Chem. Pharm. Bull.
33(12)5404—5412(1985)

Increased Sensitivity of Aged Erythrocytes to Drugs and Age-Related Loss of Cell Components

TARO OGISO,* MASAHIRO IWAKI, TOSHIHIKO TAKAGI, ITSUKO HIRAI and TOKIKO KASHIYAMA

Faculty of Pharmaceutical Sciences, Kinki University, Kowakae 3-4-1, Higashi-Osaka 577, Japan

(Received April 3, 1985)

Human erythrocytes were separated into fractions of different age by centrifugation. The effect of some drugs on the separated cells, the levels of some components and enzymes in the cells and the fatty acid composition of membrane phospholipids were estimated to obtain insight into the lifespan of the cells. The bottom (old) cells were relatively more sensitive to the drugs, and aged cells pretreated with chlorpromazine had an increased osmotic fragility and increased viscous properties compared with the top (young) cells, although the differences were relatively small. Significantly lower levels of antioxidant, glutathione and tocopherol, and decreased activities of enzymes (superoxide dismutase, glutathione peroxidase and catalase) involved in peroxide metabolism were observed in old cells as compared with young ones. The proportion of arachidonic acid and the unsaturated/saturated ratio of fatty acids of phospholipids in old cells were significantly lower than those of young cells. The treatment of cells with hydrogen peroxide *in vitro* produced a dramatic decrease in the proportions of polyenoic fatty acids in all cell fractions, although the decrease in arachidonic acid was less in old cells. The causal relationships among these changes and the lifespan of erythrocytes are discussed.

Keywords——human erythrocyte; aged cell; viscous property; peroxide-metabolizing enzyme; antioxidant; fatty acid composition

It has long been known that the *in vivo* lifespan of erythrocytes is limited[1,2]; human erythrocytes have a lifespan of about 120 d.[2] There is a general agreement that the ultimate recognition and phagocytosis of aged erythrocytes are initiated by alterations in their mechanical properties.[3-5] In the process of aging, cellular deformability decreases[5-7] and the physical, biochemical and physiological properties of red blood cells change.[8-10] The decrease in cellular deformability, which leads to enhancement of the rate of mechanical entrapment, may be a consequence of one or more events, *e.g.* an increase in the internal viscosity[11] and a decrease in the flexibility of the cell membrane.[12] On the other hand, many kinds of drugs are known to exert various effects on erythrocytes, but little is known about the differences in the effect of drugs on old and young erythrocytes.

The experiments described in this paper were undertaken to determine whether or not older cells with decreased enzyme activities[13] and adenosine triphosphate (ATP) level[9,10] exhibit increased sensitivity to the cytolytic actions of some drugs. Chlorpromazine and clemastine, having a severe hemolytic activity,[14] were chosen as representative drugs. In addition, the levels of some components and enzymes involved in peroxide metabolism were estimated in aged and young cells.

Experimental

Materials——Chlorpromazine hydrochloride (Nihon Shinyaku Co.) and clemastine fumarate (Sankyo Co.) were used throughout this experiment. Reduced glutathione (GSH), ATP disodium salt, glutathione reductase from

NII-Electronic Library Service

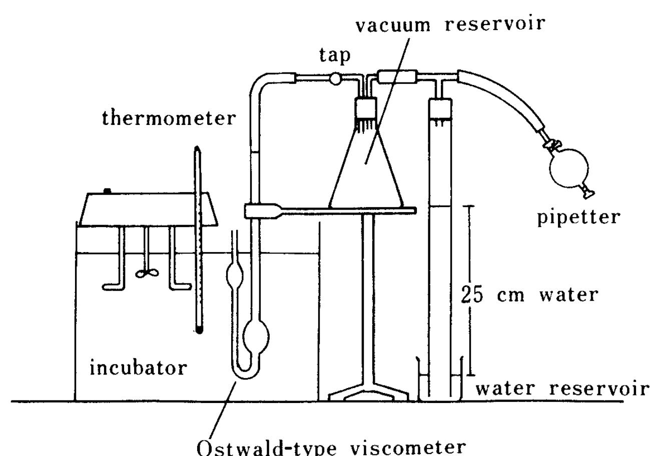

Fig. 1. A Schematic Representation of the Apparatus for Measuring Viscous Properties of Erythrocytes

bakers' yeast and ouabain were obtained from Sigma Chemical Co. and E. Merck A. G., respectively. Hexokinase from yeast was purchased from Miles-Seravac. Nicotinamide adenine dinucleotide phosphate (NADP), reduced NADP (NADPH) and glucose 6-phosphate dehydrogenase (G-6-PD) from yeast were obtained from Oriental Yeast Co.

Separation of Erythrocytes of Different Ages and Preparation of Erythrocyte Suspension——Blood was obtained from healthy adults by utilizing sodium citrate as an anticoagulant. The method for separating the cells was essentially the same as described by Murphy,[15] except that bovine serum albumin (5% (w/v)) dissolved in buffered-saline–glucose (8.12 g NaCl, 1.22 g Na_2HPO_4, 0.219 g $NaH_2PO_4 \cdot 2H_2O$ and 2.0 g glucose/l, pH 7.4) was used instead of serum. After centrifugation, the desired fractions were removed carefully from the tube. Usually the fractions taken were the top 10%, middle 10% and bottom 10%. These cells were washed 3 times with isotonic NaCl–phosphate buffer, pH 7.4. The washed erythrocytes were resuspended in the washing solution to make finally $40 \pm 1\%$ hematocrit value and in some experiments to make $5.0 \pm 0.2\%$.

Density Measurement of Various Fractions——Each fraction obtained was further separated by a discontinuous Dextran 40 density gradient centrifugation as described by Abraham *et al.*[16]

Drug-Induced Hemolysis——Drug-induced hemolysis of erythrocytes was measured as described in a previous paper,[14] by using the cell suspension of $40 \pm 1\%$ hematocrit value.

Osmotic Fragility——The cells treated with drug were washed once with the isotonic buffer and readjusted to $40 \pm 1\%$ hematocrit value. The osmotic fragility was measured as described in a previous paper.[14]

Measurement of Viscous Properties——For the measurement of viscous properties of erythrocytes, an Ostwald type viscometer (having a flow time of 93.4 ± 1.4 s with deionized water at 37 °C) was used. Figure 1 shows a schematic representation of the apparatus. The viscometer is connected to a vacuum reservoir and a pipetter fitted to the vacuum reservoir is used to evacuate the system by drawing up a column of water from a second reservoir. The erythrocytes, untreated or treated with the drug at 37 °C for 30 min, were resuspended in isotonic NaCl phosphate buffer to make finally $40 \pm 1\%$ hematocrit value, and 10 ml of the suspension was poured into the viscometer. The measurement of viscous properties was done under a negative pressure of 25 cm of water at 37 °C.

Peroxidation of Erythrocyte Lipids by Hydrogen Peroxide——Sodium azide was added to washed cell suspension (hematocrit value, $5.0 \pm 0.2\%$) to give a concentration of 2 mM. Equal volumes (2.0 ml each) of the cell suspension and 20 mM hydrogen peroxide were mixed and shaken for 60 min at 37 °C. A 2 ml aliquot of trichloroacetic acid–arsenite[17] was added to the incubation mixture and the whole was centrifuged. An aliquot (4 ml) of the supernatant was mixed with 1 ml of 0.7% thiobarbituric acid (TBA) solution, and the mixture was heated in a boiling water-bath for 15 min, then immediately cooled under tap water. The malondialdehyde (MDA) concentration was determined at 535 nm, using the molar extinction coefficient of MDA, $1.56 \times 10^5\ M^{-1} \cdot cm^{-1}$.[18]

Measurement of Reduced Glutathione and Tocopherol——The cells, separated and washed three times with isotonic NaCl–phosphate buffer, pH 7.4, were diluted with the same volume of water and lysed by freezing and thawing twice. The lysate (5.0 ml) was mixed with ice-cold 25% trichloroacetic acid (1.5 ml) and the supernatant obtained was used for the measurement of GSH. GSH and tocopherol in erythrocytes were determined by the methods of Morson *et al.*[19] and Taylor *et al.*,[20] respectively.

Enzyme Assays——The cells, separated and washed three times, were lysed by freezing and thawing twice. Hemoglobin was removed from the hemolysate by chloroform–ethanol–water extraction.[21] Aliquots of the final supernatants were used for assays. Activities of superoxide dismutase (SOD) [EC 1.15.1.1], glutathione peroxidase [EC 1.11.1.9] and catalase [EC 1.11.1.6] were assayed by the methods of Mirsa and Fridovich,[22] Sinet *et al.*[23] and Beers and Sizer,[24] respectively. One unit of activity was defined as the amount causing 50% inhibition of activity for SOD, as 1 μmol of NADPH oxidized per min for glutathione peroxidase and as the amount causing dispropor-

NII-Electronic Library Service

tionation at a rate of $10^{-3} \cdot s^{-1}$ for catalase.

Separation of Phospholipids and Fatty Acid Analysis——Extraction of the ghost membrane lipids and separation of phospholipids were done by the procedure of Colbeau *et al.*,[25] using a column of silicic acid–Celite 545 (2 : 1, by weight). The fatty acids of phospholipids, after hydrolysis in 0.5 N NaOH–methanol at 60 °C, were methylated with BCl_3–methanol.[26] The methyl esters were analyzed by gas-liquid chromatography (GLC) on a Shimadzu gas chromatograph, model GC-4BM, with a hydrogen flame ionization detector (15% ethylene glycol adipate, 60—80 mesh Chromosorb WAW DMCS, 3 mm × 2 m). In the case of erythrocytes treated with hydrogen peroxide, the fatty acids were analyzed by the same method. Because only relative values for each fatty acid methyl ester of a given sample were sought, a value of 18% of the total was assigned arbitrarily to methyl palmitate.

Electron Microscopy——Normal and drug-treated erythrocytes were fixed with 1.5% glutaraldehyde in isotonic sodium phosphate buffer, pH 7.2. The cells were washed 4 times with the phosphate buffer and dried with increasing concentrations of acetone (60 to 100% (v/v)). The specimens were coated at continuously varying angles with platinum and viewed with a Hitachi scanning electron microscope, model S-405.

Protein Determination——Protein concentration was determined by the procedure described by Lowry *et al.*[27] with bovine albumin, fraction V, as a standard.

Statistical Analysis——The means of all data are presented with their standard deviation (mean ± S.D.). Student's *t*-test was utilized to determine the significance of differences between the data, $p < 0.05$ being taken as the criterion of significance.

Results

Density Distribution of Separated Cells

Density distributions of erythrocytes separated into fractions of different ages are shown in Fig. 2. The cells of the top, middle and bottom fractions were mainly distributed among density ranges of 1.078 to 1.086, 1.086 to 1.091 and 1.096 to 1.102, respectively. The results presented in Fig. 2 showed that the separation of cells on various dextran mixtures was satisfactory. Reproducible distribution patterns were obtained in repeated experiments using different sources of blood. The main fraction obtained in each case was collected and the percentage with respect to applied cells was estimated after lysis with water. The percentages (total OD_{543}) were approximately 77, 54 and 65% for top, middle and bottom fractions, respectively. The relatively small percentage for the middle fraction was due to the presence of a subfraction in the density range of 1.091 to 1.096.

Drug-Induced Hemolysis

Results from the measurement of percentage hemolysis are plotted against drug concentration for the top and bottom fractions in Fig. 3A. Both chlorpromazine and clemastine were found to show slightly different hemolytic actions on the separated cells; the bottom (old) cells were more sensitive to the drugs as compared with the top (young) cells. The time course of hemolysis in the presence of clemastine (4 and 5×10^{-4} M) is depicted in Fig. 3B. At 5×10^{-4} M drug concentration, the rate of hemolysis of old cells was slightly faster than that of young cells, but the difference was not significant except for the 60 min data at 5×10^{-4} M.

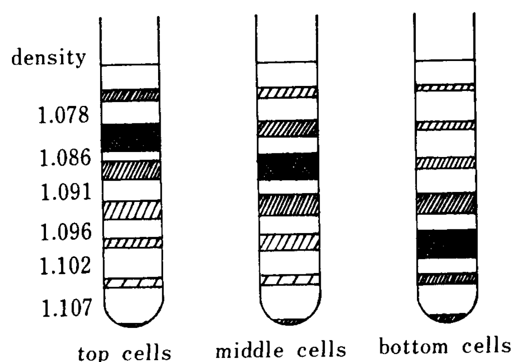

Fig. 2. Dextran 40 Discontinuous Density Gradient Centrifugation of Separated Cells

The appropriate dextran solutions were prepared by mixing Dextran 40 (in g), standard medium (in ml) and distilled water (in ml) in the following quantities: 24–80.0–20.0; 26–76.7–23.3; 28–73.3–26.7; 30–70.0–30.0; 32–66.7–33.3 and 34–63.3–36.7. The cell suspension (0.5 ml) was layered over the gradient and the tubes were centrifuged for 90 min at 90000 × *g* at 5 °C.

NII-Electronic Library Service

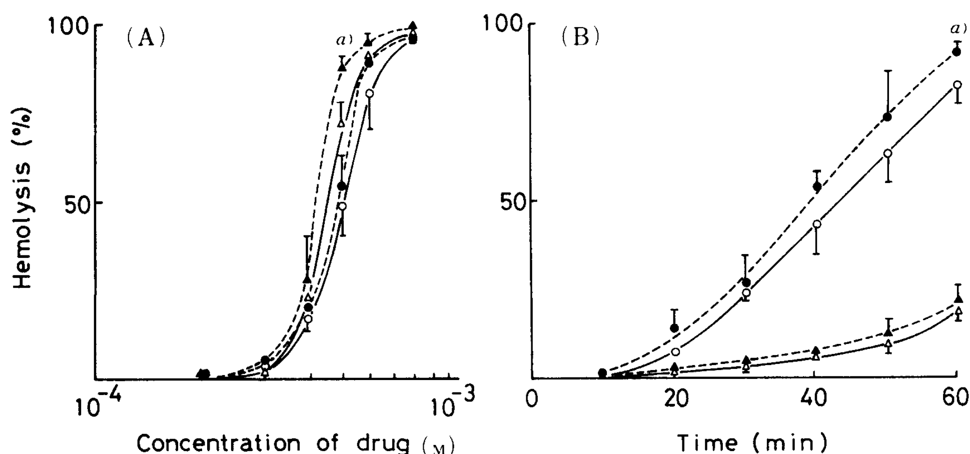

Fig. 3. Hemolytic Effect of Drugs on Young and Old Erythrocytes

A 0.3 ml aliquot of erythrocyte suspension (hematocrit value, $40 \pm 1\%$) was added to 3.0 ml of the test solution. The mixture was incubated for 60 min (A) or 10 to 60 min (B) and then centrifuged for 3 min. The percentage hemolysis was determined. Open and closed symbols are data for young and old cells, respectively. (A) chlorpromazine (○, ●); clemastine (△, ▲). (B) clemastine 4×10^{-4} M (△, ▲); 5×10^{-4} M (○, ●). *a*) $p < 0.05$ in young *vs.* old.

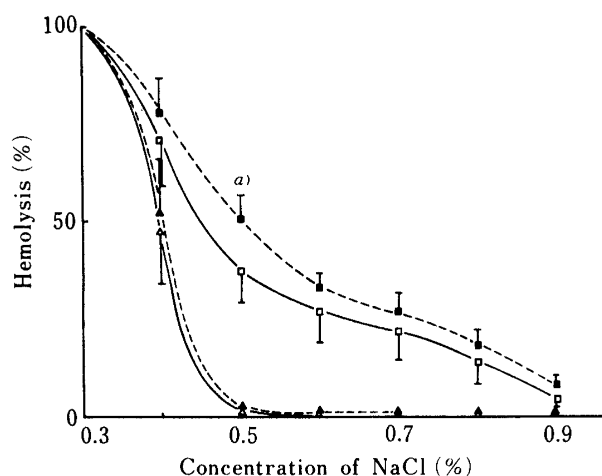

Fig. 4. Osmotic Fragility of Young and Old Erythrocytes Treated with Chlorpromazine

The cells were incubated in the drug solution at the indicated concentration for 30 min at 37 °C and washed once with isotonic buffer. A 0.3 ml aliquot of the cell suspension (hematocrit value, $40 \pm 1\%$) was added to 3 ml of hypotonic NaCl solution. The mixture was incubated for 30 min at 25 °C, and after centrifugation, the percentage hemolysis was determined. Open and closed symbols are data for young and old cells, respectively. Untreated (△, ▲); 5×10^{-5} M chlorpromazine (□, ■). *a*) $p < 0.05$ in young *vs.* old.

Osmotic Fragility of Erythrocytes Treated with Chlorpromazine

As shown in Fig. 4, the osmotic fragility of the old cells treated with chlorpromazine at 5×10^{-5} M was slightly increased as compared with that of young cells, although the difference was not statistically significant except for the 0.5% data, while there was no significant difference in fragility between young and old cells untreated with the drug.

Viscous Properties of Erythrocytes

The viscous properties of separated cells, untreated or treated with 10^{-4} M chlorpromazine, were measured by a simple method using a viscometer. The result is shown in Table I as the time necessary to flow through the viscometer. The young cells flowed more rapidly than old ones, indicating that the old cells were more viscous. The drug treatment, however, increased the flow time of young cells more than that of old cells, as shown in the "difference" column of the table. Thus, both young and old cells became more viscous when exposed to chlorpromazine.

Scanning Electron Microscopic Observations

The result of microscopic observations of separated cells is shown in Fig. 5 together with those of chlorpromazine-treated cells. Both young and middle cells showed typical biconcave

NII-Electronic Library Service

TABLE I. Viscous Properties of Erythrocytes Treated and Untreated with Chlorpromazine

Fraction	Drug treatment	Flow time (s)	Difference (s)
Young	None	74.0 ± 2.2	5.0
	Treated	$79.0 \pm 1.6^{a)}$	
Middle	None	$78.9 \pm 2.3^{b)}$	3.8
	Treated	82.7 ± 3.2	
Old	None	$91.5 \pm 4.2^{b,c)}$	3.2
	Treated	$94.7 \pm 3.9^{d)}$	

The viscous properties of young, middle and old cells and drug-treated cells, which had been incubated in chlorpromazine solution (1×10^{-4} M) for 30 min at 37 °C and resuspended in isotonic NaCl–phosphate buffer, were determined by using an Ostwald-type viscometer. Values are means ± S.D. of 4 experiments. *a)* $p < 0.01$ in none *vs.* treated in young cells. *b)* $p < 0.05$ in young *vs.* middle or old. *c)* $p < 0.01$ in middle *vs.* old. *d)* $p < 0.05$ in middle *vs.* old.

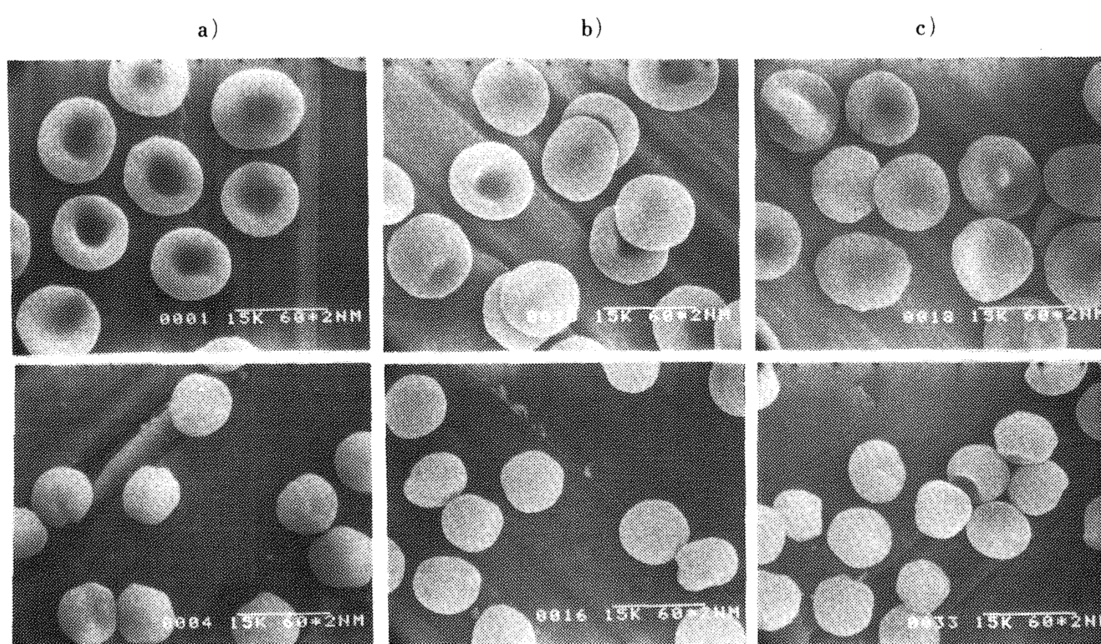

Fig. 5. Scanning Electron Micrographs of Erythrocytes Treated and Untreated with Chlorpromazine

a) Young cells, b) middle cells and c) old cells. The upper row shows cells with no drug treatment and the lower row shows the cells treated with 1.5×10^{-4} M chlorpromazine for 30 min at 37 °C (bar = 6 μm).

disc form, while some of the old cells appeared as irregular discs or were slightly transformed in shape. The drug treatment at 1.5×10^{-4} M induced shape change to spherocytes in all cells.

Lipid Peroxidation of Erythrocytes by Hydrogen Peroxide

Unsaturated lipid peroxidation by hydrogen peroxide *in vitro* was estimated in separated cells by means of the TBA reaction. As shown in Table II, old cells were peroxidized much less than young and middle cells, suggesting that the lipids of old cells may be less sensitive to oxidative stress produced by hydrogen peroxide than those of young cells under the conditions tested.

Antioxidant Levels and Activities of Enzymes of Peroxide Metabolism in Erythrocytes

The levels of antioxidant, GSH and tocopherol, in separated cells are shown in Table III.

NII-Electronic Library Service

TABLE II. Peroxidation of Erythrocytes by Hydrogen Peroxide

Fraction	Malondialdehyde concentration	
	nmol/ml cell suspension	nmol/g hemoglobin[a]
Young	5.75 ± 0.11	807 ± 38
Middle	4.42 ± 0.08[b]	638 ± 77[c]
Old	3.98 ± 0.17[b]	486 ± 67[b]

Equal volumes of the cell suspension (hematocrit value, 5.0 ± 0.2) and 0.068% (20 mM) hydrogen peroxide solution were incubated for 1 h at 37 °C under shaking. Lipid peroxidation was determined by means of the TBA reaction. *a*) Hemoglobin concentration was determined by the cyanmethemoglobin method. *b*) $p < 0.01$. *c*) $p < 0.05$ in young *vs.* middle or old.

TABLE III. Antioxidant Levels and Enzyme Activities in Young, Middle and Old Erythrocytes

Component	Young	Middle	Old
Reduced glutathione (nmol/mg protein[a])	2.86 ± 0.02	2.39 ± 0.13[c]	2.06 ± 0.04[d]
Tocopherol (μg/mg protein[a])	5.39 ± 0.17	3.98 ± 0.26[c]	2.77 ± 0.18[d]
Superoxide dismutase (unit/mg protein[b])	6.18 ± 0.49	4.24 ± 0.19[c]	1.88 ± 0.10[d]
Glutathione peroxidase (unit/mg protein[b])	3.23 ± 0.16	2.29 ± 0.17[c]	1.43 ± 0.10[d]
Catalase (unit/mg protein[b])	1.46 ± 0.08	1.14 ± 0.18[c]	0.78 ± 0.04[e]

Each value represents the mean $\pm$ S.D. of 4 experiments. *a*) Protein in hemolysate containing hemoglobin. *b*) Protein in supernatant after removing hemoglobin. *c*) $p < 0.005$ in young *vs.* middle. *d*) $p < 0.005$ in young or middle *vs.* old. *e*) $p < 0.01$ in middle *vs.* old.

The levels of components of peroxidative metabolism were significantly lower in old cells than in young ones, and in particular, the tocopherol content was dramatically decreased in old cells to half of that in young cells. The activities of enzymes of peroxide metabolism, SOD, catalase and glutathione peroxidase, were assayed in separated cells after the removal of hemoglobin. As shown in Table III, these enzyme activities were significantly lower in old cells than in the young cells. SOD activity in old cells was 30% of that in young ones. The decreased activities in old cells indicate that aging makes erythrocytes more sensitive to peroxide-induced cellular damage.

Fatty Acid Composition of Phospholipids in Erythrocyte Membrane

The percentage composition of major fatty acids in ghost membrane phospholipids was estimated for separated fractions. The proportion ($11.4 \pm 1.6\%$) of arachidonic acid in old cells was significantly lower than that ($13.9 \pm 1.1\%$) in young cells ($p < 0.05$). In addition, the percentages of polyenoic fatty acids other than arachidonic acid in old cells were slightly lower (but not statistically significant) than those in young ones. The values of the ratio of unsaturated/saturated fatty acids were 1.55 ± 0.20, 1.48 ± 0.26 and 1.39 ± 0.20 in young, middle and old cells, respectively.

Fatty Acid Composition of Phospholipids Following Peroxidation by Hydrogen Peroxide

The fatty acid composition of phospholipids following treatment of cells with hydrogen peroxide is shown in Table IV. The treatment with hydrogen peroxide dramatically reduced

NII-Electronic Library Service

TABLE IV. Fatty Acid Composition of Phospholipids in Erythrocytes Treated and Untreated with Hydrogen Peroxide

Fatty acid	Treated composition (%)			Untreated composition (%)
	Young	Middle	Old	Whole cells
$C_{16=0}$	18.4 ± 1.5	18.0 ± 1.2	18.7 ± 3.6	18.0 ± 1.1
$C_{18=0}$	13.5 ± 1.3	12.9 ± 1.9	13.5 ± 2.0	14.2 ± 1.1
$C_{18=1}$	15.4 ± 0.4	14.5 ± 1.2	15.8 ± 4.3	15.2 ± 0.2
$C_{18=2}$	9.7 ± 1.0	9.1 ± 0.9	9.2 ± 1.8	9.6 ± 0.9
$C_{20=4}$	3.5 ± 1.1[a]	4.7 ± 1.3[a]	4.8 ± 1.1[a]	11.4 ± 0.7
$C_{20=5}$	0	0	0	1.6 ± 0.5
$C_{22=6}$	0	0	0	8.9 ± 1.0

A value of 18.0% of total methyl esters was assigned arbitrarily to methyl palmitate of middle or untreated cells. Each value represents the mean ± S.D. of 3 experiments. *a*) $p < 0.01$ in untreated *vs.* young, middle or old.

the proportion of polyunsaturated fatty acids; $C_{20=5}$ and $C_{22=6}$ fatty acids were completely lost and arachidonic acid was greatly reduced in all separated cells. However, the reduction of arachidonic acid was much greater in young cells than in old cells. The values of the ratio of unsaturated/saturated fatty acids following hydrogen peroxide were 0.90 ± 0.12, 0.92 ± 0.14 and 0.93 ± 0.24 in young, middle and old cells, respectively. These results suggested that the polyunsaturated fatty acids of young cell membrane are much more sensitive to hydrogen peroxide-induced peroxidation than are those of old cells under the conditions tested. This result is consistent with the higher MDA concentrations following peroxidation by hydrogen peroxide in young cells, as shown in Table II.

Discussion

The present study indicated that old cells were hemolyzed at relatively lower drug concentrations than young cells, and that old cells exposed to chloropromazine at 5×10^{-5} M showed enhanced osmotic fragility (Figs. 3 and 4). These results suggest that aged cells have an increased sensitivity to drugs. The increase in drug sensitivity in aged cells may be a result of partial loss of intracellular components, such as ATP[9,10] and G-6-PD,[13] and alteration of membrane structure.[28]

It has been shown that the lifespan of erythrocytes is not determined by factors related to membrane flexibility or cell shape but may depend on changes in their viscous properties,[29] based on micropipette analysis. The increase in viscous properties of cells may be related to the removal of senescent cells by phagocytosis. The increased viscous properties of old cells may be associated with an increase in the internal viscosity. The data obtained were approximately consistent with the increase in elastic shear reported by Nash and Wyard.[29] The aged cells having increased viscous properties will thus be trapped in the spleen and removed from the blood. When exposed to a higher concentration of drug, old cells also showed the highest viscosity. This suggested that treatment of old cells with drugs would further shorten the lifespan. The increase in the viscous properties of cells induced by drug treatment might be partly due to the transformation (Fig. 5) and aggregation of cells. On the other hand, it has also been shown that the sialic acid content of circulating erythrocytes decreases with the age of the cells[30,31] (in old cells the sialic acid content is 10—15% less than in younger cells[30]), and that the decrease in content is closely related to the elimination from the circulation of senescent cells.[32]

Electron microscopic observations indicated that most older normal cells were partially transformed and that the drug treatment transformed all cells to spherocytes. The "difference"

NII-Electronic Library Service

in the flow time (viscous properties) caused by the drug treatment was less in old cells than in young ones (Table I). This is explained by the reduced transformation capacity of old cells.

Of particular interest was the result of lipid peroxidation by hydrogen peroxide; the lipids of old cells were significantly less peroxidized than those of young cells (Table II). This phenomenon may be partly related to lower percentages of unsaturated fatty acids in older cells. This interpretation is consistent with a larger loss of polyunsaturated fatty acids of phospholipids in young cells as compared with that of old cells after the *in vitro* hydrogen peroxide treatment (Table IV).

The old cells had a significantly lower concentration of arachidonic acid in membrane phospholipids as compared with young ones. Although the effect was not statistically significant, a slight decrease in docosahexaenoic acid was observed in old cells, as reported by Phillips *et al.*[33] The differences in phospholipid fatty acids of cells may be of importance in relation to membrane functions. The fluidity and permeability of the cell membrane are strongly affected by the fatty acid composition of phospholipids.[10,34,35] Therefore, the lower degree of unsaturation of the phospholipid fatty acids in old cells may be related to the decreased deformability of cells and to the reduced permeability of the membrane.

GSH is an important constituent of erythrocytes. Its destruction causes hemolysis of G-6-PD-deficient red blood cells, and hereditary deficiencies in the enzymes of GSH synthesis are associated with shortened erythrocyte lifespan.[36] Tocopherol abolishes the effects of peroxide treatment on fatty acid peroxidation and the disturbance of phospholipid organization in the erythrocyte membrane.[37] On the other hand, SOD, catalase and glutathione peroxidase are believed to form the primary enzymatic defence against oxygen toxicity and lipid peroxidation.[38] It is therefore of special interest to estimate the levels of these antioxidants and enzymes in relation to the protection against peroxide-induced cellular damage. In old cells, allowing for the increased cell density, these antioxidant levels and enzyme activities were greatly diminished as compared with those of young cells (Table III), suggesting that the initiation and propagation of free-radical reactions might proceed relatively rapidly, consequently promoting the aging of cells. This concept is supported by the observation that lipid peroxidation is implicated in membrane alterations associated with aging of cells and tissues.[28,39]

References

1) P. Rous, *Physiol. Rev.*, **3**, 75 (1923).
2) Y. Tanimoto, "Ketsuekigaku, Hito to Dobutsu no Setten," Seishi Shoin, Tokyo, 1982, pp. 58—60.
3) G. J. Johnson, D. W. Allen, S. Cadman, V. F. Fairbanks, J. G. White, B. C. Lampkin and M. E. Kaplan, *N. Engl. J. Med.*, **301**, 522 (1979).
4) P. Teitel, *Blood Cells*, **3**, 55 (1977).
5) R. I. Weed, *Am. J. Med.*, **49**, 147 (1970).
6) P. L. Lacelle and B. Arkin, *Blood*, **36**, 837 (1970).
7) P. B. Canham, *Circ. Res.*, **25**, 39 (1969).
8) M. Wintrobe, "Clinical Hematology," 7th ed., Lea and Febiger, Philadelphia, 1967, pp. 200—204.
9) N. S. Cohen, J. E. Ekholm, M. G. Luthra and D. J. Hanahan, *Biochim. Biophys. Acta*, **419**, 229 (1976).
10) T. Shiga, N. Maeda, T. Suda, K. Kon and M. Sekiya, *Biochim. Biophys. Acta*, **553**, 84 (1979).
11) N. Mohandas, W. M. Phillips and M. Besis, *Semin. Hematol.*, **16**, 95 (1979).
12) P. L. Lacelle, *Biorheology*, **9**, 51 (1972).
13) B. M. Turner, R. A. Fisher and H. Harris, *Clin. Chim. Acta*, **50**, 85 (1974).
14) T. Ogiso, S. Imai, R. Hozumi, M. Kurobe and Y. Kato, *Chem. Pharm. Bull.*, **24**, 479 (1976).
15) J. R. Murphy, *J. Lab. Clin. Med.*, **82**, 334 (1973).
16) E. C. Abraham, D. Walker, M. Gravely and T. H. J. Huisman, *Biochem. Med.*, **13**, 56 (1975).
17) J. Stocks and T. L. Dormandy, *Br. J. Haematol.*, **20**, 95 (1971).
18) E. D. Wills, *Biochem. J.*, **113**, 315 (1969).
19) M. S. Morson, J. W. Depierre and B. Mannervik, *Biochim. Biophys. Acta*, **582**, 67 (1979).
20) S. L. Taylor, M. P. Lamden and A. L. Tappel, *Lipids*, **11**, 530 (1976).

NII-Electronic Library Service

21) A. Concetti, P. Massei, G. Rotilio, M. Bruneri and E. Rachmilewitz, *J. Lab. Clin. Med.*, **87**, 1057 (1976).
22) H. P. Mirsa and I. Fridovich, *J. Biol. Chem.*, **247**, 3170 (1972).
23) P. M. Sinet, A. M. Michelson, A. Bazin, J. Lejeune and H. Jerome, *Biochem. Biophys. Res. Commun.*, **67**, 910 (1975).
24) R. F. Beers, Jr. and I. W. Sizer, *J. Biol. Chem.*, **195**, 133 (1952).
25) A. Colbeau, J. Nachbaur and P. M. Vignais, *Biochim. Biophys. Acta*, **249**, 462 (1971).
26) L. D. Metcalfe, A. A. Schmitz and J. R. Pelka, *Anal. Chem.*, **38**, 514 (1966).
27) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
28) P. Hochstein and S. K. Jain, *Fed. Proc.*, **40**, 183 (1981).
29) G. B. Nash and S. J. Wyard, *Biochim. Biophys. Acta*, **643**, 269 (1981).
30) A. Yaari, *Blood*, **33**, 159 (1969).
31) A. Baxter and J. G. Beeley, *Biochem. Soc. Trans.*, **3**, 134 (1975).
32) D. Aminoff, W. F. V. Bruegge, W. C. Bell, K. Sarpolis and R. Williams, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 1521 (1977).
33) G. B. Phillips, J. T. Dodge and C. Howe, *Lipids*, **4**, 544 (1969).
34) G. A. Thompson, Jr. and Y. Nozawa, *Biochim. Biophys. Acta*, **472**, 55 (1977).
35) A. D. Keith, B. J. Wisnieski, S. Henry and J. C. Williams, "Lipids and Biomembranes of Eukaryotic Microorganisms," ed. by J. A. Erwin, Academic Press, New York, 1973, p. 259.
36) E. Beutler, "The Metabolic Basis of Inherited Disease," 4th ed., ed. by J. B. Stanbury, J. B. Wyngaarden, D. S. Fredrickson, J. L. Goldstein and M. S. Brown, McGraw-Hill, New York, 1983, pp. 1629—1953.
37) S. K. Jain, *Biomed. Biochim. Acta*, **42**, S43 (1983).
38) R. W. Carrell, C. C. Winterboun and E. A. Rachmilewitz, *Br. J. Haematol.*, **30**, 259 (1975).
39) G. Cohen and P. Hochstein, *Biochemistry*, **3**, 895 (1964).

NII-Electronic Library Service