in the plasma membrane fraction is low, an indication of purification of the plasma membrane fraction from endoplasmic reticulum compared to other fractions. Similar order of purification was found in the membrane preparations from both unimmunized and immunized animals.

The chemical and enzymatic analyses of the membranes from the ACI strain are shown in Table II, and those from the F344 strain are shown in Table III. There are no significant differences between normal animals of the two strains but there are some differences between the membranes from nonimmunized and immunized animals, the most striking being the statistically significant increase in the phospholipid content and decrease in the carbohydrate content in the membranes of immunized F344 rats.

*Sodium dodecylsulfate polyacrylamide gel electrophoresis*

The patterns of isolated membrane components obtained by sodium dodecylsulfate-polyacrylamide gel electrophoresis in unimmunized and immunized rats are illustrated in Figs 2 and 3 for ACI male rats. A large number of protein components were found, and the major ones are identified by number. There is no significant difference in the protein patterns of the membranes from the spleens or thymuses when comparing unimmunized and immunized animals. In both strains, the thymic and the splenic membrane patterns differ at bands 5, 7, 9, 11, 19 and 25. In thymic membranes, bands 9 and 11 are much smaller but band 25 is much more prominent. The latter band is a glycoprotein giving an intense reaction with periodic acid Schiff

TABLE IV

COMPARISON OF SURFACE $^{125}$I-LABELED COMPONENTS OF LYMPHOCYTE MEMBRANES PREPARED BY DIFFERENT SOLUBILIZATION PROCEDURES (cpm $\times 10^{-3}$)

| Component number* | 3 | 5 | 7 | 11 | 14 | 16 | 17 | 18 | 20 | 21 | 24–27 | 30 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Apparent molecular weight $\times 10^{-3}$ | 200 | 200 | 172 | 138 | 110 | 100 | 92 | 80 | 65 | 58 | 35–20 | 10** |
| Spleen, solubilized in | | | | | | | | | | | | |
| SDS | | 2.5 | 2.0 | 1.6 | 2.8 | | | 1.6 | 1.9 | | | 3.0–2.5 |
| NP-40 | 2.9 | | 2.8 | 2.1 | 3.5 | | 4.5 | | 3.4 | | 3.1–3.5 | 10–15 |
| Triton X-100 | | | 2.7 | 4.2 | 3.5 | | 3.0 | | 3.4 | 3.2 | 3.3–3.5 | 10–20 |
| Thymus, solubilized in | | | | | | | | | | | | |
| SDS | | | 1.8 | 3.2 | 1.3 | 1.3 | | | 1.6 | 1.7 | 2.5–4.0 | 1.7–1.2 |
| NP-40 | | | 2.5 | 2.5 | 3.2 | 2.8 | 2.6 | 2.6 | 3.2 | | | 15–25 |
| Triton X-100 | | 1.1 | 1.4 | 2.1 | 2.0 | 1.6 | 1.3 | 1.4 | 1.7 | 1.4 | | 15–20 |

* Refers to component number on polyacrylamide gels stained with Coomassie blue as in Figs 2 and 3.

** Lipid or glycolipid.

reagent. Similar results were obtained with ACI females and with both sexes of the F344 strain.

*Surface radioiodination of lymphocytes and analyses of the surface components*

The solubilization of radioiodinated intact lymphocytes was done with NP-40, Triton X-100 or acid-urea. After incubation with NP-40 or Triton X-100 and centrifugation at $1300 \times g$, the supernatants contained 57 % of the label, and the nuclear and mitochondrial pellets contained 43 %. Acid-urea treatment solubilized 90 % of the label, but when the supernatant was dialyzed, 85 % of the label appeared in the dialysate and 5 % was nondialyzable: one-half of the nondialyzable material was sedimented after centrifugation at $1300 \times g$ for 45 min.

Two procedures were employed for the examination of the cell surface components by sodium dodecylsulfate polyacrylamide gel electrophoresis. In the first, intact radioiodinated lymphocytes were solubilized with 0.5 % NP-40 or 0.5 % Triton X-100. In the second, radioiodinated membranes were isolated by sucrose gradient centrifugation, and the membrane components were solubilized by heating in 2 % sodium dode-

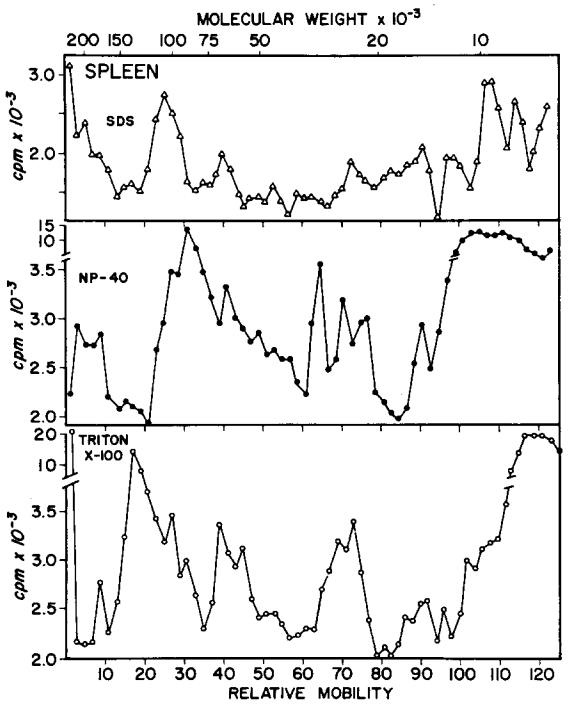


Fig. 4. Electrophoretic analysis of $^{125}$I-labeled surface components of splenic lymphocytes from ACI female rats resolved in sodium dodecylsulfate-containing polyacrylamide gel (7.5 %). Upper panel, solubilization of isolated membranes by sodium dodecylsulfate; middle panel, solubilization of intact cells by NP-40; and lower panel, solubilization of intact cells by Triton X-100. In all three graphs, the material placed on the gel was obtained from $5\text{–}10 \times 10^7$ cells. After solubilization and electrophoresis, the gels were sliced into 60–64 pieces and counted. The background was subtracted in each case (50–100 cpm). The break in the scales refers to the peak with 100–120 mm relative mobility only.

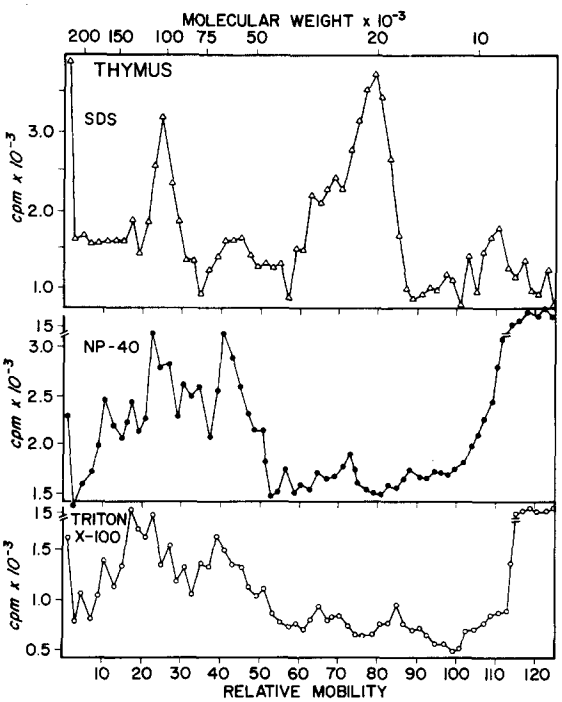


Fig. 5. Electrophoretic analysis of $^{125}$I-labeled surface components of thymic lymphocytes from ACI female rats. The conditions are the same as in Fig. 4.

cylsulfate. Figs 4 and 5 represent the consistent patterns of at least five experiments in which the various preparations were analyzed by polyacrylamide gel electrophoresis. A summary of the major bands and their relative intensities following the different solubilization procedures is given in Table IV. The electrophoretic patterns of the labeled membrane proteins from surface iodinated cells solubilized in sodium dodecylsulfate, NP-40 or Triton X-100 show quantitative differences, but there is a remarkable similarity with respect to the prominent bands (Figs 4 and 5). The most remarkable finding is that the thymus specific glycoprotein (band 25, Fig.2) is readily solubilized from the isolated membranes by sodium dodecylsulfate, but it cannot be extracted by Triton X-100 or NP-40 (shown in band region 24 to 27 in Table IV). As shown in Table IV, the prominent common surface component in all these samples has an *M*r of 110000 (band 14). There are some differences between the patterns of splenic and thymic cell membranes in some minor components as well as in the intensity of labeling. (Table IV). There were no significant differences in external membrane proteins between unimmunized and immunized animals of either the ACI or F344 strains.

*Immunoprecipitation studies on $^{125}$I-labeled surface components*

Variable results were obtained with immunoprecipitation of NP-40 or Triton X-100 solubilized cells. When labeled membrane components with the mobilities of immunoglobulins were obtained, the counts were generally low. When cells were solubilized in acid-urea and precipitation performed with anti-rat immunoglobulins, la-

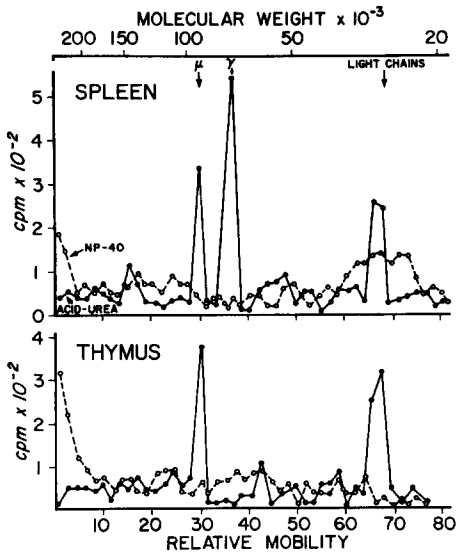


Fig. 6. Electrophoretic analysis of reduced, $^{125}$I-labeled surface immunoglobulin in sodium dodecyl-sulfate-polyacrylamide gel (7.5 %). The labeled cells ($5 \cdot 10^7$) were solubilized with NP-40 or acid-urea, and the preparations were then dialyzed. The precipitations were performed with anti-rat IgM and anti-rat IgG or anti-rat $\gamma$-chain. The symbols $\gamma$, $\mu$ and light chains refer to relative mobilities of rat immunoglobulin chains under the same conditions. The background was subtracted, as above.

beled $\mu$ and $\gamma$-chains were present in splenic lymphocytes but only $\mu$-chains were present in the thymus; labeled light chains were present in both preparations. The results are illustrated in Fig. 6. No differences were found between ACI or F344 strains when comparing either unimmunized or immunized animals.

*Immunodiffusion studies*

The results of the double diffusion using heavy-chain specific rabbit anti-rat IgG and anti-rat IgM antisera and solubilized lymphocyte membranes are illustrated in Figs 7 and 8. IgM is readily detected in the membranes of thymic and splenic cells from both unimmunized and immunized animals. The same amount of anti-IgM serum was used with 300 $\mu$g of splenic membrane protein or 800 $\mu$g of thymic cell membrane protein. Nevertheless, the thymic membrane IgM band is more prominent in the immunized than in control animals (Figs 7 and 8, A and D). This finding suggests that the IgM concentration in the thymic lymphocyte membranes is higher in the immunized animals than in the control animals. The most surprising result is that anti-IgG antiserum gave prominent precipitin lines against thymic as well as splenic membranes from both unimmunized and immunized animals (Figs 7 and 8, B and E). Splenic and thymic lymphocyte membranes from animals after primary immunization only also showed IgG (Figs 7 and 8, F). In addition, anti-$\gamma$-chain antiserum gave precipitin lines with both thymic and splenic cell membrane preparations from unimmunized and immunized animals (Figs 7 and 8, C and G), but they were weaker in the unimmunized animals. When solubilized plasma membranes were assayed by immunodiffusion against various sera and proteins in order to test for nonspecific reactivity, no immu-

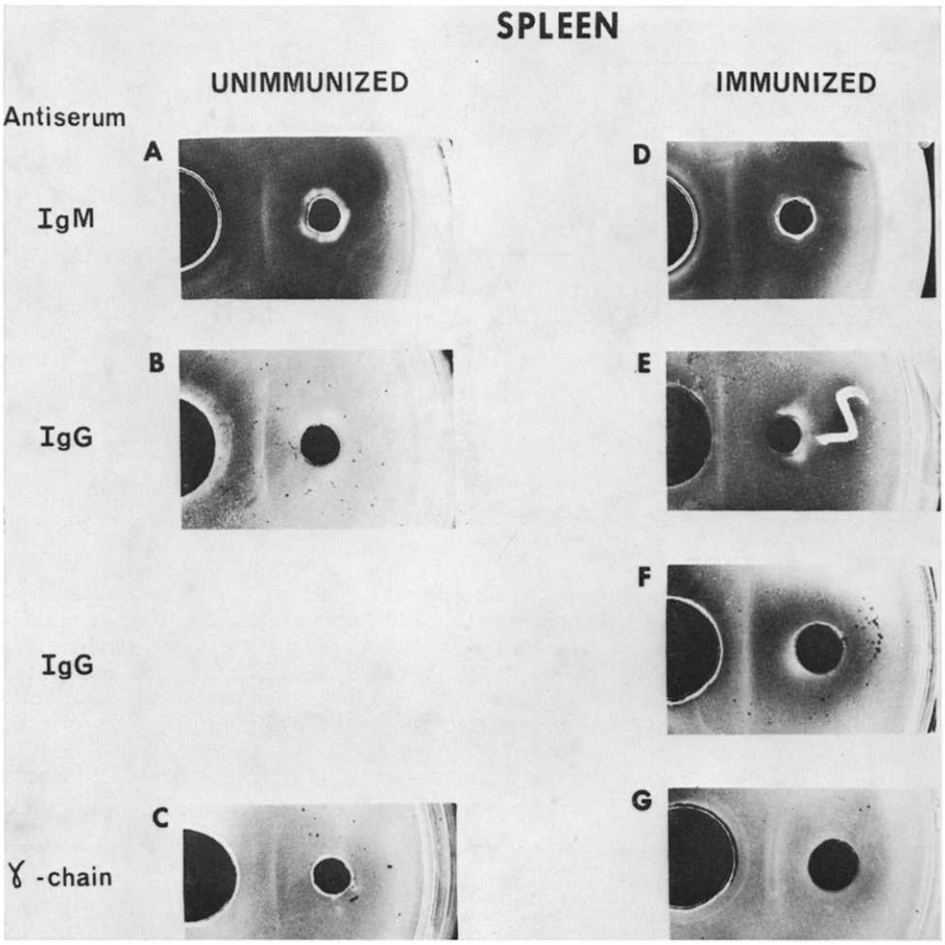


Fig. 7. Double diffusion in 1.5–2.0 % agarose gel of isolated splenic lymphocyte membranes. The isolated membrane (300 $\mu$g protein) was solubilized in 1 % Triton X-100 and was placed in the large wells (necessary to allow the proteins to be in dilute solution). The membrane preparations were: A, B, and C, membranes from unimmunized rats; D, E and G, membranes from rats after standard immunization with the synthetic polypeptide (see text); and F, membranes from rats immunized with a single injection of synthetic polypeptide in Freund's complete adjuvant. The antisera were: A and D, antiserum monospecific for IgM; B, E and F antiserum prepared against IgG; and C and G, antiserum monospecific for $\gamma$-chain. Adjacent wells, where seen, contain varying dilutions of the same antiserum.

nodiffusion bands appeared (Fig. 9). Hence, there is no artefactual precipitation of solubilized membrane components in the agarose gel, and these results confirm the specificity of the immunodiffusion patterns obtained with antisera to the membrane surface immunoglobulins.

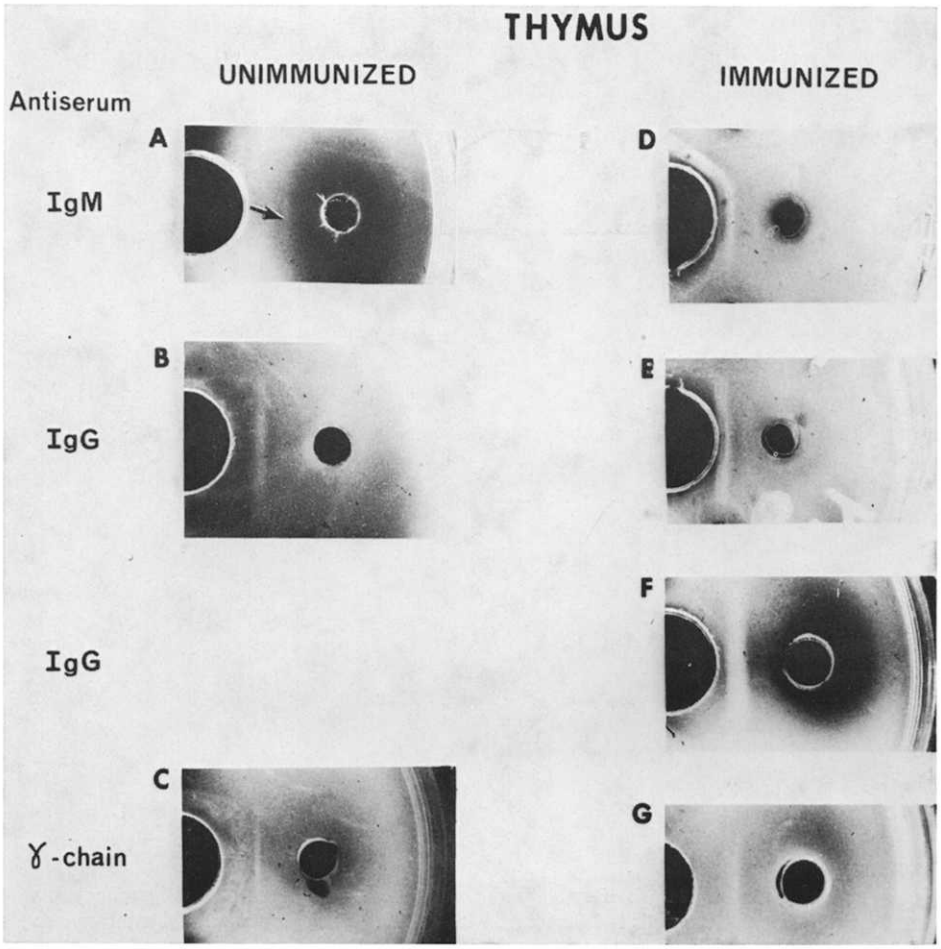


Fig. 8. Double diffusion in 1.5–2.0 % agarose gel of isolated thymic lymphocyte membranes (800 μg protein). Antiserum and membrane components as in Fig. 7.

## DISCUSSION

Since the thymus gland preparations were free of lymph node contamination histologically and the immunofluorescence analyses demonstrated a completely homogeneous population, the thymic cell suspensions were highly purified. The spleen cell suspensions consisted of an approximately equal mixture of thymus-derived and marrow-derived lymphocytes judged by immunologic criteria.

The purity of our isolated membranes was demonstrated by chemical and enzymatic analyses and by electron microscopy. The low concentrations of DNA, RNA and contaminant enzymes were also indicative of highly purified plasma membrane

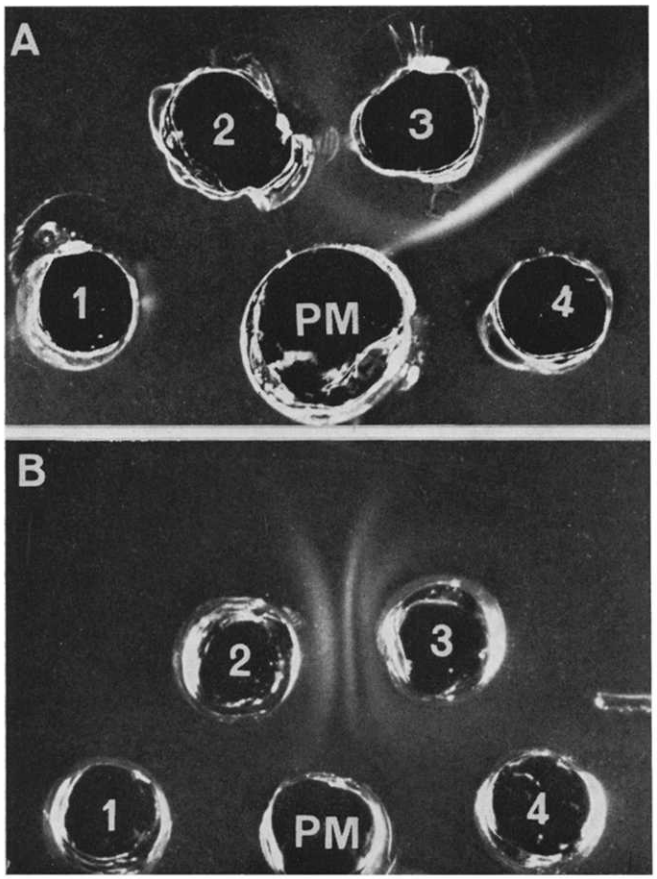


Fig. 9. Immunodiffusion analysis of solubilized plasma membranes from thymic lymphocytes with various sera. In panel A, the wells 1–4 were filled with normal guinea pig serum, rabbit anti-guinea pig fibrinogen, monospecific rabbit anti-rat IgG serum and normal rat IgG, respectively. Partial identity of membrane with IgG is demonstrated. In panel B, the wells 1 to 4 were filled with rat IgM myeloma protein, rabbit anti-sheep hemolysin, sheep erythrocyte ghosts solubilized in 0.5 % Triton X-100 and normal rat IgG, respectively. In both panels the central wells were filled with thymic lymphocyte plasma membrane solubilized in 1 % Triton X-100.

preparations. The primary change in the chemical composition of lymphocyte membranes after immunization (Tables II and III) was an increase in the phospholipid to cholesterol ratio. In immunized rats, there was a statistically significant increase in the phospholipid content in both the thymic and splenic lymphocyte membranes. In F344 animals, the phospholipid to cholesterol weight ratio increased from 2.30 to 2.69 in thymic lymphocytes, but it did not change significantly in splenic lymphocytes (2.19 to 2.27). In ACI animals, the phospholipid to cholesterol weight ratio increased after immunization from 2.21 to 2.97 in thymic lymphocytes and from 2.28 to 2.71 in splenic lymphocytes. Since this effect occurred in both strains, it may be due to the immunization with complete Freund's adjuvant rather than being related to genetically controlled strain differences in responsiveness to poly (Glu $^{52}$Lys$^{33}$Tyr$^{15}$). In support of this

conclusion, other studies [9, 18, 19] showed increased phospholipid synthesis in both thymic and splenic lymphocytes nonspecifically stimulated by mitogens.

The gel patterns of isolated membranes and of their $^{125}$I-labeled surface components did not show any marked difference between unimmunized and immunized rats. This finding may be due to the qualitative nature of these techniques, since only one spleen cell in $10^3$ in the high responding ACI strain makes antibody at the peak of the immune response, as measured by the plaque-forming technique [20]. Recently, Schmidt-Ullrich et al. [21] reported that there were no significant changes in the pattern of membrane proteins between normal and Concanavalin A-stimulated lymphocytes but that stimulation induced the rapid turnover of membrane proteins. Therefore, our findings of similar membrane protein patterns in lymphocytes from unimmunized and immunized animals does not preclude the possibility that membrane protein turnover is augmented in immunized animals.

There were no significant differences between unimmunized and immunized rats when comparing bands with the mobilities of surface immunoglobulins. In contrast, others [22–24] found that transformed T-cells have more readily detectable surface immunoglobulin molecules than nontransformed cells. Since we examined whole membrane preparations and not just surface macromolecules, we could identify both IgM and IgG immunoglobulins in unstimulated as well as stimulated T-cells which would be masked from detection by immunofluorescent techniques.

The evidence for the presence of IgM and IgG on splenic and thymic lymphocytes of unimmunized and immunized rats is: (a) immunodiffusion demonstrated both IgG and IgM immunoglobulins in Triton X-100 solubilized plasma membrane fractions and (b) acid-urea extraction of surface labeled lymphocytes followed by specific immunoprecipitation demonstrated $\mu$-chains and light chains on thymic and splenic membrane preparations and $\gamma$-chains also on splenic membrane preparations.

The demonstration of IgG on thymic lymphocyte membranes by double diffusion in gel was surprising, since surface labeling and immunoprecipitation detected only $\mu$-chains in the thymic lymphocyte preparations (100 % $\Theta$-positive cells). Any recirculating B-cells in the thymus would be less than 1 % and not sufficient to account for the quantity of IgG present. Furthermore, the preparation of thymic lymphocytes for both surface labeling experiments and for membrane isolation was identical to the point of detergent solubilization. Others [25–27] have demonstrated the presence of a receptor for aggregated and unaggregated IgG in $\Theta$-positive murine and human lymphoma lines and in peripheral murine T-cells, but they have not demonstrated IgG to be an integral component of the membrane. Our results suggest that the IgG may indeed be a membrane component but one which is not generally exposed on the outer surface of the membrane.

The possibility that cytophilic immunoglobulin is present on the lymphocytes cannot be excluded, but the conclusion that such adsorbed immunoglobulins can account for the findings of immunoglobulin in the isolated membrane fractions seems unlikely. Firstly, the samples tested by immunodiffusion are purified plasma membrane fractions subjected to extensive washing during the fractionation and purification procedures. We expect that the membrane fractions therefore would be entirely free of contamination by adsorbed immunoglobulins or other proteins. Secondly, our recent efforts have shown that detectable immunodiffusion bands are obtained using diluted anti-immunoglobulin antisera and as little as 50 $\mu$g plasma membrane protein.

Since the limit of sensitivity of the immunodiffusion assay method is above 1 $\mu$g protein [28], the immunoglobulin content is at least 2 % of the total plasma membrane proteins. This finding is difficult to reconcile with contamination of purified membranes by adsorbed immunoglobulin.

Since proteins and glycoproteins are integral components of the plasma membrane, their characterization often depends upon solubilization into an aqueous solution. Ionic detergents (sodium dodecylsulfate), nonionic detergents (NP-40 and Triton X-100), which bind predominantly to lipids [29, 30], and chaotropic agents (acid-urea) were employed in this study. The results showed quantitatively and qualitatively different solubilization by the various agents (Figs 4 and 5, Table IV). For example, there is a prominent external glycoprotein in thymic lymphocytes with an apparent molecular weight of 27 000. This band is demonstrable in membranes solubilized by sodium dodecylsulfate but not by Triton X-100 or NP-40. Therefore, comparison of results obtained by different methods of solubilization is not valid, and each system must be tested individually for the type and extent of solubilization. This conclusion is illustrated by the results of specific immunoprecipitation after solubilization with either NP-40 or acid-urea. Immunoprecipitation of the NP-40 solubilized membranes yielded variable results in that bands with mobilities of immunoglobulins could not be demonstrated consistently. In contrast, immunoglobulins were consistently detected in acid-urea solubilized membranes. Although we used NP-40 in concentrations similar to those reported by Vitetta et al. [8] and by Grey et al. [25], who demonstrated the presence of immunoglobulins on the surface of B-cells but not T-cells, we analyzed the samples immediately after solubilization rather than after dialysis. This methodological difference may account for the variable results we obtained by NP-40 solubilization and immunoprecipitation.

Much controversy has surrounded the issue of immunoglobulins on the surface of thymic lymphocytes. Marchalonis and his colleagues have reported [7] that IgM immunoglobulin is detectable after acid-urea extraction, but poorly recovered after NP-40 solubilization. They concluded therefore that the failure to detect immunoglobulins using NP-40 detergent [8] is due to the differences in detergent solubilization methods. In this context our results in rat thymic or splenic lymphocytes clearly indicate that solubilization of membrane proteins by different detergents gives different results and support the contention that differences in solubilization methods account for apparent differences in the immunoglobulin content of thymic lymphocyte membranes.

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