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Studies of Density-Fractionated Human Erythrocyte Membranes

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Membranes obtained from the normal human RBC population were separated by continuous sucrose density gradient centrifugation and the density-fractionated membranes were then examined for changes in molecular markers. This study focuses on changes of (i) the membrane protein profile, (ii) differences in membrane-associated enzyme activities, and (iii) the amount of autologous IgG bound. The following observations were made: (i) ratios for band 4.1a over the sum of bands (4. 1a + 4.1b) ranged from 0.58 to 0.79 for membranes of lowest density; (ii) significant changes in bound glyceraldehyde-3-phosphate dehydrogenase and acetylcholinesterase activities were found; (iii) the amount of autologous IgG's attached to the red blood cells was highest in the membrane fraction of lowest density.

KEY WORDS: erythrocyte membrane, density-fractionation, band 4.1, membrane-bound enzymes, autologous IgG.

Mature erythrocytes transport oxygen throughout the body, and are removed from circulation after a well-defined lifespan, which varies from species to species (Shemin and Rittenberg, 1946; Allison, 1960). Because of their quantity,as well as ease and reproducibility in processing, and because they lack subcellular organelles in adult mammamls, erythrocytes have been used as a model for both cell membrane and cell aging studies (Fairbanks *et al.*, 1971; Borum *et al.*, 1957). Moreover, mature mammalian erythrocytes perform their biological functions without benefits of transcriptional and translational directions for the resynthesis of new materials. Though the mammalian erythrocytes have been used frequently to understand the mechanism of cell aging and sequestration from the circulation, the nature of the biological clock which determines the lifespan is still uncertain.

In 1957, Borum *et al.*, (1957) showed the progressive changes in density distribution of Fe^{59}-labeled erythrocytes throughout their lifespan. Since then, many investigators have used similar density-dependent separation of red blood cells, based on the assumption that cell density increases with cell aging, and myriad observations concerning the properties of density-separated cell have appeared. However, the relationship between cell age and density has been seriously questioned (Mueller *et al.*, 1987; Morrison *et al.*, 1988).

The red blood cell (RBC) membrane is composed of a lipid bilayer and integral membrane proteins attached to an underlying membrane skeleton (Fairbanks *et al.*, 1971; Branton *et al.*, 1981; Elgsaeter *et al.*, 1986; Bennett, 1985; Ferrel and Huestis, 1984; Steck, 1974). The ratio of lipid to protein in the red blood cell membranes is approximately 1:1 (Ferrel *et al.*, 1984). Smaller quantities of minor glycolipids, free fatty acids, and phosphatidic acids are also present (Ferrel *et al.*, 1984). The 10-12 major membrane proteins have

been separated by SDS-PAGE and numbered (Steck, 1974; Fairbanks *et al.*, 1971). The flexibility as well as the durability of red blood cells is largely determined by the properties of their membranes, controlled by the membrane protein skeleton.

Here, instead of density fractionation of cells, I initiated studies for fractionating cell membranes on suitable density gradients. This study focuses on changes of (i) the membrane protein profile, (ii) differences in membrane-associated enzyme activities, and (iii) the amount of autologous IgG bound.

Materials and methods

Preparation of human erythrocyte membranes

Fresh human blood collected in CPDA-1 anticoagulant was obtained and was processed within 1 day. Red blood cells were separated by centrifugation (4 × 20 ml of blood; 3,000g × 10 min, 4°C) and cells in each tube were washed two times with 35 ml of buffer K (100 mM KCl, 60 mM NaCl, 10 mM glucose, and 10 mM Tris-HCl, pH 7.4) by centrifugation. RBC suspension of 20% hematocrit in buffer K was passed through cotton wool (Johnson & Johnson, New Brunswick, NJ) column (3.3 × 7 cm) to remove white cells and the red cells were washed four times further by centrifugation. Aliquots (2 ml) of packed cells in each tube were lysed with 35 ml of ice-cold 5 mM sodium phosphate buffer, pH 8.0 (5P8) and were centrifuged. After removing supernatant and red button by aspiration, the membranes derived from 2 ml of packed cells were washed three times with 30 ml of ice-cold 10 mM Tris-HCl, pH 8.0.

Membrane fractionation by continuous sucrose density gradient centrifugation

Continuous sucrose (Sigma, Saint Louis, MO) density gradient (30 ml, 30%-39% in 5 mM Tris-HCl, pH 8.0) was prepared in transparent centrifuge tube (cellulose nitrate, 25 × 89 mm, Beckman) and 5 ml of RBC membranes (6-7 mg of protein) were loaded. After centrifugation (18,000rpm × 17 h, 4°C, Beckman SW-28 rotor; without the use of brake), fractions of 15 drops were collected from the bottom of centrifuge tube and kept on ice. Protein concentration in each fraction was determined by the Miller-modified Lowry assay (Miller, 1959) and sucrose density was determined by a Zeiss refractometer. Fractions were pooled into 7 groups (pool A to G, see Fig. 1) and washed with approximately 10 volumes of ice-cold 10 mM Tris-HCl, pH 8.0 by centrifugation (35,000g × 15 min, 4°C).

Analysis of band 4.1

Membrane proteins (35 μg) were incubated at 40°C for 30 min with gel sample buffer (25% sucrose, 2.5% SDS, 2.5 mM EDTA, 100 mM dithiothreitol, 10 μg pyronin Y/ml, and 25 mM Tris-HCl, pH 6.8) and separated by SDS-polyacrylamide gel (5%/10%) electrophoresis using the Laemmli buffer system (Laemmli, 1970).

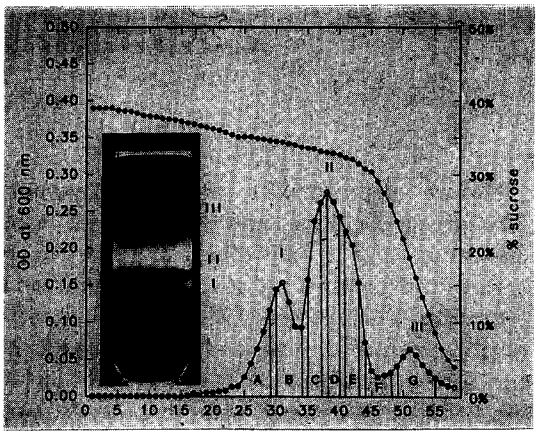

Fig. 1. Fractionation of human RBC membranes using a continuous sucrose density gradient. Human erythrocyte membranes were prepared from freshly washed red blood cells with buffer K, followed by hypotonic lysis with ice-cold 5mM sodium phosphate buffer, pH 8.0. Membranes (5 ml each) were loaded onto 30 ml of continuous sucrose density gradient (30%-39%) and centrifuged at 18,000rpm, 4°C for 17 hours in a Beckman Ultracentrifuge using an SW-28 rotor. Fractions of 15 drops each were collected starting from the bottom of the centrifuge tube (abscissa). After 10 fold dilution with 10 mM Tris-HCl, pH 8.0, protein was assayed (left ordinate) and sucrose concentration was measured by refractometry (right ordinate).

Gels were stained with 0.05% (w/v) Coomassie blue R in 25% (v/v) methanol and 18% (v/v) acetic acid. Stained bands were scanned by an LKB laser densitometer (LKB, Bromma, Sweden) using LKB Ultrascan XL program.

Measurement of membrane-associated enzyme activity

Sucrose density-separated membranes (pool A to G) were diluted 1/2-1/10 with 10 mM Tris-HCl, pH 8.0 to determine the membrane-associated enzyme activities of glyceraldehyde-3-phosphate dehydrogenase and acetylcholinesterase (Steck and Kant, 1974). For the glyceraldehyde-3-phosphate dehydrogenase activity, aliquots (50 μl) of diluted membranes were preincubated at room temperature with 50 μl of 0.2% (v/v) Triton X-100 in 5 mM sodium phosphate buffer, pH 8.0. Sodium pyrophosphate (4 mM in 4 mM cysteine-HCl, pH 8.4) was added to 720 μl followed by 30 μl of 0.4 M sodium arsenate and 50 μl of 20 mM β-NAD. The enzyme reaction was initiated with the addition and quick mixing of 100 μl of 15 mM DL-glyceraldehyde-3-phosphate, pH 7.0. The enzymatic reaction was followed spectrophotometrically at 340 nm, at room temperature. Enzymatic activity was measured by increase in optical density between the first and the second minute of reaction (ΔOD_{min}). In order to measure the membrane-associated acetylcholinesterase, 50 μl of diluted membranes was also preincubated for 1 min with 50 μl of 0.2% (v/v) Triton X-100 in 5P8 (5 mM sodium phosphate buffer, pH8.0). Sodium phosphate (100 mM, pH 7.5) was added to a volume of 0.9 ml, and 0.05 ml of each of 10 mM DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)] in 100 mM sodium phosphate, pH 7.0, and 12.5 mM acetylthiocholine chloride were then mixed. Since acetylcholinesterase catalyzes the hydrolysis of acetylthiocholine to thiocholine, the rate of thiocholine production was measured by following the reaction of thiocholine with DTNB, which produces a yellow color with the formation of 5-thio-2-nitro-benzoic acid. The rate of formation of the yellow anion was detected at 412 nm.

Measuring autologous IgG's attached to red blood cells

Buffer K-washed cells at approximately 20% hematocrit, prepared as above, were incubated 15 h at 4°C with 1/25 or 1/50 diluted in buffer K of goat anti-human IgG (γ-chain specific)-alkaline phosphatase (Ab-AP) conjugate (Sigma). Ab-AP conjugate-treated cells were washed five times with 20 volumes of buffer K bycentrifugation (3,000g × 5 min, 4°C) until the supernatant had no alkaline phosphatase activity. Sucrose density separation of Ab-AP conjugate-decorated membranes was carried out by the same method described above. Aliquots (20 μl) from each pool were mixed with 980 μl of ρ-nitrophenyl phosphate (5 mg per ml of 100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.5) at room temperature (22°-23°C) and color developed during 6 to 12 min was measured at 409 nm by Beckman DU64 spectrophotometer. Molar extinction coefficient at pH 9.5 for the ρ-nitrophenol released by alkaline phosphatase is 18,255 (Dawson *et al.*, 1984).

Results and Discussion

Fractionation of RBC membranes by continuous sucrose density gradient centrifugation is illustrated in Fig. 1. Two main bands (I and II) and one minor band (III) were obtained. Sucrose densities at these peaks were 34.5% ± 0.2% (I), 33.5% ± 0.2% (II), and 19.5% ± 0.5% (III). The sucrose densities decreased rapidly from 30% to 4%-5% near the top of the tube because of contact with 5 ml of RBC membranes in 10 mM Tris-HCl, pH 8.0. The sucrose density-separated membranes from the highest density to the lowest were subdivided into pool A to G, and membranes in each pool with appropriate proportions of proteins were washed once with ice-cold 10 mM Tris-HCl, pH 8.0 by centrifugation and saved for the further studies. Since peak III contained a large amount of hemoglobin, sufficient membrane proteins in pool G were not available for analysis by SDS-PAGE.

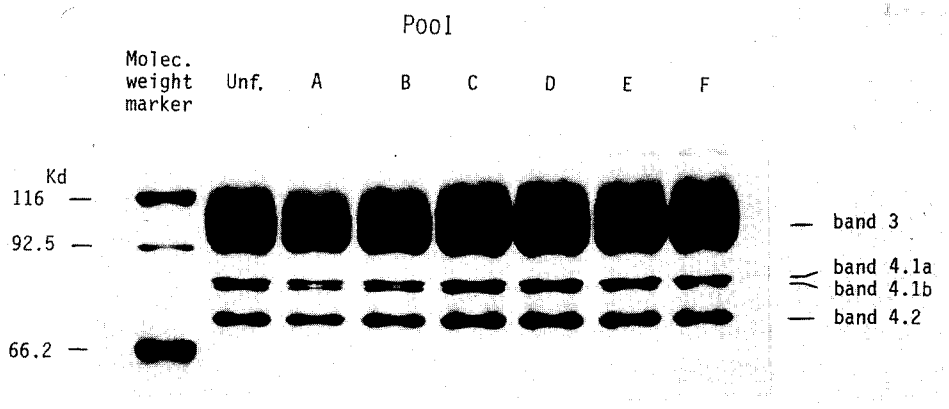

Fig. 2. SDS-PAGE of the band 3 to 4.2 region for the density-fractionated RBC membranes. After pooling RBC membranes, each pool was washed with 10 volumes of 10 mM Tris-HCl, pH 8.0 and was centrifuged (35,000g, 4°C for 15 min using Beckman JA rotor). Aliquots (35 μg) of RBC membrane proteins were separated by running SDS-polyacrylamide gel (5%/10%) electrophoresis with the Laemmli buffer system (1970).

Differences in 4.1a/(4.1a + 4.1b) ratios

Differences in the membrane protein profile were examined by discontinuous SDS-PAGE (Fig. 2). Since pool G contained a large amount of hemoglobin, very little membrane proteins were available for the SDS-PAGE. A readily noticeable alteration was observed in regard to band 4.1 which is usually present as a doublet, designated 4.1a (Mr 80,000) and 4.1b (Mr 78,000). The amount of 4.1b seemed to have diminished in the membranes of lower densities. These changes are documented by the gel scans shown in panel A of Fig. 3. Using the program of LKB Ultrascan XL, individual peak areas were obtained from which the 4.1a/(4.1a + 4.1b) ratio could be calculated. This ratio seems to progressively increases from a value of 0.58 in pool B to 0.79 in pool F (panel B in Fig. 3). In measurement with unfractionated membranes, band 4.1a accounted for approximately 64% of the total band 4.1. This result described above was clearly reproducible with little variation to show changes of the pattern of band 4.1a and b. In the human, Sauberman *et al.* (1979) have found that band 4.1a is deficient in the red blood cells of patients with a variety of hemolytic anemias, and they suggested that younger red cells are deficient in 4.1a. The use of TEC (transient erythroblastopenia of childhood) erythrocytes has illustrated clearly that during the period of cessation of erythroid cell production, most of the band 4.1 is in the form of "a" form (Ravindranath *et al.*, 1987). Results obtained in the mouse (Mueller *et al.*, 1987) and rabbit (Suzuki and Dale, 1988) lend further support to the contention that the band 4.1a/(4.1a + 4.1b) ratio is a valid marker for cell senescence. The ratio of protein 4.1a/4.1b of erythrocyte membranes prepared from various healthy mammals has been studied by Inabe and Maeda (1988). Their results showed that the 4.1a/4.1b ratio in erythrocytes from various mammals correlate to the mean cell age in circulating blood. It is not certain at this point that membrane density is related to cell aging. Nevertheless, it is quite interesting that the density-separated membranes also show changes in the band 4.1a/(4.1a + 4.1b) ratio, which has been observed and correlated with cell aging. Several posttranslational modifications of band 4.1 such as glycosylation, phosphorylation, methylation, and acylation have been suggested. However, it is premature to conclude that the same posttranslational modification of band 4.1 during cell aging occurs in the density-fractionated cell membranes.

Changes in glyceraldehyde-3-phosphate dehydrogenase and acetylcholinesterase activities

It has been suggested that *in vivo* cell aging is

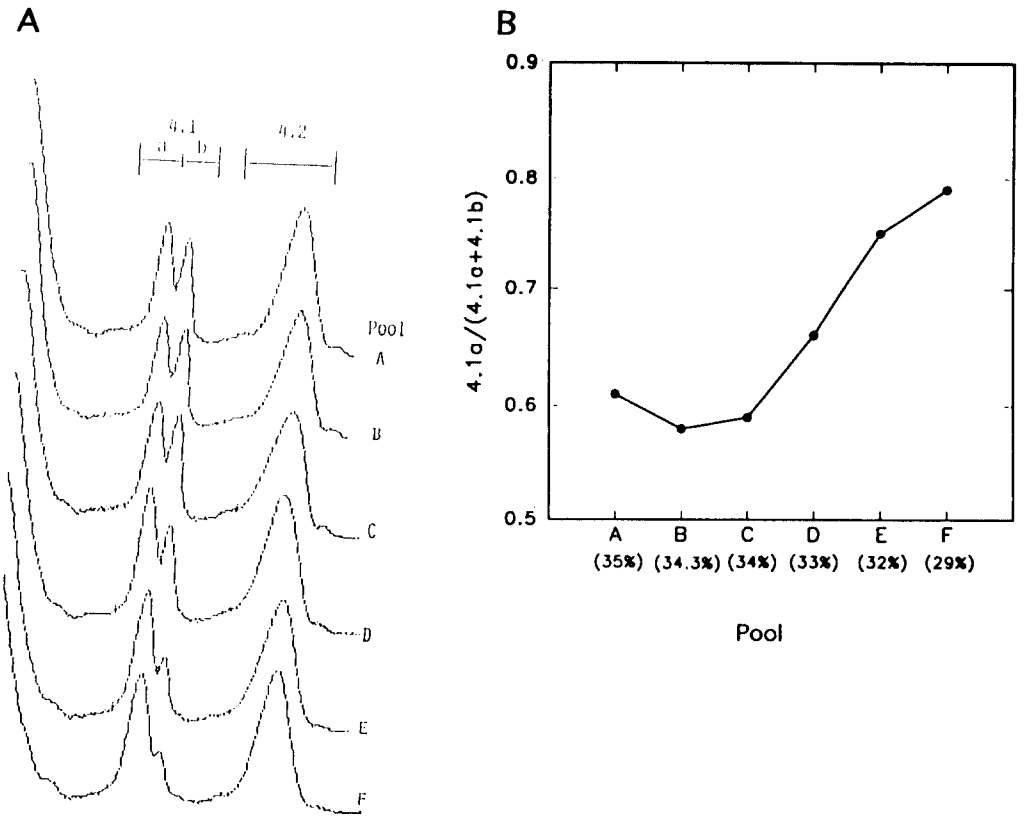

Fig. 3. Changes of the band 4.1a/(4.1a + 4.1b) ratio in the sucrose density-fractionated erythrocyte membranes. The Coomassie blue-stained gel shown in Fig. 2 was scanned (LKB Laser Densitometer with the program of LKB UltrascanXL program) (Panel A). Individual peak areas were obtained by the same programand calculated to give the 4.1a/(4.1a + 4.1b) ratio in Panel B.

associated with a significant decrease in the specific activity of acetylcholinesterase (approximately 20%)(Kadlubowski and Agutter, 1977). Bosman and Kay (1988) also reached the same conclusion on the basis of a decrease in glyceraldehyde-3-phosphatedehydrogenase activity (approximately 60%). Fig. 4 shows that both acetylcholinesterase and glyceraldehyde-3-phosphate dehydrogenase activities are reduced in the lightest membranes. It is not immediately possible to conclude that any of these decreases indicates a reduction of catalytic activity, according to different cell membrane density. An alternative explanation may be the *in situ* loss of enzyme proteins from the membrane into the cytoplasm while the specific enzyme activities remain constant. Nevertheless, membrane density-dependent changes in membrane-associated enzyme activities are related to the changes in the band 4.1a/(4.1a + 4.1b) ratio.

Comparison of attached autologous IgG's

Comparison of the distribution of autologous IgG's on the density-separated human RBC membranes indicate that there is almost twice the amount of IgG on the lightest membranes in comparison to the heaviest. Since the molar ratio of Ab-AP conjugate (alkaline phosphatase molecules per goat anti-human IgG) was not determined, it was not possible to calculate the absolute number of autologous IgG molecules per cell. Kay *et al.* (1989) have reported that human mononuclear phagocytes distinguish IgG's to the altered form of membrane protein, band 3.

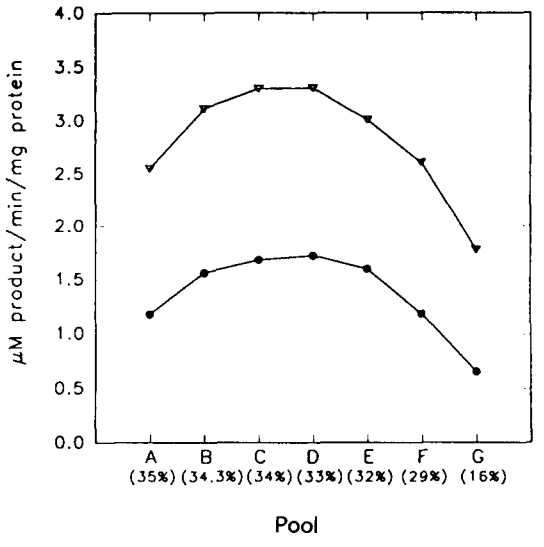

Fig. 4. Analysis of enzyme activities bound to density-fractionated RBC membranes. Two enzyme activities: glyceraldehyde-3-phosphate dehydrogenase (●) and acetylcholinesterase (▽) were assayed by the methods of Steck and Kant (1974).

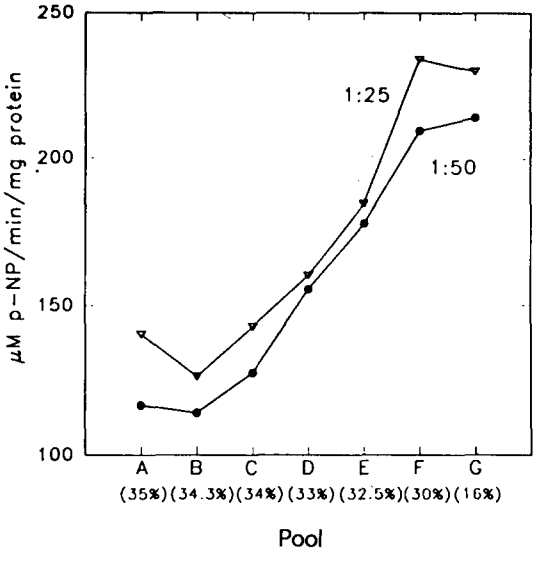

Fig. 5. Distribution of IgG's on the density-separated RBC membrane fractions. Washed RBC's were incubated with a goat anti-human IgG-alkaline phosphatase conjugate at two dilutions (1/25: ▽, 1/50: ●; membranes were obtained by 5P8 lysis and fractionated by sucrose density centrifugation as described in Fig. 1. The alkaline phosphatase activity of each pool was measured, which represented the amount of autologous IgG's attached to the populations of erythrocyte membranes.

However, it is not clear if the sequestration of aged cells from the circulation is performed solely by this autologous immunological reaction.

The separation of human red blood cells over the discontinuous Stractan (arabinogalactan) density gradients did not show any changes in band 4.1 (data not shown). If the band 4.1a/(4.1a + 4.1b) ratio reflects *in vivo* cell aging, it may be concluded that density-dependent cell fractionation does not correlate with cell aging. As to membrane fractionation based on density, it is still uncertain whether there is a real correlationship between cell aging and membrane density. The clue to this question may be obtained by combining two results of Lorand *et al.* (1987) and Sauberman *et al.* (1979). Lorand *et al.* observed that erythrocyte membranes prepared from Hb-Köln disease have a density of about 1.18, as compared to normal controls of about 1.16. The result obtained by Sauberman *et al.* indicated that band 4.1b is prominent in the red blood cells of patients with a variety of hemolytic anemias. Since results in this report show that the heavy membrane fraction has more 4.1b relative to the light membranes, it may lead to the hypothesis that cell membrane density decreases with cell aging. In order to confirm this hypothesis, density-dependent fractionation of membranes from pathological red cells (Hb-Köln, sickle cells, thalassemia cells) followed by the characterization of density-separated membranes should be carried out. Inasmuch as such pathological erythrocytes have a reduced lifespan, it can be expected that the heavier membrane fraction might be present in higher proportions, and the overall band 4.1a/(4.1a + 4.1b) ratio might be lower than in normal cells.

More direct evidence can be obtained by using membranes from TEC (transient erythroblastopenia of childhood) erythrocytes. Comparison of the membrane density with the band 4.1a/(4.1a + 4.1b) ratio at different stage of diagnosis and during recovery should be performed.

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농도분배에 따라 분리한 사람 적혈구 막에 관한 연구

정종문(수원대학교 생물학과)

정상인의 적혈구에서 추출한 세포막이 연속 밀도기울기를 갖는 설탕용액에서 원심분리되었다. 이렇게 밀도차에 의해 분리된 적혈구의 세포막을 대상으로, 여러가지 분자수준에서의 변화양상을 조사하였다. 여기에서는 주로 (1) 막단백질의 분포상황, (2) 세포막에 결합되어 있는 효소들의 활성도 조사, (3) 세포막에 붙어 있는 자기 항체량의 변화 등을 중점조사하였다. 얻은 결과로는 첫째, 막단백질의 한가지인 band 4.1중 band 4.1a/(4.1a + 4.1b)의 비율이 0.58에서부터 시작하여 가장 낮은 밀도를 갖는 세포막에서는 0.79까지 증가하였다. 둘째, 세포막에 결합된 glyceraldehyde-3-phosphate dehydrogenase와 acetylcholinesterase의 효소활성도가 세포막의 밀도차에 따라 현저한 변화를 보였다. 셋째, 가장 낮은 밀도를 갖는 적혈구막에서 막에 결합된 자기항체가 최대로 검출되었다.