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Peroxidation of liposomes in the presence of human erythrocytes and induction of membrane damage of erythrocytes by peroxidized liposomes

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Hemolysis (Kobayashi, T., Takahashi, K., Yamada, A., Nojima, S. and Inoue, K. (1983) J. Biochem. 93, 675–680) and shedding of acetylcholinesterase-enriched membrane vesicles (diameter 150–200 nm) were observed when human erythrocytes were incubated with liposomes of phosphatidylcholine which contained polyunsaturated fatty acyl chains. These events occurring on erythrocyte membrane were inhibited by radical scavengers or incorporation of α-tocopherol into liposomes, suggesting that lipid peroxidation is involved in the process leading to membrane vesiculation and hemolysis. The idea was supported by findings that generation of chemiluminescence, formation of thiobarbituric acid reactive substance, accumulation of conjugated diene compounds in liposomes and decrease of polyunsaturated fatty acids in liposomes occurred concomitantly during incubation. Hemolysis was also suppressed by the addition of extra liposomes, insensitive to peroxidation, or of serum albumin even after the completion of peroxidation of liposomes. These results suggest that peroxidized lipids, responsible for vesiculation and hemolysis, may be formed first in liposomes and then gradually transferred to erythrocyte membranes. The accumulation of these lipids peroxides may eventually cause membrane vesiculation followed by hemolysis.

Introduction

Studies on liposome-cell interactions provide models of various physiological processes [1–3]. Erythrocytes are cells which have been employed widely to examine the interactions with liposomes. Several ways by which liposomes or proteoliposomes interact with erythrocytes have been reported. They include adhesion [4], fusion [5,6], transfer of membrane proteins from liposomes to erythrocytes [7,8] or vice versa [9], and alteration of cholesterol content of erythrocyte membrane [10]. Recently, Ott et al. [11] showed that the incubation of human erythrocytes with dimyristoylglycerophosphocholine liposomes caused the release of plasma membrane vesicles. We have demonstrated that erythrocytes of various species of animals shed membrane vesicles without any appreciable hemolysis when incubated with sonicated liposomes of dilauroylglycerophosphocholine [12].

In a previous paper, we showed that liposomes composed of phosphatidylcholine, which contained polyunsaturated fatty acyl chains, induced lysis of erythrocytes from various animals [13]. During a study on the mechanism of hemolysis by the phosphatidylcholine liposomes, we observed that vesiculation of erythrocyte membrane was also induced. The involvement of formation of lipid peroxides in liposomes during incubation with erythrocytes was suggested in the process leading to such membrane changes of erythrocytes. The

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sequential events leading to vesiculation as well as hemolysis during incubation of erythrocytes with liposomes will be discussed.

Materials and Methods

Buffer. Phosphate-buffered saline [13] was used throughout.

Erythrocytes. The human erythrocytes were prepared as described previously [13].

Lipids. Egg yolk and rat liver phosphatidylcholines were prepared by chromatography on neutral aluminium oxide and Unisil. Cholesterol, L-α-dimyristoylglycerophosphocholine, DL-α-dipalmitoylglycerophosphocholine, L-α-distearoylglycerophosphocholine and L-α-dioleoylglycerophosphocholine were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., and used without further purification. L-α-Dilinoleoylglycerophosphocholine was purchased from Nippon Shoji Co., Osaka, Japan, and used after the removal of BHT (2,6-di(*tert*-butyl)-*p*-cresol). All lipid preparations showed a single spot on silica-gel thin-layer chromatograms. Sonicated liposomes of egg yolk phosphatidylcholine were prepared as described previously [13]. Multilamellar liposomes were prepared by suspending a dried sample of a lipid in the medium used in the experiments.

Treatment of erythrocytes with sonicated liposomes. A liposome suspension (900 μl) was preincubated for 15 min at the required temperature and the reaction was started by adding 100 μl of erythrocyte suspension. The mixture was occasionally gently shaken during the reaction. After incubation, the generation of chemiluminescence, degree of lipid peroxidation, the release of acetylcholinesterase activity and hemolysis were determined.

Measurement of chemiluminescence. Chemiluminescence was measured after the transfer of 950 μl of the reaction mixture to the prewarmed cuvette containing 50 μl of saturated solution of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione). The cuvette was constantly stirred and the chemiluminescence was measured with Chemiluminescence Reader (Aloka, BLR-102, counting rate, 30KC/M; time constant, 30 s). In some experiments, an appropriate amount of a phosphatidylcholine suspension containing luminol (900 μl) was first preincubated for 15 min at the required temperature in the cuvette stirred at a constant speed and the reaction was started by adding 100 μl of erythrocyte suspension.

Measurement of lipid peroxidation. The amount of thiobarbituric acid reactive substance was estimated by the method of Buege and Aust [14] and expressed as equivalents of malondialdehyde. For the assay of the formation of conjugated dienes in liposome fraction, liposomes were separated from erythrocytes by the centrifugation of reaction mixture at $12000 \times g$ for 15 min. Then, lipids in liposome fraction were extracted by the method of Bligh and Dyer [15]. Lipids were dissolved in ethanol and ultraviolet absorption was measured with a Hitachi 200-10 recording spectrometer. The increase in absorbance at 233 nm of extracted lipid reflects the formation of conjugated dienes [14]. The fatty acid composition of phosphatidylcholine was determined by gas-liquid chromatography as described earlier [16].

Other analytical methods. The release of acetylcholinesterase activity from erythrocytes was determined as described previously [12]. Hemolysis was assayed using ^{51}Cr-labeled erythrocytes [13].

Other chemicals. Bovine superoxide dismutase (2900 units/mg), bovine catalase (2200 units/mg) and crystallized bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Luminol was purchased from Wako Chemical Co., Osaka, Japan. α-Tocopherol was a gift from Eisai Co., Tokyo, Japan. Other chemicals were all of reagent grade.

Results

Serial events observed upon exposure of washed erythrocytes to egg yolk phosphatidylcholine liposomes

When liposomes composed of egg yolk phosphatidylcholine were incubated with human erythrocytes at 37°C, chemiluminescence was generated (Fig. 1A). Either liposomes or erythrocytes alone produced no appreciable chemiluminescence under the present conditions. Chemiluminescence reached peak within 10 min and thereafter declined sharply. Liposomes prepared from rat liver phosphatidylcholine or dilinoleoylglycerophosphocho-

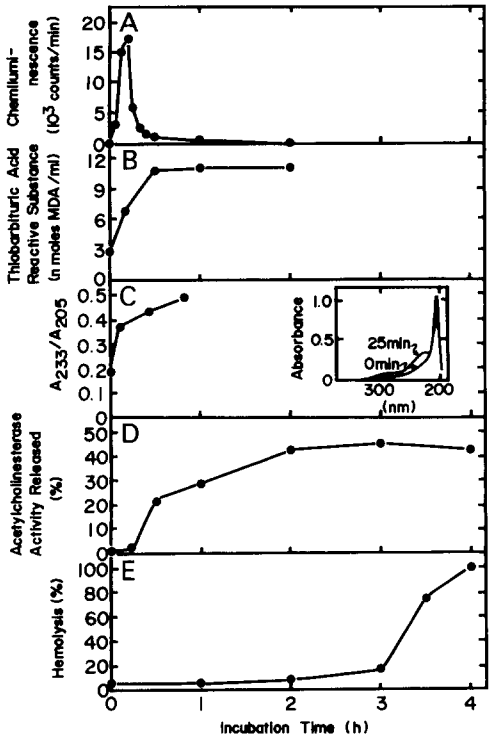

Fig. 1. Serial events observed upon exposure of washed erythrocytes to egg yolk phosphatidylcholine liposomes. (A) Human erythrocytes ($1 \cdot 10^7$ cells/ml) were incubated with 100 μM egg yolk phosphatidylcholine at 37°C. At appropriate intervals, chemiluminescence was determined as described in Materials and Methods. (B) Human erythrocytes ($1 \cdot 10^8$ cells/ml) were incubated with 1 mM egg yolk phosphatidylcholine at 37°C. At appropriate intervals, thiobarbituric acid reactive substance in the reaction mixture was determined as described in Materials and Methods. MDA; malondialdehyde. (C) Human erythrocytes were incubated with egg yolk phosphatidylcholine as described in (B). At appropriate intervals, an aliquot of the reaction mixture was centrifuged to separate liposomes from erythrocytes. Lipids of the liposome fraction were extracted and ultraviolet absorption was determined as described in Materials and Methods. Inset shows the optical spectrum of lipids of the liposome fraction at time 0 and after 25 min incubation with erythrocytes. (D) Human erythrocytes ($1 \cdot 10^8$ cells/ml) were incubated with 100 μM egg yolk phosphatidylcholine at 37°C. Acetylcholinesterase activity in the $300 \times g$ supernatant was determined as described in Materials and Methods. Acetylcholinesterase activity of the whole incubation mixtures at the beginning of the experiment was taken as 100%. (E) Erythrocytes ($1 \cdot 10^7$ cells/ml) were incubated with 200 μM egg yolk phosphatidylcholine at 37°C. The percentage of hemolysis was determined at appropriate intervals using ^{51}Cr-labeled erythrocytes as described in Materials and Methods.

line were also active for generating chemiluminescence, whereas those prepared from dioleoylglycerophosphocholine were not (data not shown).

The amount of thiobarbituric acid reactive substance in the reaction mixture increased almost linearly during 30 min (Fig. 1B). The conjugated diene concentration in liposomes increased simultaneously when incubated with erythrocytes (Fig. 1C).

Change of fatty acid composition of phosphatidylcholine recovered from the liposome fraction was also examined (Table I). Throughout the experiment, the ratio of palmitic acid, stearic acid or oleic acid to an internal standard, pentadecanoic acid, was not changed significantly, whereas those of polyunsaturated fatty acids, such as linoleic acid and arachidonic acid decreased. These findings are consistent with the idea that some molecular species containing polyunsaturated fatty acid in phosphatidylcholine was peroxidized during incubation with erythrocytes.

Exposure of washed erythrocytes to egg yolk phosphatidylcholine liposomes caused the release of acetylcholinesterase activity from erythrocytes preceding hemolysis (Fig. 1D). Release of acetylcholinesterase could be observed after a lag of 15 min and became a plateau within 2 h. Total acetylcholinesterase activity in reaction mixture remained constant under the present conditions. The acetylcholinesterase activity released to the medium was separated from the liposomes by a discontinuous Dextran density gradient centrifugation (data not shown). When the medium was centrifuged further at $12\,000 \times g$ for 15 min, about 80% of the enzyme activity was recovered from the sediment. Under scanning electron microscopy, vesicles with an average diameter of 150–200 nm were observed in the sediment fraction. These findings indicate that the release of acetylcholinesterase activity was indicative of the release of acetylcholinesterase-enriched vesicles from erythrocytes.

Erythrocytes were eventually hemolyzed after a lag of 3 h when incubated with the liposomes (Fig. 1E).

The production of chemiluminescence was temperature-dependent and could be observed significantly above 31°C (Table II). The increase of thiobarbituric acid reactive substance was also

TABLE I

CHANGE OF FATTY ACID COMPOSITION OF EGG YOLK PHOSPHATIDYLCHOLINE DURING INCUBATION WITH ERYTHROCYTES

Human erythrocytes ($1 \cdot 10^8$ cells/ml) were incubated with 1 mM egg yolk phosphatidylcholine. At appropriate intervals, the reaction mixture was centrifuged and liposomes were separated from erythrocytes. Lipids of the liposome fraction were extracted and fatty acid composition was determined as described in Materials and Methods.

Incubation time (min)	Fatty acid composition (%)							
	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4
0	3.1	31.9	1.6	13.0	29.6	17.3	0.1	3.4
5	2.4	33.7	1.4	12.8	30.5	15.9	0.9	2.3
15	2.2	35.8	1.4	12.4	30.5	14.8	0.5	2.4
30	4.1	33.9	1.4	15.7	31.2	11.9	0.7	1.3
60	2.4	35.5	1.2	14.1	31.6	13.3	0.4	1.5
60 (without erythrocytes)	1.8	33.7	1.3	12.5	29.5	17.3	0.3	3.6

temperature-dependent. The increase observed after 1 h incubation at 25°C was 16.2% of that observed at 37°C. At 10°C, the increase of thiobarbituric acid reactive substance was negligible.

Release of membrane vesicles (membrane vesiculation) also occurred above 25°C; significant release of acetylcholinesterase activity was observed at 25°C after 2 h incubation, but no appreciable release occurred below 10°C. Hemolysis was again dependent on the incubation temperature. The time required for 40% hemolysis was 3.2 h at 37°C and 5.8 h at 31°C. Below 25°C, no appreciable hemolysis was observed even after 6 h incubation.

Effect of lipid composition of liposomes on their sensitivity to peroxidation and activity to induce chemiluminescence production and erythrocyte damage

Egg yolk phosphatidylcholine liposomes have been shown to remove cholesterol from erythrocyte membranes [10]. The removal of cholesterol from erythrocyte membranes is dependent on the cholesterol content of the liposomes; liposomes

TABLE II

TEMPERATURE DEPENDENCE OF GENERATION OF CHEMILUMINESCENCE, INCREASE OF THIOBARBITURIC ACID REACTIVE SUBSTANCE, VESICULATION AND HEMOLYSIS

Human erythrocytes were incubated with egg yolk phosphatidylcholine liposomes under the same conditions described in the legend to Fig. 1 at various temperatures. Then chemiluminescence produced in the first 12 min, the increase of thiobarbituric acid reactive substance after 1 h, the release of acetylcholinesterase activity after 2 h and degree of hemolysis after 6 h were determined. The values at 37°C were taken as 100%.

Temp. (°C)	Percentage			
	Relative chemiluminescence	Relative amount of thiobarbituric acid reactive substance	Relative amount of acetylcholinesterase activity released	Relative hemolysis
0	0	0	0	0
10	0	2.7	0	0
25	0.3	16.2	69.1	0
31	10.0	67.4	83.9	43.0
37	100	100	100	100

composed of egg yolk phosphatidylcholine/cholesterol (molar ratio, 1:1) do not appreciably remove cholesterol from erythrocyte membranes [10]. The generation of chemiluminescence and the increase of thiobarbituric acid reactive substance as well as membrane vesiculation and hemolysis were, however, not significantly affected by incorporation of even equimolar cholesterol into egg yolk phosphatidylcholine liposomes (data not shown). The incorporation of α-tocopherol into egg yolk phosphatidylcholine liposomes (molar ratio, 1 : 100) almost completely suppressed the generation of chemiluminescence, the increase of thiobarbituric acid reactive substance, vesiculation and hemolysis. The inhibition of hemolysis by α-tocopherol could not be observed when α-tocopherol was added as emulsion to the mixtures of liposomes and erythrocytes (data not shown).

Inhibition of production of chemiluminescence and thiobarbituric acid reactive substance and induction of erythrocyte damage by radical scavengers and some enzymes

Generation of chemiluminescence observed upon incubation of erythrocytes with liposomes was suppressed by the presence of enzymes such as superoxide dismutase, catalase and scavengers of active oxygen species such as hydroquinone, thiourea, tryptophan and ascorbate in medium (Table III). The increase of thiobarbituric acid reactive substance was also suppressed by the presence of thiourea and dimethylthiourea. The presence of superoxide dismutase did not affect the increase of thiobarbituric acid reactive substance, whereas catalase partially suppressed the increase.

Vesiculation and hemolysis were suppressed by the presence of thiourea and dimethylthiourea. Catalase suppressed hemolysis, but it did not significantly affect vesiculation.

Effect of concentration of egg yolk phosphatidylcholine liposomes on inducing erythrocyte damage

Almost 100% hemolysis was observed when erythrocytes were incubated with 100–200 μM of phosphatidylcholine for 4 h at 37°C (Fig. 2D). With more than 500 μM of phosphatidylcholine, hemolysis was, however, rather suppressed. The amount of chemiluminescence generated increased with increase of phosphatidylcholine added and became a plateau at 500 μM phosphatidylcholine (Fig. 2A, inset). Dose dependence of chemiluminescence generation is similar to that of hemolysis when the amount of chemiluminescence was plotted per unit of phosphatidylcholine (μM) (Fig. 2A). The increase of conjugated diene in

TABLE III

EFFECTS OF 'PEROXIDATION INHIBITORS' ON THE PRODUCTION OF CHEMILUMINESCENCE, INCREASE OF THIOBARBITURIC ACID REACTIVE SUBSTANCE, VESICULATION AND HEMOLYSIS

Human erythrocytes were incubated with egg yolk phosphatidylcholine liposomes under the same conditions as described in the legend to Fig. 1 in the presence of various 'inhibitors'. Then chemiluminescence produced in the first 12 min, the increase of thiobarbituric acid reactive substance after 1 h, the release of acetylcholinesterase activity after 1.5 h and degree of hemolysis after 4 h were determined. The values without inhibitors were taken as 100%. n.d., not done.

Inhibitor added	Percentage			
	Relative chemiluminescence	Relative amount of thiobarbituric acid reactive substance	Relative amount of acetylcholinesterase activity released	Relative hemolysis
None	100	100	100	100
Superoxide dismutase (200 U/ml)	0.9	79.8	89.6	87.6
Catalase (2700 U/ml)	0.4	89.1	95.8	6.3
Cytochrome *c* (30 μM)	0.4	n.d.	n.d.	61.2
Thiourea (100 mM)	0.7	0	0	0
Dimethylthiourea (100 mM)	0.1	0	2.0	0
Urea (100 mM)	76.3	n.d.	96.9	100
Mannitol (100 mM)	31.3	n.d.	n.d.	95.1

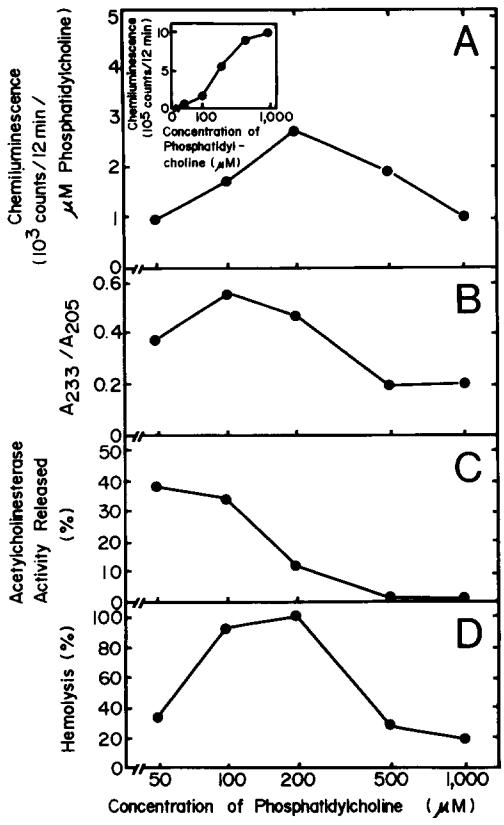

Fig. 2. Effect of concentration of egg yolk phosphatidylcholine on production of chemiluminescence, increase of conjugated diene in liposomes, membrane vesiculation and hemolysis. Human erythrocytes ($1 \cdot 10^7$ cells/ml) were incubated with various concentrations of egg yolk phosphatidylcholine at 37°C. Then, generation of chemiluminescence in the first 12 min (A), A_{233}/A_{205} ratio of the liposome fraction after 1 h incubation (B), release of acetylcholinesterase activity after 1.5 h incubation (C) and hemolysis after 4 h incubation (D) were determined.

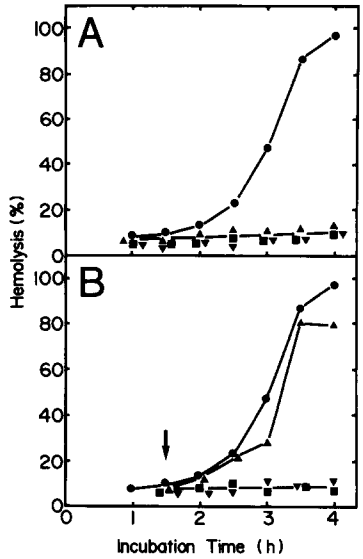

Fig. 3. Effects of thiourea, bovine serum albumin and dioleoylglycerophosphocholine on hemolysis induced by egg yolk phosphatidylcholine. (A) Human erythrocytes ($1 \cdot 10^7$ cells/ml) were incubated with 100 μM egg yolk phosphatidylcholine in the absence (●) or presence of thiourea (final concentration, 10 mM) (▲), bovine serum albumin (4 mg/ml) (■) or multilamellar liposomes composed of dioleoylglycerophosphocholine (500 μM) (▼). In (B) Thiourea (▲), bovine serum albumin (■) or liposomes composed of dioleoylglycerophosphocholine (▼) was added 1.5 h after incubation of erythrocytes with egg yolk phosphatidylcholine liposomes. Arrow indicates the time when these reagents were added.

liposomes (A_{233}/A_{205}) showed about the same dose dependence (Fig. 2B). Vesiculation was also suppressed with high concentrations of phosphatidylcholine (Fig. 2C).

Inhibition of egg yolk phosphatidylcholine liposome-induced hemolysis by serum albumin and by the presence of extra liposomes insensitive to peroxidation

The hemolysis by egg yolk phosphatidylcholine was inhibited by the presence of serum albumin or liposomes composed of dioleoylglycerophosphocholine (Fig. 3A). Dioleoylglycerophosphocholine liposomes did not affect the generation of chemiluminescence, whereas thiourea suppressed the generation completely. Formation of thiobarbituric acid reactive substance was also not inhibited by serum albumin and dioleoylglycerophosphocholine (data not shown). Albumin and dioleoylglycerophosphocholine showed suppressive effect even after erythrocytes and egg yolk phosphatidylcholine liposomes were previously incubated for 1.5 h at 37°C (Fig. 3B). The findings suggest that the mechanism of inhibition by these compounds is different from that of thiourea, since thiourea showed inhibition only when it was added to the reaction mixtures at the time of mixing of erythrocytes with egg yolk phosphatidylcholine. Albumin and dioleoylglycerophosphocholine may inhibit the process after generation of active oxygen and peroxidation of liposomal lipids. Vesiculation was also inhibited when albumin or dioleoyl-

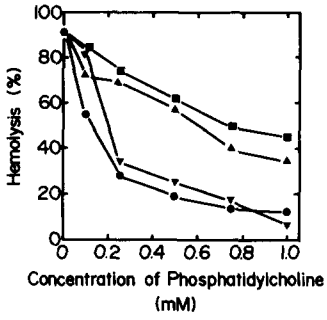

Fig. 4. Effects of various phosphatidylcholines on hemolysis induced by egg yolk phosphatidylcholine. Human erythrocytes ($1 \cdot 10^7$ cells/ml) were incubated with 100 μM egg yolk phosphatidylcholine in the presence of various concentration of multilamellar liposomes composed of dimyristoylglycerophosphocholine (●), dipalmitoylglycerophosphocholine (▲), distearoylglycerophosphocholine (■) or dioleoylglycerophosphocholine (▼) at 37°C for 4 h. After incubation, percentage of hemolysis was determined as described in Materials and Methods.

glycerophosphocholine liposomes were present in the reaction mixture (data not shown).

Fig. 4 shows the effect of fatty acid composition of liposomes on their inhibitory activity to hemolysis. Dimyristoylglycerophosphocholine liposomes were equally active to dioleoylglycerophosphocholine liposomes, whereas distearoylglycerophosphocholine and dipalmitoylglycerophosphocholine liposomes were much less active at 37°C.

Discussion

Incubation of washed erythrocytes with egg yolk phosphatidylcholine liposomes resulted in membrane vesiculation as well as hemolysis. It should be stated that complete hemolysis was observed when erythrocytes were incubated with very high concentrations of phosphatidylcholine (100–200 nmol liposome lipids/4 nmol erythrocyte membrane lipids). Incubation of egg yolk phosphatidylcholine liposomes with erythrocytes can cause removal of cholesterol from erythrocyte membranes [10]. It was also reported that cholesterol depletion increased the osmotic fragility of erythrocytes [17]. However, membrane damage of erythrocytes observed in the present experiment may not be due to cholesterol depletion from erythrocytes, since incorporation of cholesterol into liposomes did not significantly affect both vesiculation and hemolytic activity of phosphatidylcholine liposomes.

We postulate that some products arising in liposomes, which were peroxidized during incubation with erythrocytes, participate in erythrocyte membrane damage for following reasons: (1) Generation of chemiluminescence, formation of thiobarbituric acid reactive substance, increase of conjugated diene and decrease of polyunsaturated fatty acid content in liposomes preceded erythrocyte membrane damage. (2) Generation of chemiluminescence, formation of thiobarbituric acid reactive substance, membrane vesiculation as well as hemolysis showed temperature dependence. (3) α-Tocopherol and several radical scavengers inhibited all of the reactions described above. (4) The amount of chemiluminescence plotted per unit of phosphatidylcholine, the increase of conjugated diene in liposomes, membrane vesiculation and hemolysis showed about the same dose dependence of phosphatidylcholine.

Although oxygen radicals have been known to cause damaging effects on erythrocyte membranes [18–22], erythrocyte membrane damage observed in the present study was not induced by oxygen radical itself. The amount of chemiluminescence generated, which reflects the production of oxygen radicals, increased with the increase of the concentration of phosphatidylcholine, whereas vesiculation and hemolysis were suppressed with high concentrations of phosphatidylcholine. O'Mally et al. [23] previously reported that erythrocyte acetylcholinesterase activity was inhibited by peroxides, but in our experiments, total acetylcholinesterase activity in the reaction mixture remained constant even when erythrocytes were incubated with high concentrations of egg yolk phosphatidylcholine.

Hemolysis was inhibited by serum albumin or by the presence of extra liposomes insensitive to peroxidation even after the completion of lipid peroxidation. These results together with the findings that membrane vesiculation and hemolysis occurred after a certain lag period, suggest that some product(s), which is responsible for membrane vesiculation and/or hemolysis, was pre-

formed in the liposomal membrane and could transfer to the erythrocyte membrane during the reaction. The accumulation of these lipid peroxides in erythrocyte membranes may eventually cause membrane vesiculation followed by hemolysis.

The precise mechanism, by which liposomes are peroxidized during incubation with intact erythrocytes, is not clarified at present. Hematin compounds have long been known to accelerate peroxidation of lipids [24–26]. In our preliminarily experiments, neither the production of chemiluminescence nor the formation of thiobarbituric acid reactive substance was observed when liposomes were incubated with the erythrocyte membrane fraction, suggesting that a small amount of the cytosol components, most probably hemoglobin, which was leaked out from intact erythrocytes may be responsible for peroxidation of liposomes. Experiments using erythrocyte lysate or hemoglobin are now in progress. ·OH and/or $^1O_2$ may be involved in peroxidation of liposomes, since thiourea, dimethylthiourea and α-tocopherol inhibited lipid peroxidation.

Previously, it was demonstrated that active products derived from a peroxidation of liver microsomal fraction were capable of inducing damage in cells [27,28]. Recently, the structures of some of the cytotoxic products were identified [29,30]. At present, the chemical identity of the toxic product(s) arising in peroxidized liposomes in our experiments is not known. Although malondialdehyde, an end-product of lipid peroxidation, has been known to affect erythrocyte membrane organization [31,32], the exposure of erythrocytes to exogenous malondialdehyde did not cause membrane vesiculation nor hemolysis (Kobayashi, T., unpublished observation). The fact that serum albumin and extra liposomes suppressed damage of erythrocyte membrane suggests that the peroxidized product(s) can easily associate with serum albumin and/or lipid membranes. These properties are similar to those of dilauroylglycerophosphocholine [33,34], which can induce membrane vesiculation [12] as well as hemolysis [35] of erythrocytes. The chemical property of the peroxidized product(s) may be similar to short chain phosphatidylcholines. The optimum concentration of egg yolk phosphatidylcholine required to induce membrane vesiculation and hemolysis were rather different. The temperature dependence of vesiculation was also different from that of hemolysis; appreciable vesiculation, but no hemolysis, was observed at 25°C. This difference was also observed following addition of catalase which inhibited the homolysis but not vesiculation. These findings suggest that different products may participate in the process leading to vesiculation and hemolysis.

The membrane vesiculation of erythrocyte occurs under various conditions, such as depletion of endogenous ATP [36,37] or reoxygenation of sickled erythrocytes [38] and is considered to be involved in the process of erythrocyte aging [36]. Our experiments suggest that lipid peroxidation or acquirement of peroxidized lipids during circulation may be responsible in erythrocyte aging.

Egg yolk phosphatidylcholine liposomes have been used widespreadly as a delivery package for various molecules. Care should be taken to protect liposomes from peroxidation in vivo when these liposomes are employed.

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