A GLYCOPROTEIN ASSOCIATED WITH THE MEMBRANE
FRACTION OF HUMAN B BUT NOT T LYMPHOCYTES*

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Surface markers and functional properties of human bursa-derived (B) lymphocytes and thymus-dependent (T) lymphocytes have been carefully studied and compared (1). Differences in lymphocyte cell surface properties and morphology should reflect specific and identifiable membrane components. Several laboratories have reported differences in membrane preparations of T lymphocytes from various tissues (2, 3). Recently, B- and T-lymphocyte-specific membrane proteins have been reported on mouse lymphocytes using autoradiography (4). A human B-cell membrane component, possibly related to the stimulator antigen of the mixed lymphocyte culture reaction, has been identified using antibodies present in pregnancy sera (5).

This paper describes a method for analysis of human lymphocyte plasma membranes by centrifugation followed by polyacrylamide gel electrophoresis and reports on the differences observed in the glycoprotein associated with the membrane fraction of B and T lymphocytes. Of particular interest is the presence, in all of the B-lymphocyte preparations, of a large amount of a glycoprotein of approximately 30,000 daltons.

Materials and Methods

Human Lymphoid Cell Lines. Lines HSB-2, CEM, and MOLT-4 were obtained from the culture stock of Dr. M. Weksler, Cornell University Medical College, New York. HSB-2 and CEM (6) were originally gifts from Dr. J. Kaplan, Child Research Center of Michigan. MOLT-4 (7, 8) was originally a gift from Dr. J. Minowada, Roswell Park Memorial Institute, Buffalo, N. Y. HSB-2, CEM, and MOLT-4 were classified as T lymphocytes on the basis of the following surface properties: ability to form rosettes with sheep erythrocytes, absence of surface immunoglobulins, absence of complement (C) receptors, and absence of Epstein-Barr virus antigens (6–8). Lines HT and JR were established by Doctors I. Jack and C. S. Hoskins, Royal Children's Hospital, Parkville, Victoria, Australia (9) and were gifts to this laboratory. Lines HH, CL, and MW were established in this laboratory from the blood of immunologically normal donors by Dr. T. H. Hutteroth, Cornell University Medical College, New York. HT, JR, HH, CL, and MW were classified as B lymphocytes because of the presence of surface and cellular immunoglobulin and C receptors (10, 11). All cell lines were grown in RPMI 1640 containing 17% (vol/vol) heat-inactivated fetal calf serum containing 100 μg/ml streptomycin and 100 U/ml penicillin. Cultured cells were harvested in logarithmic growth phase. Cells were washed four times with 10 mM Tris-HCl-0.15 M NaCl, pH 7.4, and kept at −20°C until the membrane preparations were made.

Thymus Lymphocytes and Tonsillar Lymphocytes. Cell suspensions were prepared from fresh human thymus and tonsils by teasing cells into 10 mM Tris-HCl-0.15 M NaCl. Lymphocytes were

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separated from erythrocytes by Ficoll-Hypaque density gradient centrifugation (12), washed four times with 10 mM Tris-HCl-0.15 M NaCl at pH 7.4, and frozen.

**T Lymphocytes from Peripheral Blood.** A unit of heparinized whole blood was centrifuged at 100 g for 10 min to sediment erythrocytes. The supernate was then mixed with a half volume of lymphocyte separator reagent (Technicon Instruments Corp., Tarrytown, N. Y.), incubated at 37°C for 60 min with gentle agitation, and applied to a Ficoll-Hypaque gradient (12). Lymphocytes were recovered from the interphase, washed twice with Hanks' solution, resuspended at a concentration of 0.5 x 10^8 cells/ml in Hanks' solution containing 10% fetal calf serum, and applied to a nylon fiber column to remove B lymphocytes (13). 95% of the remaining lymphocytes formed rosettes with sheep erythrocytes (14). There was minor contamination with platelets. Cells were then washed four times with 10 mM Tris-HCl-0.15 M NaCl, pH 7.4, and frozen.

**Lymphocytes from a Patient with Chronic Lymphocytic Leukemia.** Blood from a patient with chronic lymphocytic leukemia (leukocyte count 66 x 10^9) was provided by Memorial-Sloan Kettering Tumor Procurement Center, New York. Lymphocytes were separated by Ficoll-Hypaque gradient centrifugation (12), washed four times with 10 mM Tris-HCl-0.15 M NaCl, and frozen.

**Preparation of a Plasma Membrane Fraction.** The procedure used was modified from the methods of Allan and Crumpton (15), Demus (16), and Marique and Hildebrand (17). Cells were thawed at 37°C; thereafter, all manipulations were performed at 0°-4°C. A lymphocyte suspension at a concentration of 20 x 10^8 cells/ml in 10 mM Tris-HCl buffer, pH 7.4, was chilled in crushed ice for 5 min, disrupted by five to eight strokes of a tight fitting pestle in a tissue grinder (PYREX) and restored to isotonicity by adding to the lysate approximately one tenth of its volume of 10 mM Tris-HCl-1.5 M NaCl, pH 7.4. The lysate was centrifuged at 4000 g for 15 min. The supernate was then centrifuged at 7,000 g for 20 min; both pellet and supernate were collected. The pellet was resuspended in 10 mM Tris-HCl-0.15 M NaCl and centrifuged at 37,000 g for 60 min, the supernate discarded, and the pellet suspended in 10 mM Tris-HCl buffer, pH 7.4. An equal volume of 48% (wt/vol) sucrose in 10 mM Tris-HCl buffer, pH 7.4, was mixed with the pellet and overlayed on 2 ml of 35% (wt/vol) sucrose in 10 mM Tris-HCl buffer, pH 7.4. Centrifugation was performed at 80,000 g for 18 h. The interphase was collected, diluted four times with 10 mM Tris-HCl buffer at pH 7.4, and centrifuged at 37,000 g for 60 min. The pellet (plasma membrane fraction) was now suspended in an equal volume of 48% (wt/vol) sucrose in 10 mM Tris-HCl buffer, pH 7.4, and stored at −20°C. 5'-mononucleotidase (18) was assayed according to the method described by Mitchell and Hawthorne (19). In three experiments on tonsillar lymphocyte preparations, 5'-mononucleotidase activity was increased by an average of 37-fold in the membrane preparations.

**Analytical Polyacrylamide Gel Electrophoresis.** To two volumes of the plasma membrane fraction (25-150 µg protein) was added one volume of a solution of 6% sodium dodecyl sulfate (SDS), 3% mercaptoethanol, 30% glycerol, 0.003% bromphenol blue (BPB), and 30 mM sodium phosphate buffer. The final vol ranged from 150 to 300 µl. The mixture was incubated at 70°C for 20 min, and applied to the top of a polyacrylamide gel. 0.5% SDS-7.5% polyacrylamide gels were prepared in 5 mm (inner diameter) x 12 cm (length) glass tubing. Electrophoresis was performed in 0.1 M sodium phosphate buffer-0.5% SDS, pH 7.1. A current of 5 mA/gel was applied for the first 30 min followed by 10 mA/gel until the BPB reached a mark 8 cm from the origin. Gels were stained with Coomasie Brilliant Blue for protein and with periodic acid-Schiff reagent (PAS) for carbohydrate (20).

To estimate the molecular weight of the components associated with the plasma membrane fraction the following proteins were used as markers: phosphorylase A (94 x 10^3 daltons), bovine serum albumin (66 x 10^3 daltons), IgG heavy chain (55 x 10^3 daltons), ovalbumin (45 x 10^3 daltons), α-chymotrypsinogen A (25 x 10^3 daltons), Ig light chain (24 x 10^3 daltons), and ribonuclease A (14 x 10^3 daltons).

**Immunologic testing for β2-Microglobulin and Kappa and Mu Chains of Immunoglobulin.** Each gel was sliced into 11 elution fractions starting at the top and ending at the BPB band 8 cm from the origin. The protein bands in the tonsillar membrane preparations, elution fractions I-XI, and the position of the molecular weight markers are shown in a diagram on the right side of Figs. 1a and 16. Each slice was soaked in 0.5% SDS overnight in order to elute membrane

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1 *Abbreviations used in this paper: BPB, bromphenol blue; SDS, sodium dodecyl sulfate.*
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Results

Isolation of the Plasma Membrane Fraction. An average of 0.59% of the protein in the cell lysate was recovered in the plasma membrane fraction. The protein yield varied with the cell type: for tonsillar lymphocytes it averaged 115 μg from 10⁶ cells (10 preparations); for cultured lymphoid cell lines it averaged...
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356 μg from 10⁹ cells (8 preparations); for peripheral leukemic lymphocytes it was 75 μg from 10⁹ cells (1 preparation), for peripheral blood T lymphocytes it was 322 μg from 10⁹ cells (1 preparation), and for thymus lymphocytes it was 63 μg from 10⁹ cells (1 preparation).

Analysis of the Membrane Fraction on SDS-Polyacrylamide Gels. Figs. 1a and 1b show the components associated with the membrane fraction stained for protein. Strong bands are evident at approximately 30,000, 48,000, and 83,000 daltons. The multiple protein-banding pattern can be visualized best in the tonsillar membrane preparations and in the diagrams drawn from them, which are the last two patterns in Figs. 1a and 1b. The gels from different experiments were photographed under different conditions and in these figures are visually lined up with respect to the major identifiable protein and carbohydrate bands.

PAS staining for carbohydrate is shown in Figs. 2a and 2b. The PAS-positive band, which moved ahead of the tracking dye, was thought to be glycolipid analogous to results seen in erythrocyte membranes (20).

When the same preparation, stained for protein and carbohydrate respectively, is compared, the glycoprotein components associated with the membrane fraction can be identified. This is best accomplished by comparing the same positions in gels of Fig. 1a vs. Fig. 2a, and the same position in 1b vs. 2b. A glycoprotein of approximately 30,000 daltons can be readily identified in the preparations of Figs. 1a and 2a. It has a high carbohydrate content, and as judged by staining intensity constitutes a major component in the membrane fraction. In fresh gels, PAS-positive bands were also observed at approximately 48,000 daltons but, unfortunately, these are not easily discernible in the photographs. Since there is a strong protein component of the same approximate molecular weight (see Figs. 1a and b), this may represent a glycoprotein.

All of the B-lymphocyte membrane preparations possessed the approximately 30,000 dalton glycoprotein component. This included four B-cell lymphoid lines, blood lymphocytes from a subject with chronic lymphocytic leukemia, and tonsillar lymphocytes—all seen in Figs. 1a (protein staining) and 2a (PAS-staining). 10 different tonsil preparations had this band; only one is shown in the figures. The 30,000 dalton glycoprotein was absent or at very low concentration in T-lymphocyte membrane preparations which included T-cell lymphoid lines, thymocytes, and normal peripheral blood depleted of B lymphocytes. (see Figs. 1b and 2b).

T-cell lymphoid line MOLT-4 had a unique banding pattern (see Figs. 1b and 3). A strong protein band was present at approximately 30,000 daltons; however, this band was PAS negative and slightly narrower and higher in position than the band found in B-lymphocyte preparations. In addition, it was noted in MOLT-4 that the protein band at approximately 48,000 daltons, which was present in all lymphoid preparations, was weak in staining intensity.

In the higher mol wt region (80,000–100,000 daltons) the T-lymphocyte preparations appeared to have more PAS-positive material than B-lymphocyte preparations. These differences were not definitive.

Immunologic Testing for β₂-Microglobulin and Immunoglobulin Antigens. Two tonsillar membrane preparations were tested for β₂-microglobulin, and mu and kappa antigens by double agar diffusion. Only kappa antigens were
(a) Carbohydrate banding patterns of B lymphocytes on 0.5% SDS-7.5% polyacrylamide gels are shown. Gels were stained with PAS reagent: (1) HH, 36 μg; (2) CL, 150 μg; (3) MW, 72 μg; (4) HT, 104 μg; (5) peripheral blood lymphocytes from a patient with chronic lymphocytic leukemia, 72 μg; and (6) tonsillar lymphocytes, 112 μg. On the far right is shown a schematic of protein banding for tonsillar lymphocytes. (b) Carbohydrate banding patterns of T lymphocytes on 0.5% SDS-7.5% polyacrylamide gels are shown. Gels were stained with PAS reagent: (1) CEM, 68 μg; (2) HSB-2, 150 μg; (3) MOLT-4, 146 μg; (4) peripheral blood T lymphocytes, 116 μg; (5) thymocytes, 124 μg; and (6) tonsillar lymphocytes, 112 μg. On the far right is shown a schematic of protein banding for tonsillar lymphocytes.

Discussion

Recently, several laboratories have developed methods for the isolation of plasma membranes from lymphocytes (15–17). Antigenically identifiable membrane components, in particular H-2 and HL-A histocompatibility antigens, have been prepared by the use of enzymes, detergents, or other chemical reagents (22–25). The present approach represents an adaptation of previous
methods (15–17) to various types of human lymphoblastoid cells and tissue lymphocytes using differential centrifugation followed by density gradient centrifugation. The purity of the membrane preparations was comparable to results in previous reports using a similar methodology (17) as monitored by the increase in specific activity of 5′-mononucleotidase, a membrane-associated enzyme. The banding patterns for protein and carbohydrate on SDS-polyacrylamide gels were reproducible for membrane preparations from the same line or tissue prepared at different times. Ten different tonsillar lymphocyte preparations all had the major 30,000 dalton glycoprotein and other components in similar positions and of comparable intensity. The major limitation of the technique was the small amount of plasma membrane fraction obtained; it averaged 0.59% of the original cell lysate protein. This necessitated the use of over 10⁹ cells/preparation and limited the isolation and characterization of isolated membrane fraction-associated components.

It is not clear whether each protein band represents an intrinsic membrane component, a protein associated with the membrane fraction, or a fragment of either of the above. The isolation technique of homogenization and centrifugation is relatively gentle and should produce little if any cleavage of covalent bonds to yield fragments. The use of both detergent and reducing agents lessen the risk of polymer formation. However, the presence of cell-associated proteases which could act on the membrane preparations, even at low temperatures, cannot be excluded.

There were as many as 7 PAS-positive bands and 20 protein bands evident in fresh gels. At least two of the PAS-positive components, those at 30,000 daltons and at 48,000 daltons, coincide with strong protein staining. There would appear to be a limited number of major glycoproteins associated with the membrane fraction of lymphocytes, a finding analogous in certain respects to those made by Hamaguchi and Cleve (26) and by Fujita and Cleve (27) using a similar method to study human erythrocytes.
The most important discovery of this study was the 30,000 dalton glycoprotein easily detected in the membrane preparations from B but not T lymphocytes. This glycoprotein is of particular interest both because of the large amount that is present (it represents the major carbohydrate component and is a high percentage of the total protein), and because, as a glycoprotein, it is of possible biological significance as a membrane antigen and/or receptor. There is no information available at present on the immunologic or biologic properties of the 30,000 dalton glycoprotein. It could represent the Fc receptor molecule or the mixed lymphocyte culture stimulator antigen discussed by Wernet et al. (5) although the molecular weights appear to differ. In mouse lymphocytes, Ia antigen has been reported to have a mol wt near 30,000 daltons and to be present on B lymphocytes (28). No equivalent to the mouse Ia antigen has yet been detected on human lymphocyte membranes. The biochemical characterization of the B-lymphocyte-specific 30,000 dalton glycoprotein is being undertaken.

T-lymphocyte membrane preparations appear to have larger amounts of PAS-positive material in the 80,000–100,000 dalton region. The crowding of multiple bands in this area of the gel make this finding hard to interpret. B-lymphocyte- and T-lymphocyte-specific proteins with mol wt of 170,000 daltons or greater have been reported on mouse lymphocyte membranes (4).

The major difference between MOLT-4 and the other T-lymphocyte preparations was a protein band near 30,000 daltons which failed to stain for carbohydrate and which differed slightly in position and width from the B-lymphocyte 30,000 dalton glycoprotein. MOLT-4 has been previously reported to lack the capacity to stimulate allogeneic cells in mixed lymphocyte culture testing (8). MOLT-4, CEM, and HSB-2, the three T-lymphocyte lines (6–8), were all established from patients with acute lymphocytic leukemia (7, 29, 30), and all fail to stimulate allogeneic cells (M. Weksler, and M. M. Kuntz, personal communication). The protein band at approximately 48,000 daltons was present but diminished in MOLT-4 as compared with other preparations in which the same or higher loads of membrane preparation had been placed on the gel. This component may be associated with the HL-Aβ2-microglobulin complex which has been reported to have a mol wt of 44,000 (22). Unfortunately, we were unable to confirm this with immunologic testing for β2-microglobulin, perhaps due to the low concentration of this component and/or the possibility of denaturation caused by SDS or the ethanol used to precipitate the protein from each elution fraction. Among the possible explanations for the differences in the banding pattern obtained with MOLT-4 are an alteration of the membrane, associated with malignant transformation, or the existence of T-lymphocyte subgroups.

**Summary**

A method is described which employs differential centrifugation and sucrose density gradient centrifugation to isolate a membrane fraction from human lymphocytes. Membrane preparations from long-term human cultured B- and T-lymphoid lines, peripheral blood lymphocytes, tonsillar lymphocytes, and thymocytes were analyzed on 0.5% sodium dodecyl sulfate-7.5% polyacrylamide gels stained for protein and carbohydrate. The most important finding was a major glycoprotein of approximately 30,000 daltons associated with the membrane
preparations from B lymphocytes. T-lymphocyte preparations did not contain readily detectable amounts of this membrane-associated component. The T-cell lymphoid line MOLT-4 was unique in that it had a narrow protein band at approximately 30,000 daltons which did not contain carbohydrate.

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