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Research Article

Role of serum components in the binding and phagocytosis of oxidatively damaged erythrocytes by autologous mouse macrophages

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Abstract. To investigate the role of autologous serum components in the recognition of damaged cells by macrophages, we examined the binding and phagocytosis of damage oxidatively damaged red blood cells with Cu^{2+} and ascorbate (oxRBCs) by autologous resident mouse peritoneal macrophages. The binding of oxRBCs by macrophages was independent of the presence of serum. However, phagocytosis by macrophages increased with serum concentration, and macrophages showed little ingestion of oxRBCs in a serum-free medium. Macrophages neither bound nor appreciably ingested native RBCs (before oxidation) in either the absence or presence of autologous serum. Mouse macrophages ingested significantly more native as well as oxRBCs in the presence of heat-inactivated fetal calf serum than in the presence of heat-inactivated mouse serum. Pretreated oxRBCs with normal serum were rarely ingested by macrophages in a serum-free medium. Phagocytosis of oxRBCs was significantly inhibited by depletion of IgG or calcium from serum, by heat inactivation of complement, or by antiserum against mouse C3. These results demonstrate that serum components such as IgG, C3, and calcium are involved in phagocytosis of oxRBCs by autologous macrophages.

Key words. Calcium; complement; erythrocyte; macrophage; phagocytosis.

Damaged and old erythrocytes are cleared rapidly from the circulation. Every day, 360 billion red blood cells (RBCs), or 5 million per second are phagocytosed [1]. Damaged cells are assumed to be removed quickly and appropriately, although the changes that RBCs undergo are not well characterized. Damaged and senescent erythrocytes have been reported to bind autologous IgGs on the protein [2, 3] or the carbohydrate chain of membrane protein, band 3 [4], the bound IgGs being recognized by macrophages through their Fc receptors. Lutz [5] suggested that erythrocyte clearance is mediated by autologous IgG and complement in serum. Beppu et al. [6] reported that macrophages bound oxidized RBCs (oxRBCs) in the absence of serum through scavenger receptors whose ligand was the sialosaccharide chain of glycophorin. Horn et al. [7] reported that phagocytosis of glucose-6-phosphate dehydrogenase-deficient RBCs or phenylhydrazine-oxidized RBCs occurred in the absence of serum, and involved the specific lectin-like and Fc receptors of macrophages. However, another signal of oxRBC binding to macrophages is reported to be the exposure of phosphatidylserine on the outer membrane leaflet, and recognition occurs through oxidized low-density lipoprotein receptors.

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Many investigators have attempted to elucidate the cellular and molecular mechanisms of erythrophagocytosis, and these studies were performed under various experimental conditions. As with RBCs, in vivo or in vitro senescent RBCs, and variously damaged RBCs, for example, RBCs oxidized in vitro [6, 10] and in vivo [11], desialylated [12], and damaged in hemolytic anemia [13], have been used. With regard to the role of macrophages, macrophages of different origins, for example, resident and activated peritoneal macrophages [14] and monocytes collected from human blood and THP-1 cells (activated monocyte cell lines) [15] have been reported to demonstrate different characteristics during phagocytosis of aged erythrocytes [14] and apoptotic cells [15]. Furthermore, various experiments have combined of RBCs and macrophages of different species, for example human RBCs and mouse macrophages [8, 9], or sheep RBCs and mouse macrophages [14]. To investigate the mechanism of physiological erythrophagocytosis systematically, an autologous system is necessary. Therefore, in this study, we applied an entirely autologous system to eliminate nonphysiological effects caused by species differences and used resident peritoneal mouse macrophages because these had not been exposed to inflammatory stimulus. We also performed the first evaluation of the role of serum components in binding and phagocytosis of autologous macrophages.

Materials and methods

Materials

RPMI 1640 medium (No. 22400), Dulbecco's modified Eagle's medium (DMEM, No. 12430), fetal calf serum (FCS), and penicillin-streptomycin were purchased from GIBCO BRL (Rockville, Md.). Goat anti-mouse complement C3 antiserum was purchased from ICN Biomed. (Aurora, Ohio). Immobilized protein A/G gel was purchased from Pierce (Rockford, Ill.). Normal goat serum was purchased from Vector Laboratories (Burlingame, Calif.). All other reagents were purchased from Nacalai Tesque (Kyoto, Japan).

Cells

Guidelines from the Prime Minister's Office of Japan (No. 6 of 27 March 1980) for the care and use of laboratory animals were followed. Resident peritoneal macrophages were collected using phosphate-buffered saline (PBS) from the peritoneal cavity of 5- to 6-week-old ICR male mice (Japan SLC, Shizuoka, Japan). Macrophages were resuspended in RPMI 1640 medium, containing 10% heat-inactivated FCS, penicillin (50 units/ml), and streptomycin (50 μg/ml), and $4-5 \times 10^5$ cells were placed in each well of 48-well dishes. After 3–4 h, nonadherent cells were removed by washing three times with DMEM. Macrophages were used immediately after the washing step because overnight incubation results in the acquisition of the ability to bind even normal human RBCs via a sialic acid-dependent mechanism [10, 16].

Autologous RBCs and serum were separated from heparinized blood and nonheparinized blood, respectively. These heparinized and nonheparinized blood specimens were collected on the day of use by direct cardiac puncture of the same mouse from which macrophages were obtained. After washing three times with PBS, RBCs were resuspended at 4% hematocrit in PBS, then treated with 0.2 mM $CuSO_4$ and 5 mM ascorbate at 37 °C for 60 min as described elsewhere [9]. Native RBCs were taken from the 4% hematocrit suspension before the addition of $CuSO_4$ and ascorbate. oxRBCs were subsequently washed twice with PBS containing 0.2% EDTA (pH 7.4) to remove Cu^{2+}, and twice with PBS.

Effect of autologous serum

Complement-inactivated serum was prepared by a heat treatment at 56 °C for 30 min in a water bath. IgGs were eliminated from serum by an immobilized protein A/G gel. More than 90% of IgG was depleted as confirmed by immunoblotting.

For the binding and phagocytosis experiment, washed RBCs were resuspended in 0.1% hematocrit with DMEM. To RBCs were added 1–20% serum, IgG-depleted serum, or complement-inactivated serum. RBCs were added to macrophages immediately after resuspension.

For the preincubation experiment, washed oxRBCs were pretreated in DMEM at 0.1% hematocrit with 10% serum at 37 °C for 1 h, then washed twice and resuspended with DMEM.

Binding and phagocytosis assay

RBCs (hematocrit at 0.1% in DMEM) were incubated with macrophages at 37 °C for 1 h [9] with 0–20% autologous serum or heat-inactivated FCS at 10%. After washing unbound RBCs with DMEM, the percentage of macrophages binding at least one RBC was determined. Each well was counted three to four times (100–200 macrophages were counted each time) and the mean value was obtained. Macrophage-bound RBCs were removed by hypotonic lysis with 5 mM phosphate buffer (PB) at pH 7.4 for 30 s and macrophages were fixed with 80 mM PB containing 2% paraformaldehyde and 0.5% glutaraldehyde. After fixation, wells were washed five times with 50 mM PB and reacted with 0.1% 3,3′-diaminobenzidine solution containing 0.8 mM H_2O_2 in 0.1 M PB (pH 6.5) to stain hemoglobin in RBCs which were ingested in macrophages. After 15 min, wells were washed with PBS to stop the reaction. Phagocytosis was also measured as the percentage of macrophages ingest-

ing at least one RBC, which was clearly stained red or brown. Each well was counted three to four times (100–200 macrophages were counted each time) and the mean value was obtained. This method can be considered sufficiently accurate for these determinations since similar counting has been previously used for these purposes [10].

The percentage of macrophages ingesting RBCs was expressed as the mean ± SD for more than three independent runs (one mouse was used for one run) and analyzed by ANOVA using StatView software (Abacus Concepts, Berkeley, Calif.). Differences between group means were analyzed using Bonferroni/Dunn (Dunn's procedure as a multiple-comparison procedure) generated by the same program. Differences were considered significant at $p < 0.05$.

Effect of goat anti-mouse C3 antiserum on the binding and phagocytosis of RBCs by autologous macrophages

Goat anti-mouse C3 was dissolved with 2 ml of water to make a solution of 24.8 mg protein/ml as described by the supplier, and the resulting solution was diluted ten-fold with DMEM. After filtration with a 0.45-µm filter (Millipore, Tokyo, Japan), 90 µl of the diluted solution was added to 810 µl of the suspension of oxRBCs (final hematocrit at 0.1% in DMEM containing 90 µl of autologous serum). Eight hundred microliters of the resulting suspension was treated with macrophages at 37 °C for 1 h. For comparison, normal goat serum at the same protein concentration was added instead of anti-C3 serum. The percentage of binding and phagocytosis was determined as described above.

Effect of EDTA, and EGTA and Mg^{2+}

EDTA · 2Na solution (100 mM, dissolved in PBS) was adjusted to pH 7.4. After filtration with a 0.45-µm filter (Millipore), 90 µl of this solution was added to 810 µl of the suspension of oxRBCs (final hematocrit at 0.1% in DMEM containing 90 µl of autologous serum). Eight hundred microliters of the resulting suspension was treated with macrophages at 37 °C for 1 h. EGTA solution (150 mM) in PBS at pH 7.4 and 1.5 M $MgCl_2$ solution in PBS were prepared and both solutions were filtered through a 0.45-µm filter (Millipore). The filtered EGTA solution (90 µl) and the filtered $MgCl_2$ solution (9 µl) were added to 801 µl of the oxRBCs suspension (final hematocrit at 0.1% in DMEM containing 90 µl of autologous serum). Eight hundred microliters of the resulting suspension was treated with macrophages at 37 °C for 1 h.

Results

Effect of serum on the binding and phagocytosis of oxRBCs by autologous macrophages

To investigate the mechanism of physiological erythrophagocytosis, in the present study we used oxRBCs as a model of in vivo-damaged cells. To obtain oxRBCs, mouse erythrocytes were treated with 0.2 mM $CuSO_4$ and 5 mM ascorbate at 37 °C for 60 min, since RBCs oxidized under this condition have been reported to be effectively recognized by macrophages. The oxRBCs obtained this way were treated with autologous mouse peritoneal macrophages in the presence of various concentrations of autologous serum. Around 90% of mouse peritoneal macrophages showed extensive binding to autologous oxRBCs independent of the serum concentration (fig. 1). However, macrophages did not ingest oxRBCs appreciably in the absence of serum (1.8 ± 1.4%), but the percentage of macrophages ingesting oxRBCs increased as the serum concentration increased (fig. 1). Since phagocytosis reached a plateau at 10% serum, the following experiments were carried out with 10% serum. Native RBCs (before oxidation) were slightly bound by macrophages in the absence (4.2 ± 3.4%) and presence (4.1 ± 1.3%) of serum. Macrophages did not appreciably ingest native RBCs in the absence of serum (0.3 ± 0.6%), but slightly ingested these cells in the presence of serum (4.2 ± 2.7%).

Effect of serum from different species

We also used heat-inactivated FCS at 10% as a typical example to compare the effect of serum from different species on phagocytosis of oxRBCs. We found that significantly more mouse macrophages ingested oxRBCs in the presence of heat-inactivated FCS (60.8 ± 5.7%) than

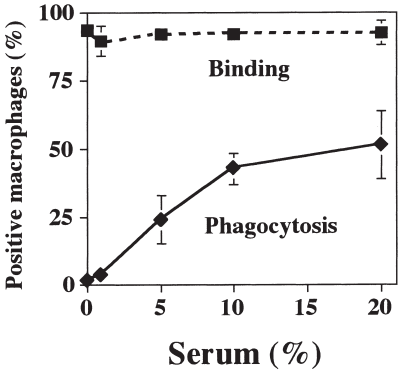

Figure 1. Serum concentration dependence of macrophage binding and phagocytosis of oxidized RBCs. RBCs oxidized by Cu^{2+}/ascorbate (0.1% final hematocrit) were added to autologous macrophages and treated for 1 h at 37 °C. Each value represents the percentage of macrophages that bound (*squares*) or phagocytosed (*diamonds*) one or more RBCs. Each point is the mean ± SD of four or more independent runs. Where no whisker is shown, the SD is smaller than the symbol.

in the presence of heat-inactivated autologous serum (14.3 ± 2.0%). Mouse macrophages even ingested native RBCs at 14.6 ± 6.2% in the presence of FCS, while only 4.2 ± 2.7% ingested native RBCs in the presence of autologous serum as described above.

Effect of autoantibody and complement

To investigate relative contributions of serum components to phagocytosis of oxRBCs, we compared macrophage phagocytosis using autologous, IgG-depleted, and complement-inactivated serums. The percentage of macrophages that bound oxRBCs in the presence of IgG-depleted serum at 10% or complement-inactivated serum (treated at 56 °C for 30 min) at 10% were around 90% and similar to that in the presence of normal serum at 10% (fig. 2). However, phagocytosis was decreased significantly to 27.5 ± 7.7% when IgG-depleted serum was used (fig. 2). Phagocytosis was further decreased to 14.3 ± 2.0% when complement-inactivated serum was used, whereas in the presence of normal serum, 42.7 ± 5.6% macrophages ingested oxRBCs (fig. 2).

Effect of goat anti-C3 antiserum on binding and phagocytosis by macrophages

To gain more insight into the function of complement, the effect of anti-C3 antiserum was examined. Goat anti-C3 antiserum inhibited phagocytosis of oxRBCs by macrophages (fig. 2) to a level similar to that using serum complement inactivated by heat treatment (table 1). The addition of normal (nonimmunized with C3) goat serum at the same protein concentration did not affect phagocytosis of oxRBCs by macrophages (table 1). These results indicate that C3 of complement is involved in the phagocytosis of oxRBCs by autologous macrophages.

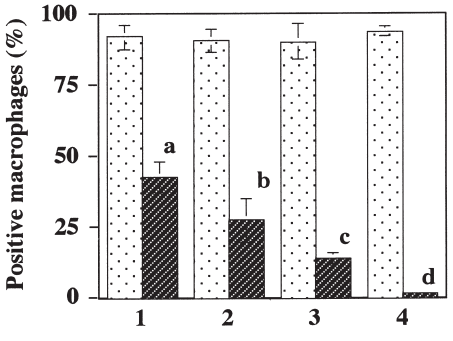

Figure 2. Effect of serum treatments on macrophage binding and phagocytosis of oxidized RBCs. RBCs oxidized by Cu^{2+}/ascorbate (0.1% final hematocrit) were added to macrophages and treated for 1 h at 37 °C in the presence of 10% normal serum (1), IgG-depleted serum at 10% (2), complement-inactivated serum at 10% (3), and in the absence of serum (4). Each value represents the percentage of macrophages that bound (stippled bar) or phagocytosed (hatched bar) one or more RBCs. Each point is the mean ± SD of more than seven independent runs. Where no whisker is shown, the SD is smaller than the symbol. Different letters above the bars indicate significant differences among groups by Bonferroni/Dunn protected least significant difference test ($p < 0.01$).

Table 1. Effect of goat anti-mouse C3 antiserum on macrophage binding and phagocytosis of mouse oxRBCs by autologous macrophages.

Condition	Binding (%)	Phagocytosis (%)
+ Serum (9)	94.4 ± 4.7	49.0 ± 9.3
+ Serum + goat anti-C3 antiserum (4)	94.9 ± 4.2	16.2 ± 5.1*
+ Serum + normal goat serum (3)	94.2 ± 3.7	51.8 ± 10.1

Oxidized RBCs were added to autologous macrophages and treated for 1 h at 37 °C in the presence of autologous serum at 10%. Each value represents the mean ± SD percentage of macrophages that bound or phagocytosed one or more RBCs. The number of independent runs is shown in parentheses. The asterisk indicates a significant difference from the control (+ serum) group (ANOVA Bonferroni/Dunn procedure, $p < 0.01$).

Effect of calcium and magnesium ions on binding and phagocytosis by macrophages

Both binding and phagocytosis of oxRBCs were significantly inhibited by EDTA (a chelator of both calcium and magnesium) (table 2). Addition of EGTA (a calcium-specific chelator) and $MgCl_2$ showed similar results (table 2). In the absence of serum, there was no influence on binding of oxRBCs to macrophages as described above, but EDTA significantly inhibited binding of oxRBCs (table 2). Mg^{2+} did not remove the inhibition (table 2).

Effect on binding and phagocytosis by macrophages of pretreating oxRBCs with serum

To estimate the contribution of prebinding reactions to macrophages, oxRBCs were pretreated with 10% autologous serum for 1 h. After washing with serum-free medium, the cells were treated with macrophages in the absence of serum. The binding of macrophages to the pre-

Table 2. Effect of calcium chelators on macrophage binding and phagocytosis of mouse oxRBCs by autologous macrophage.

Condition	Binding (%)	Phagocytosis (%)
+ Serum (4)	94.7 ± 2.3[a]	43.7 ±12.4[a]
+ Serum + EDTA (4)	43.9 ± 12.8[b]	0.5 ± 0.6[b]
+ Serum + EGTA + $MgCl_2$ (3)	27.0 ± 3.2[b]	0.9 ± 0.6[b]
– Serum (4)	92.5 ± 2.5[a]	3.7 ± 2.4[b]
– Serum + EDTA (3)	44.7 ± 2.5[b]	0.1 ± 0.2[b]
– Serum + EDTA + $MgCl_2$ (3)	53.8 ± 10.7[b]	0.3 ± 0.3[b]

Oxidized RBCs were added to autologous macrophages and treated for 1 h at 37 °C under the conditions indicated. Each value represents the mean ± SD percentage of macrophages that bound or phagocytosed one or more RBCs. The number of independent runs is shown in parentheses. Different superscripts within each column indicate significant differences (ANOVA Bonferroni/Dunn procedure, $p < 0.01$).

treated oxRBCs was similar to that without pretreatment. However, little ingestion of pretreated oxRBCs by macrophages was observed (2.9 ± 2.2%), whereas in the presence of serum, macrophages effectively ingested oxRBCs (42.7 ± 5.6%).

Discussion

One of the possible causes of in vivo damage to erythrocytes is assumed to be oxidative stress [17]. In living tissue, reactive oxygen species such as superoxide anion, hydrogen peroxide, and the hydroxyl radical cause free radical damage of various components, including lipids, proteins, and sugars [18–23]. Oxidative stress is also known to cause RBC injury, such as membrane lipid peroxidation [24, 25], hemoglobin denaturation [26], membrane protein aggregation [26, 27], and inhibition of membrane enzyme activity [28, 29]. Changes caused by these oxidative reactions are found in senescent RBCs [30–33], and oxRBCs have been used as a model of in vivo aged RBCs [1, 34–36]. We therefore used oxRBCs as damaged cells and determined both binding and phagocytosis of oxRBCs by autologous macrophages in the present study.

Mouse peritoneal macrophages showed extensive binding to autologous oxRBCs independent of serum concentration, while native RBCs (before oxidation) were only slightly bound by macrophages in either the absence or presence of serum (fig. 1). These results suggest that serum components are not involved in generating the signals for macrophage binding to oxRBCs, and that oxidative changes in RBCs themselves constitute the binding signal(s).

The percentage of macrophages ingesting oxRBCs was serum concentration dependent (fig. 1). Sambrano et al. [10] reported that about 40% mouse peritoneal macrophages ingested Cu^{2+}/ascorbate-oxidized human RBCs in a serum-free medium. The difference between our and their results can probably be attributed to different experimental conditions. We used RBCs and macrophages from the same mouse, whereas they used human RBCs and mouse macrophages. These results suggest that autologous RBCs and macrophages must be used for studies of physiological erythrophagocytosis.

Moreover, mouse macrophages ingested significantly more mouse oxRBCs in the presence of heat-inactivated FCS than in the presence of heat-inactivated autologous serum. Mouse macrophages were also observed to ingest more native RBCs in the presence of FCS. These results also demonstrate that the degree of phagocytosis of RBCs by macrophages in the presence of autologous serum is significantly different from that in the presence of FCS. These observations also indicate that autologous serum should be applied to exclude nonphysiological recognition caused by species differences in the study of physiological erythrophagocytosis.

As shown in figure 2, IgG and complement did not appear to be involved in the binding of oxRBCs to macrophages. However, phagocytosis was significantly affected by the almost complete depletion of IgG by a protein A/G column and heat inactivation of complement (fig. 2). Since the addition of goat anti-C3 antiserum inhibited phagocytosis to 30% of the control, i.e., to a similar extent as heat treatment (table 1), C3 seems to play a key role for 70% of total phagocytosis. Matsui et al. [37] reported that anti-Fas-induced apoptotic Jurkat T cells were attacked by the alternative pathway of human complement. Tsuji et al. [38] also reported that apoptotic human umbilical vein endothelial cells activated the alternative pathway based on Mg^{2+} dependence. The present results obtained by experiments similar to those in the literature [37, 38] show that phagocytosis of oxRBCs by autologous macrophages is dependent on Ca^{2+} and independent of Mg^{2+} (table 2), suggesting that the alternative pathway is not involved in phagocytosis of oxRBCs. The classical pathway requires both Ca^{2+} and Mg^{2+}, and antibody. Since the present results demonstrate the partial contribution of IgG (fig. 2) and Mg^{2+} independence (table 2) in phagocytosis of oxRBCs, the mechanism of C3 function remains to be clarified. That the binding of oxRBCs to macrophages was significantly influenced by Ca^{2+} depletion either in the presence or absence of serum (table 2) is worth noting.

Beppu et al. [4] suggested that autologous IgGs, which recognized oxRBCs, caused binding by macrophages. Lutz et al. [39] reported that complement deposition to autologous IgG-bound RBCs gave rise to phagocytosis. These studies suggested that damaged RBCs were designated to be recognized and/or ingested before contacting macrophages, for example, by clustering of band 3 protein [40], binding of autologous IgGs to clustered band 3 [26], or complement deposition [39]. In this study, pretreatment of oxRBCs with autologous serum did not affect macrophage binding. However, little ingestion of pretreated oxRBCs by macrophages was observed in the absence of serum. This result contradicts the report [41] that the preincubation of RBCs with autologous serum caused macrophage ingestion, although there were differences in experimental conditions. Kay [41] used in vitro aged human RBCs and macrophages isolated on Lymphoprep from human blood, and determined the percentage of phagocytosis based on the difference in the number of RBCs before and after treatment with macrophages. These results suggest that binding of macrophages to oxRBCs is mediated by serum-independent mechanisms, for example, by the carbohydrate chain on RBCs and the lectin-like receptor of macrophage [29], or by phosphatidylserine on the outer membrane leaflet of RBCs and the oxidized low-density lipoprotein receptor

of macrophage [8]. However, as with phagocytosis, our results suggest that serum components are needed by macrophages for ingestion process of oxRBCs, rather than as a signal caused by the reaction of serum components with oxRBCs.

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1 Bratosin D., Mazurier J., Tissier J. P., Estaquier J., Huart J. J., Ameisen J. C. et al. (1998) Cellular and molecular mechanisms of senescent erythrocyte phagocytosis by macrophages: a review. Biochimie **80:** 173–195
2 Kay,M. M. B., Goodman S. R., Sorensen K., Whitfield C. F., Wong P., Zaki L. et al. (1983) Senescent cell antigen is immunologically related to band 3. Proc. Natl. Acad. Sci. USA **80:** 1631–1635
3 Lutz H. U., Gianora, O., Nater, M., Schweizer, E. and Stammler, P. (1993) Naturally occurring anti-band 3 antibodies bind to protein rather than to carbohydrate on band 3. J. Biol. Chem. **268:** 23562–23566
4 Beppu M., Takahashi T., Kashiwada M., Masukawa S. and Kikugawa K. (1994) Macrophage recognition of saccharide chains on the erythrocytes damaged by iron-catalyzed oxidation. Arch. Biochem. Biophys. **312:** 189–197
5 Lutz H. U. (1990) Erythrocyte clearance. In: Blood Cell Biochemistry. I: Erythroid Cells, pp. 81–120, Harris J. R. (ed.), Plenum, New York
6 Beppu M., Hayashi T., Hasegawa T. and Kikugawa K. (1995) Recognition of sialosaccharide chains of glycophorin on damaged erythrocytes by macrophage scavenger receptors. Biochim. Biophys. Acta **1268:** 9–19
7 Horn S., Bashan N. and Gopas J. (1991) Phagocytosis of phenylhydrazine oxidized and G-6-PD-deficient red blood cells: the role of cell-bound immunoglobulins. Blood **78:** 1818–1825
8 Sambrano G.R. and Steinberg D. (1995) Recognition of oxidatively damaged and apoptotic cells by an oxidized low density lipoprotein receptor on mouse peritoneal macrophages: role of membrane phosphatidylserine. Proc. Natl. Acad. Sci. USA **92:** 1396–1400
9 Sambrano G. R., Terpstra V. and Steinberg D. (1997) Independent mechanisms for macrophage binding and macrophage phagocytosis of damaged erythrocytes: evidence of receptor cooperativity. Arterioscler. Thromb. Vasc. Biol. **17:** 3442–3448
10 Sambrano G. R., Parthasarathy S. and Steinberg D. (1994) Recognition of oxidatively damaged erythrocytes by macrophage receptor with specificity for oxidized low density lipoprotein. Proc. Natl. Acad. Sci. USA **91:** 3265–3269
11 Kay M. M. B., Bosman G. J. C. G. M., Shapiro S. S., Bendich A. and Bassel P. S. (1986) Vitamin E deficiency causes premature aging and IgG binding to erythrocytes. Proc. Natl. Acad. Sci. USA **83:** 2463–2467
12 Aminoff D., Bruegge,W. F. V., Bell W. C., Sarpolis K. and Williams R. (1977) Role of sialic acid in survival of erythrocytes in the circulation: interaction of neuraminidase-treated and untreated erythrocytes with spleen and liver at the cellular level. Proc. Natl. Acad. Sci. USA **74:** 1521–1524
13 Boas F. E., Forman L. and Beutler E. (1998) Phosphatidylserine exposure and red cell viability in red cell aging and in hemolytic anemia. Proc. Natl. Acad. Sci. USA **95:** 3077–3081
14 Mantovani B. (1987) Phagocytosis of in vitro-aged erythrocytes – a sharp distinction between activated and normal macrophages. Exp. Cell. Res. **173:** 282–286
15 Fadok V. A., Savill J. S., Haslett C., Bratton D. L., Doherty D. E., Campbell P. A. et al. (1992) Different populations of macrophages use either the vitronectin receptor or the phosphatidylserine receptor to recognize and remove apoptotic cells. J. Immunol. **149:** 4029–4035
16 Crocker P. R., Kelm S., Dubois C., Martin B., McWilliam A. S., Shotton D. M. et al. (1991) Purification and properties of sialoadhesin, a sialic acid-binding receptor of murine tissue macrophages. EMBO J. **10:** 1661–1669
17 Sies H. (ed.) (1991) Oxidative Stress, pp. xv–xxii, Academic Press, London
18 Tokumaru S., Iguchi H. and Kojo S. (1996) Change of the lipid hydroperoxide level in mouse organs on ageing. Mech. Ageing Dev. **86:** 67–74
19 Tokumaru S., Takeshita S., Nakata R., Tsukamoto I. and Kojo S. (1996) Change in the level of vitamin C and lipid peroxidation in tissues of the inherently scorbutic rat during ascorbate deficiency. J. Agric. Food Chem. **44:** 2748–2753
20 Tokumaru S., Ogino R., Shiromoto A., Iguchi H., and Kojo S. (1997) Increase of lipid hydroperoxides in tissues of vitamin E-deficient rats. Free Radic. Res. **26:** 169–174
21 Sun F., Iwaguchi K., Shudo R., Nagaki Y., Tanaka K., Ikeda K. et al. (1999) Change in tissue concentrations of lipid hydroperoxides, vitamin C and vitamin E in rats with streptozotocin-induced diabetes. Clin. Sci. **96:** 185–190
22 Tanaka K., Tokumaru S. and Kojo S. (1997) Possible involvement of radical reactions in desialylation of LDL. FEBS Lett. **413:** 202–204
23 Tanaka K., Iguchi H., Taketani S., Nakata R., Tokumaru S., Sugimoto T. et al. (1999) Facile degradation of apolipoprotein B by radical reactions and the presence of cleaved proteins in serum. J. Biochem. (Tokyo) **125:** 173–176
24 Chan P. C., Peller O. G. and Kesner L. (1982) Copper(II)-catalyzed lipid peroxidation in liposomes and erythrocyte membrane. Lipids **17:** 331–337
25 Beppu M., Murakami K. and Kikugawa K. (1987) Detection of oxidized lipid-modified erythrocyte membrane proteins by radiolabeling with tritiated borohydride. Biochim. Biophys. Acta **897:** 169–179
26 Low P. S., Waugh S. M., Zinke K. and Drenckhahn D. (1985) The role of hemoglobin denaturation and band 3 clustering in red blood cell aging. Science **227:** 531–553
27 Ando K., Kikugawa K. and Beppu M. (1997) Induction of band 3 aggregation in erythrocytes results in anti-band 3 autoantibody binding to the carbohydrate epitopes of band 3. Arch. Biochem. Biophys. **339:** 250–257
28 Rohn T. T., Hinds T. R. and Vincenzi F. F. (1993) Inhibition of the Ca pump of intact red blood cells by *t*-butyl hydroperoxide: importance of glutathione peroxidase. Biochim. Biophys. Acta **1153:** 67–76
29 Rohn T. T., Hinds T. R. and Vincenzi F. F. (1995) Inhibition by activated neutrophils of the Ca^{2+} pump ATPase of intact red blood cells. Free Radic. Biol. Med. **18:** 655–667
30 Brovelli A., Castellana M. A., Minetti G., Piccinini G., Seppi C., Renzis M. R. de et al. (1991) Conformation changes and oxidation of membrane proteins in senescent human erythrocytes. Adv. Exp. Med. Biol. **307:** 59–73
31 Seppi C., Castellana M. A., Minetti G., Piccinini G., Balduini C. and Brovelli A. (1991) Evidence for membrane protein oxidation during in vivo aging of human erythrocytes. Mech. Ageing Dev. **57:** 247–258
32 Jain S. K. (1988) Evidence for membrane lipid peroxidation during the in vivo aging of human erythrocytes. Biochim. Biophys. Acta **937:** 205–210
33 Miyazawa T., Suzuki T., Fujimoto K. and Kinoshita M. (1996) Age-related change of phosphatidylcholine hydroperoxide and phosphatidylethanolamine hydroperoxide levels in normal human red blood cells. Mech. Ageing Dev. **86:** 145–150
34 Beppu M., Ando K. and Kikugawa K. (1996) Poly-N-acetyllactosaminyl saccharide chains of band 3 as determinants for anti-

band 3 autoantibody binding to senescent and oxidized erythrocytes. Cell. Mol. Biol. **42:** 1007–1024
35 Lutz H. U., Fasler S., Stammler P., Bussolino F. and Arese P. (1988) Naturally occurring anti-band 3 antibodies and complement in phagocytosis of oxidatively stressed and in clearance of senescent red cells. Blood Cells **14:** 175–195
36 Beppu M., Mizukami A., Nagoya M. and Kikugawa K. (1990) Binding of anti-band 3 autoantibody to oxidatively damaged erythrocytes. J. Biol. Chem. **265:** 3226–3233
37 Matsui H., Tsuji S., Nishimura H. and Nagasawa S. (1994) Activation of the alternative pathway of complement by apoptotic Jurkat cells. FEBS Lett. **351:** 419–422
38 Tsuji S., Kaji K. and Nagasawa S. (1994) Activation of the alternative pathway of human complement by apoptotic human umbilical vein endothelial cells. J. Biochem. (Tokyo) **116:** 794–800
39 Lutz H. U., Bussolino F., Flepp R., Fasler S., Stammler P., Kazatchkine M. D. et al. (1987) Naturally occurring anti-band 3 antibodies and complement together mediate phagocytosis of oxidatively stressed human erythrocytes. Proc. Natl. Acad. Sci. USA **84:** 7368–7372
40 Beppu M., Eda S., Fujimaki M., Hishiyama E. and Kikugawa K. (1996) Recognition of poly-N-acetyllactosaminyl saccharide chains on iron-oxidized erythrocytes by human monocytes leukemia cell line THP-1 differentiated into macrophages. Biol. Pharm. Bull. **19:** 188–194
41 Kay M. M. B. (1975) Mechanism of removal of senescent cells by human macrophages in situ. Proc. Natl. Acad. Sci. USA **72:** 3521–3525