Lymphocyte Plasma Membranes: Analysis of Proteins and Glycoproteins by SDS-Gel Electrophoresis

By John Lopes, Martin Nachbar, Dorothea Zucker-Franklin, and Robert Silber

A method is described for the preparation of plasma membranes from human tonsil lymphocytes. The membrane preparation has been characterized by electron microscopy and by the use of 5'-nucleotidase as an enzymatic marker. Acrylamide gel electrophoresis in sodium dodecyl sulfate yields a reproducible pattern for the membrane proteins and glycoproteins. A minimum of 26 polypeptide bands were identified with the Coomassie blue stain. The glycoprotein material was distributed into at least five bands. Similarities and differences between the lymphocyte and red cell membrane are discussed.

The role of lymphocytes in mediating histocompatibility, antigen recognition, and immune adherence is well established. Despite the importance of the cell surface in these interactions, relatively little is known about the plasma membrane of lymphocytes and no information is available on the proteins of lymphocyte membranes of human origin.

Since tonsil tissue contains a fairly homogeneous population of lymphocytes, it is a convenient source of these cells in quantities adequate for biochemical studies. Tonsil lymphocytes have been previously characterized in terms of morphology, immunoglobulin secretion, and reactivity to phytohemagglutinin. The present communication reports the preparation of plasma membranes from human tonsil lymphocytes and describes the polypeptides and glycoproteins in these structures.

MATERIALS AND METHODS

The sources of chemicals were: Ficoll, ovalbumin, Pharmacia Fine Chemicals; sodium diatrizoate (Hypaque), Winthrop; acrylamide, bisacrylamide, and TEMED, Eastman; sodium dodecyl sulfate (SDS), Fisher; Coomassie brilliant blue R-250, Mann; basic fuchsin, Allied; dithiothreitol (DTT), Calbiochem; Bromophenol blue, Fisher; 5'AMP, β-glycerophosphate, Sigma; Bovine serum albumin, Pentex; Chymotrypsinogen A, Ribonuclease A, β-galactosidase, phosphorylase A, Worthington.

From the Department of Medicine, New York University School of Medicine, New York, N. Y.

Submitted June 7, 1972; accepted July 3, 1972.

This research was supported by National Institutes of Health Grants CA 06657 and CA 11655.

John Lopes, Ph.D.: Associate Research Scientist, Division of Hematology, New York University Medical Center, New York, N. Y.; Martin Nachbar, M.D.: Assistant Professor of Medicine, Department of Medicine, New York University Medical Center, New York, N. Y.; Senior Investigator, New York Heart Association. Dorothea Zucker-Franklin, M.D.: Associate Professor of Medicine, New York University Medical Center, New York, N. Y.; Robert Silber, M.D.: Associate Professor of Medicine and Director of the Division of Hematology, Department of Medicine, New York University Medical Center, New York, N. Y.
Preparation of Plasma Membrane

Human tonsil tissue was teased into 0.15 M NaCl, 10 mM Tris-HCl, pH 7.8 (NaCl-Tris), filtered through nylon fiber and washed twice. Epithelial cells and erythrocytes were removed by a modification of Boyum's Ficoll-Hypaque gradient. In this procedure, tonsil cells, suspended in NaCl-Tris buffer were layered on a solution containing 6.5% Ficoll and 10% Hypaque in 10 mM Tris-HCl, pH 7.8. The sample was centrifuged at 400 g for 30 min. The sample-gradient interphase, containing lymphocytes, was collected, diluted fourfold with NaCl-Tris; the cells were collected by centrifugation at 400 g and washed twice in the same buffer.

Subcellular fractionation was performed by a combination of the methods applied by Allan and Crumpton to the preparation of pig lymphocyte membranes and that of Atkinson and Summers for the purification of Hela cell plasma membranes. All procedures were performed at 0–4°C. The cells were subjected to osmotic swelling in 10 mM Tris-0.2 mM DTT (Tris-DTT) for 5 min, and disrupted by 4–5 strokes of a tight-fitting Dounce glass homogenizer. Isotonicity was then restored by the addition of 1 M NaCl and the homogenate was centrifuged at 4000 g for 15 min to remove nuclei and mitochondria. The supernatant fluid was adjusted to 0.5 M NaCl, layered over a discontinuous 30%–45% gradient of sucrose in 10 mM Tris, pH 7.8, and centrifuged at 33,000 rpm for 30 min in a Beckman Model L-2 preparative ultracentrifuge, using a SW 39 rotor. This rotor was also used for all subsequent centrifugations. Material from the 30%–45% sucrose interphase was collected, diluted three- to fourfold with Tris-DTT, adjusted to 0.5 M NaCl, and again centrifuged at 33,000 rpm for 30 min. The pellet was resuspended in a small volume of Tris-DTT and used for further experiments. The activity of 5'-nucleotidase, used as a marker for plasma membrane was assayed by a combination of the methods of Michell and Hawthorne, Belfield and Goldberg, and Neu. The assay, in a volume of 0.1 ml contained: Tris-HCl, pH 7.8, 5 μmoles; MgCl₂, 1 μmole; β-glycerophosphate, 2 μmoles; NaK tartrate, 1 μ mole; AMP, 0.1 μmole, and approximately 4 μg of membrane protein. After 20 min at 37°C the reaction was stopped with 50 μl of 0.5 N HCl. The Pi released was determined by a micromodification of the method of Ames and Dubin. A unit is defined as micromoles of phosphate released per hour at 37°C. Protein was determined by the method of Lowry et al., using bovine serum albumin as standard.

Electron Microscopy

Cells harvested from the Ficoll-Hypaque gradient or membranes obtained as described above were washed twice in Hank's saline and fixed in 3% phosphate-buffered glutaraldehyde overnight. Post-fixation was carried out with 2% osmium tetroxide for 2 hr, following which the specimens were dehydrated in increasing concentrations of ethanol and propylene oxide and embedded in Epon 812. Thin sections, obtained with an LKB ultratome, were contrasted with uranyl acetate and lead hydroxide. The samples were viewed with a Siemens Elmiskop I electron microscope equipped with a cooling device.

SDS-Acrylamide Gel Electrophoresis of Plasma Membranes

Prior to electrophoresis, 8 mg of SDS and 4 μmoles of DTT were added per milligram protein to the plasma membrane fraction. The mixture was incubated at room temperature for 20 min and then placed in a boiling water bath for 10 min. The SDS-acrylamide gel electrophoresis system described by Neville and Glossmann was used. Electrophoresis was stopped when the Bromophenol blue dye marker reached the end of the gel. When plasma membrane preparations were to be stained for protein, 50 µg of protein were put on each gel, while 200 µg per gel were used for the determination of glycoprotein. Proteins of known molecular weight were electrophoresed as standard markers on separate
Fig. 1. Survey electron micrograph of human tonsil cells obtained after Ficoll-Hypaque separation. The unmarked cells are lymphocytes. E, erythrocyte; P, plasma cell. Magnification × ± 2000.

gels. The markers were treated as outlined above for the plasma membrane material. About 2 µg of each of six marker proteins were generally applied per gel. Chymotrypsinogen A was included in the markers just before the boiling stage. The protein bands were stained according to Weber and Osborn.21 The gels were destained with a mixture of 10% isopropyl alcohol and 10% acetic acid for 6 hr followed by treatment with 10% acetic acid until the contrast between bands and background became sharp. Gels were stained for carbohydrate using the periodic acid Schiff (PAS) procedure detailed by Fairbanks et al.,22 after freeing the gels of SDS by the method of Glossmann and Neville.23

RESULTS

Preparation of Plasma Membranes

Suspensions prepared by teasing tonsil tissue consisted predominantly of lymphoid cells, as has been described in detail recently.7 Centrifugation on a Fiscoll-Hypaque gradient removed the bulk of contaminating erythrocytes, epithelial cells, and granulocytes. The material used for the preparation of plasma membranes was otherwise similar to the starting cell suspension. A
Fig. 2. Low power survey electron micrograph of membrane fraction. Arrows point to fibrillar material occasionally seen attached to plasma membranes. G indicates granulelike material. Magnification $\times 32,000$. 
LYMPHOCYTE PLASMA MEMBRANES

A representative survey is shown in Fig. 1. The bulk of the cells (90% or more) consisted of lymphocytes of varying size and cytoplasmic development; 5%–7% were plasma cells and the remainder was made up of monocytes, erythrocytes, or other cells which were difficult to classify.

Membrane Morphology

A representative survey electron micrograph of the membrane fraction is shown in Fig. 2. This preparation was devoid of any recognizable nuclei or cytoplasmic organelles. Although a few unidentifiable electron-dense structures ranging from 300 to 400 Å in diameter were seen (arrow in Fig. 2), the membranes were remarkably free of adhering cytoplasmic material. As has been observed by others, isolated membrane fragments usually assume the appearance of vesicles or large vacuoles in cross section. The membranes prepared from tonsil lymphocytes were of uniform thickness and their length makes it likely that they were derived primarily from the plasma membrane of the cell, since the circumference of most cytoplasmic organelles would probably be shorter. Occasionally, very fine filaments measuring less than 30 Å in thickness were associated with the inner aspect of the membranes.

As shown in Table 1, a 12-fold enrichment in the membrane marker, 5'-nucleotidase, occurs during the purification. A recovery of 7% was noted for 5'-nucleotidase activity, which is half the recovery reported by Crumpton for pig lymphocytes. About 0.7% of the total protein found in the crude homogenate was recovered in the plasma membrane fraction. The lower recovery of 5'-nucleotidase observed may reflect loss due to the smaller amounts of starting material processed in the present study.

Electrophoretic Fractionation of Plasma Membrane Protein

Electrophoresis of the lymphocyte plasma membranes yielded a clear pattern which was reproducible in nine consecutive experiments. As shown in Fig. 3, a minimum of 26 bands can be distinguished. The relative mobility of these bands in comparison with known markers was used to establish the molecular weight of the material found in the lymphocyte membrane. The molecular weights were estimated by interpolation from plots of the reciprocal log of the molecular weight against the relative mobility of the markers, Fig. 4.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg/fraction)</th>
<th>5'-Nucleotidase (Total activity units)</th>
<th>Specific activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>43.0</td>
<td>37.5</td>
<td>0.8</td>
</tr>
<tr>
<td>4000 g Supernatant</td>
<td>41.0</td>
<td>22.5</td>
<td>0.6</td>
</tr>
<tr>
<td>4000 g Pellet</td>
<td>6.8</td>
<td>6.75</td>
<td>1.0</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>0.29</td>
<td>2.85</td>
<td>10.0</td>
</tr>
</tbody>
</table>

These results are from material obtained from five pairs of tonsils and processed as described under Materials and Methods. Results shown are representative of those obtained with eight other preparations.
For the purpose of describing the bands, the following system was used: On the basis of staining intensity, as judged by visual examination and scanning spectrophotometry, a number ranging from 4 to 1 was assigned to the major bands. The next three digits apply to the molecular weight of the polypeptide in thousands of daltons. The mol wt distribution of the polypeptides ranges from 10,000 to approximately 250,000. The major polypeptides in the lymphocyte membrane (indicated with arrows in Fig. 3) are represented in a semiquantitative spectrophotometric scan in Fig. 5. The most intensively stained band was found at a position corresponding to a mol wt of 45-48,000. The erythrocyte membrane shown for comparison (Fig. 3), also contains a somewhat less prominent protein with a similar mobility. Two sharply defined
light bands and one more intensively staining band were found in the region corresponding in mobility to the prominent erythrocyte membrane polypeptide doublet in the 250,000 mol wt range. The relative staining intensity of these lymphocyte membrane polypeptides was less than that observed for the erythrocyte membrane 250,000 mol wt polypeptide doublet. In general, visual comparison of the two electrophorograms is sufficient to reveal striking differences between the polypeptide patterns obtained from the two cell membranes; a greater number of polypeptide bands with a wider distribution of molecular weights is apparent in the lymphocyte membrane.

Membrane Glycoproteins

Electrophorograms of plasma membranes gave a reproducible pattern illustrated in Fig. 6. The glycoprotein staining reactions for lymphocyte membranes were less intense than those obtained for erythrocyte ghosts. It was necessary
to put 200 μg of protein on a gel to obtain a barely detectable pattern with lymphocyte membranes, while 100 μg of erythrocyte membrane protein gave an intense staining reaction. Three major and two minor bands were observed in lymphocyte membrane. Two bands migrating close to each other were observed in the region corresponding to the 250,000 mol wt polypeptides, visualized with the protein stain. A diffuse major band was noted corresponding to a molecular weight of 110,000. Two very faint bands with migration comparable to 33,000 and 28,000 mol wt polypeptides were noted. In addition, an intense staining reaction was always observed just behind the tracking dye, as has been reported by others with erythrocytes, kidney brush border, and liver cell membranes.22,23,25

**DISCUSSION**

The methods applied in the preparation of plasma membranes from tonsil lymphocytes are relatively gentle, easy to perform, and yield a reproducible preparation. The appearance of the plasma membrane by electron microscopy indicated little, if any, contamination by mitochondria, endoplasmic reticulum, or nuclei. Further support for the purification process is offered by the 12-fold increase in the specific activity of 5′-nucleotidase, the most widely accepted marker of plasma membranes. This increase in specific activity is comparable to that reported in the preparation of membranes from other cell types.24

Polyacrylamide gel electrophoresis in the presence of a high concentration of SDS reveals a reproducible pattern when stained with Coomassie blue. Each of these bands in all likelihood represents either a single polypeptide or an unresolved group of polypeptides fractionated according to their molecular weight.26 The most striking protein band in the lymphocyte membrane has an apparent mol wt of 48,000, which corresponds to a protein of similar mobility presented in liver, kidney, and erythrocyte membranes.20 Further studies
LYMPHOCYTE PLASMA MEMBRANES

are needed to investigate the possibility that this band represents a structural protein with identical properties found in several cell types.

No major bands observed corresponded to the immunoglobulin heavy or light chains. This observation is not unexpected since only 7% of tonsil lymphocytes appear to secrete immunoglobulins, and immunoglobulins make up only 2%–3% of the surface of splenic lymphocytes, which are more active in antibody production than tonsil lymphocytes.

Since lymphocytes have receptors for antibody, antigens, and PHA which may be glycoprotein in composition, it is somewhat surprising that there appears to be considerably less glycoprotein present in the plasma membrane of lymphocytes than in that of erythrocytes. This finding is apparent from the relative amounts of protein required to obtain a PAS staining reaction. It should be stressed that the error in the determination of the molecular weight of a glycoprotein may be as high as 30%; the molecular weights indicated for the lymphocyte membrane glycoprotein in this study are presented with this reservation in mind.

Tonsil tissue provides a supply of normal human lymphocytes which can be incubated with precursors, stimulated into mitosis with PHA, and challenged immunologically. Studies to determine the effect of these processes on plasma membrane proteins are in progress.

REFERENCES


Lymphocyte Plasma Membranes: Analysis of Proteins and Glycoproteins by SDS-Gel Electrophoresis

John Lopes, Martin Nachbar, Dorothea Zucker-Franklin and Robert Silber