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Macrophage recognition of periodate-treated erythrocytes: Involvement of disulfide formation of the erythrocyte membrane proteins

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Upon exposure to 2 mM periodate at 0 °C for 15 min, mouse erythrocytes underwent membrane lipid oxidation, oxidation of cell surface sialyl residues into aldehyde-bearing derivatives, and oxidation of SH groups of the membrane proteins into disulfides. The periodate-treated erythrocytes exhibited a remarkable increase in rosette attachment to resident mouse peritoneal macrophages in the absence of serum. The relationship between the oxidation of the membrane constituents and the macrophage recognition of these cells was investigated. Periodate treatment of erythrocytes in the presence of butylated hydroxytoluene, an inhibitor of lipid oxidation, did not affect the subsequent attachment of the erythrocytes to the macrophages. Reduction of the periodate-treated erythrocytes with borohydride or cyanoborohydride did not affect the erythrocyte attachment. Neuraminidase treatment of erythrocytes before periodate did not affect the attachment either. On reduction of the disulfides of the membrane proteins with dithiothreitol, the periodate-treated erythrocytes lost their ability to attach to the macrophages. Erythrocytes treated with an SH-oxidizing agent, diamide, were then examined for the macrophage recognition. The diamide-treated cells also showed rosette attachment to the macrophages in the absence of serum, but did not when reduced with dithiothreitol. These results indicate that oxidation of the SH groups of the membrane proteins to disulfides causes reversible membrane changes that macrophages recognize, and it is this mechanism that is responsible for the macrophage recognition of the periodate-treated erythrocytes.

Introduction

It is known that periodate treatment of lymphocytes [1–4], macrophages [2] and erythrocytes [4,5] induces various cellular events of immunological importance, such as lymphocyte mitogenesis [1–3], lymphocyte cytotoxicity [4] and attachment of the treated erythrocytes to macrophages in the absence of serum [5].

Periodate-induced lymphocyte mitogenesis and cytotoxicity have been ascribed to the oxidation of sialyl residues of the cell surface glycoconjugates [4,6–9] because membrane sialic acid is readily oxidized to aldehyde-bearing derivatives [10,11] and reduction of the periodate-treated lymphocytes with borohydride prevented the subsequent activation of the cells [3,6–8]. Attachment of periodate-treated erythrocytes to macrophages [5] suggests that macrophages have an ability to recognize oxidatively damaged cells, but the damage of the erythrocytes critical to the macrophage recognition is not known.

We have previously shown that mild periodate treatment of human erythrocytes results in oxidation of not only cell surface sialyl residues but also membrane lipids and protein SH groups [12]. Our previous work has suggested that macrophages recognize erythrocytes which have undergone lipid oxidation [13]. We have undertaken the present study to determine which of the oxidation of these membrane constituents is involved in the macrophage recognition of the periodate-treated erythrocytes. We show here that oxidation of the protein SH groups, but not lipids and sialic acids, is responsible for the recognition of the periodate-treated erythrocytes. Furthermore, we demonstrate that macrophages recognize erythrocytes modified by SH-specific

Abbreviations: SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GSH, glutathione.

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oxidizing agents, which cause neither lipid oxidation nor sialic acid oxidation.

Materials and Methods

Materials

Sodium metaperiodate, sodium borohydride, dithiothreitol, GSH and DTNB were obtained from Wako Pure Chemical Industries. Diazene dicarboxylic acid bis (*N*,*N*'-dimethylamide) (diamide) was purchased from Sigma. Sodium cyanoborohydride and sodium tetrathionate were from Aldrich. Neuraminidase (EC 3.2.1.18, *Vibrio cholerae*) and galactose oxidase (EC 1.1.3.9, *Dactylium dendroides*) were obtained from Behringwerke AG and Cooper Biomedical, respectively. Butylated hydroxytoluene and desferrioxamine were from Nikki Universal and Ciba-Geigy, respectively. Hepes was obtained from Dojindo Laboratories, and RPMI 1640 medium and fetal bovine serum were from Gibco Laboratories.

Cells

Macrophages were obtained from the peritoneal cavity of 7–10-week-old Balb/c or ddY male mice with or without prior injection of 2–3 ml of 3% thioglycollate medium (Difco Laboratories) 4 days before the harvest. Resident or thioglycoliate-elicited peritoneal cells were washed and the macrophage monolayers were prepared on round glass coverslips (18 mm diameter) and used after overnight culture as described previously [13].

Erythrocytes for modification with periodate were obtained from Balb/c mice as described [13] and suspended in 10 mM acetate buffer containing 0.14 M NaCl (pH 6.0, buffer A) before modification. Erythrocytes for modification with diamide and tetrathionate were obtained from ddY male mice on the day of the use and suspended in a buffer consisting of 12.5 mM Na_2HPO_4-NaH_2PO_4/100 mM KCl/50 mM NaCl/44 mM sucrose (pH 8.0, buffer B) [14] before modification.

Modification of erythrocytes with periodate

An erythrocyte suspension (20% hematocrit) in buffer A was mixed with an equal volume of freshly prepared sodium metaperiodate solution in the same buffer, and incubated at 0°C for 15 min in the dark. The modified cells were washed three times with ice-chilled Dulbecco's phosphate-buffered saline (buffer C) by centrifugation ($375 \times g$, 7 min) at 0–4°C, resuspended in RPMI 1640 medium supplemented with 20 mM Hepes (pH 7.2), 50 U/ml penicillin and 50 μg/ml streptomycin (RPMI-Hepes medium) to make a 2% cell suspension, and assayed for macrophage recognition. When the periodate-induced lipid oxidation was to be inhibited, the cell suspensions were preincubated with 0.05 mM BHT in 0.15 M NaCl or 0.5 mM desferrioxamine in 0.15 M NaCl at 25°C for 30 min, then treated with periodate as above. For analysis of the modified cells, they were suspended in an appropriate buffer as described below.

Modification of erythrocytes with SH-oxidizing agents

An erythrocyte suspension (20% hematocrit) in buffer B was mixed with an equal volume of freshly prepared solutions of diamide or sodium tetrathionate in the same buffer, and incubated at 37°C for 1 h. The modified cells were washed four times with ice-chilled buffer B at pH 7.4 [14] by centrifugation ($375 \times g$, 7 min) at 0–4°C, resuspended in RPMI-Hepes medium to make a 2% cell suspension and assayed for macrophage recognition.

Modification of erythrocytes with enzymes

An erythrocyte suspension (20% hematocrit) in buffer C was treated with neuraminidase (25 mU/ml) or neuraminidase (25 mU/ml) plus galactose oxidase (10 U/ml) at 37°C for 30 min. The treated cells were washed three times and the cell suspensions for macrophage recognition assay were prepared in the same way as the suspensions of the periodate-treated erythrocytes.

Reduction of the modified erythrocytes

The modified erythrocytes were washed three times by centrifugation ($375 \times g$, 7 min) at 4°C with the buffer consisting of 40 mM Na_2HPO_4/7 mM KH_2PO_4/86 mM NaCl/4 mM KCl (pH 7.5) and resuspended in the same buffer to make a 20% cell suspension. The cell suspension was incubated with an equal volume of freshly prepared 10 mM $NaBH_4$ in the same buffer at 25°C for 30 min, with an equal volume of freshly prepared 60 mM $NaBH_3CN$ in the same buffer at 25°C for 2 h, or with 10 mM dithiothreitol in the same buffer at 25°C for 2 h. The cells were then washed three times with buffer C and resuspended in RPMI-Hepes medium to make 2% cell suspensions for macrophage recognition assay.

Adhesion and phagocytosis by macrophages

Adhesion and phagocytosis of the modified erythrocytes by macrophage monolayers were examined as described previously [13] using Balb/c mouse macrophages for periodate-treated erythrocytes and ddY mouse macrophages for SH-oxidized erythrocytes, and the percentage of macrophages that bound one or more erythrocytes (% adhesion) and the percentage of macrophages that ingested one or more erythrocyte (% phagocytosis) were determined. The data were presented as the mean ± S.D. of the triplicate determinations.

Measurement of lipid oxidation of the periodate-treated erythrocytes

Lipid oxidation of the modified erythrocytes was assessed by the decrease in the unsaturated fatty acid

content of the modified cell membrane. The modified cells were lysed in 5 mM phosphate buffer (pH 8.0) and the ghosts were recovered by centrifugation (11 000 × g, 20 min), and washed four times with the same buffer. Lipids were extracted from the ghosts by the method of Bligh and Dyer [15]. Butylated hydroxytoluene (0.5%) was present throughout the extraction to prevent lipid oxidation. The lipid extracts was esterified by 0.5 N sodium methoxide and the resultant fatty acid methyl esters were analyzed by a Hitachi Gas-chromatograph 263-30 equipped with a hydrogen flame detector. A glass column (3 mm i.d. × 3 m) packed with Uniport C (80-100 mesh, Gasukuro Kogyo), which had been coated with Unisole 3000 (Gasukuro Kogyo), was used. The chromatograph was operated isothermally at 220 °C (column temperature) and at 240 °C (inlet and detector temperature) with a nitrogen carrier gas flow of 30 ml/min. Relative amounts of unsaturated fatty acids against the amount of palmitic acid were determined.

Measurement of sialyl residues of the periodate-treated erythrocytes

Unoxidized sialyl residues on the periodate-treated erythrocytes were assessed by the measurement of the sialic acid hydrolyzable by neuraminidase (*V. cholerae*) as described before [12]. Briefly, periodate-treated erythrocytes (50% hematocrit) or the ghosts (1–3 mg protein/ml) obtained from the cells were incubated with neuraminidase (25 mU/ml) in buffer C at 37 °C for 1 h and the released sialic acid was purified by successive passages through Dowex 50 (H^+) and Dowex 1 (formate) columns and quantified by the method of Warren [16]. *N*-Acetyl neuraminic acid (Sigma Chemical, type VI) was used as a standard. Total amounts of sialic acid of intact erythrocytes and ghosts were determined by the measurement of the sialic acid hydrolyzed by heating the cells and the ghosts in 0.005 M sulfuric acid at 80 °C for 1 h [13].

Measurement of SH groups of the SH-oxidized erythrocytes

Intracellular GSH content of the modified erythrocytes was measured according to the method of Beutler et al. [17] after washing the cells with buffer B at pH 7.4. The amount of GSH per mg hemoglobin was determined and the results were expressed as the percentage of control. Hemoglobin concentration was determined spectrophotometrically after hypotonic lysis of the erythrocytes in water using a millimolar absorption coefficient of 7.88 (on a heme basis) at 523 nm, an isosbestic point of human oxyhemoglobin and methemoglobin.

Free SH groups of the modified cell membrane were measured according to the method of Habeeb [18]. The cells washed with buffer B at pH 7.4 were lyzed in 5 mM phosphate buffer (pH 8.0) at 0 °C and the ghosts were spun down and washed four times in the same buffer. The ghosts were then solubilized in a buffer containing 0.08 M sodium phosphate buffer/2% SDS/ 0.5 $mg \cdot ml^{-1}$ EDTA · 2Na (pH 8.0) and the concentration of free SH groups was determined using GSH as a standard. Absorbance of the respective solubilized ghost suspension was subtracted as the sample background. Protein concentration of the solubilized ghost suspensions was determined by the method of Lowry et al. [19] using bovine serum albumin as a standard.

SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed in the discontinuous buffer system of Laemmli [20], with a 7.5% separating gel and a 4% stacking gel. Reduced and nonreduced samples were prepared by solubilizing the ghosts with Laemmli's sample buffer with and without 2-mercaptoethanol, respectively. Samples containing 40 μg protein were loaded per lane.

Other analytical methods of erythrocytes

Deformability of the modified erythrocytes was measured according to the method of Reid et al. [21] with minor modifications [13]

Osmotic fragility of the modified erythrocytes was assessed by the measurement of the extent of hemolysis (hemoglobin release) in phosphate buffer (pH 7.4) containing varying concentrations of NaCl as described previously [13].

Scanning electron micrographs were taken as described earlier [13].

Results

Chemical, physical and morphological characterization of periodate-treated erythrocytes

It is known that periodate treatment of erythrocytes results in oxidation of sialyl residues of cell surface glycoconjugates to form sialic acid derivatives with aldehyde moieties [11], oxidation of unsaturated fatty acids of the membrane lipids [12,22], and oxidation of SH groups of the membrane proteins to disulfide [12,22]. In the present study, we have characterized the periodate-treated mouse erythrocytes in more detail, including the concentration-dependent chemical alterations of the membrane and some physical and morphological alterations of the cells. Mouse erythrocytes were treated with various concentrations of sodium metaperiodate at 0 °C for 15 min and the membrane and the cells were analyzed.

Gas-chromatographic analysis of the fatty acids of the erythrocyte membrane lipids indicated that arachidonic acid content decreased as the concentration of periodate used for the modification increased (Table I). About 40% of the arachidonic acid was lost on

modification with 2 mM periodate. Thus, it is evident that lipid oxidation took place under these conditions.

The degree of oxidation of cell surface sialyl residues was estimated by measurement of the sialyl residues remaining unoxidized on the cell membrane. The amount of sialic acid released by neuraminidase (*V. cholerae*) from the ghosts of the erythrocytes treated with 2 and 5 mM periodate was 9 and 1% of that of control erythrocytes, respectively (Table I). Since cell surface sialyl residues oxidized by periodate are much less susceptible to the hydrolysis by this enzyme [10], most sialic acid on the erythrocytes treated with 2 mM or higher concentrations of periodate must have been oxidized.

Disulfide formation in the membrane proteins was assessed by SDS-polyacrylamide gel electrophoresis. Gel electrophoresis of the ghosts from the erythrocytes treated with 2 mM periodate under nonreducing conditions showed high-molecular-weight protein bands not penetrating the gel, with concurrent disappearance of the lower-molecular-weight protein bands (Fig. 1, lane 2) present in the ghosts from the unoxidized cells (lane 1). Gel electrophoresis of the ghosts under reducing conditions resulted in disappearance of the high-molecular weight proteins (lane 5), and its electrophoretogram was essentially the same as that of the ghosts from the unoxidized cells (lane 4). These results indicate that SH groups of the membrane proteins were oxidized and disulfide-mediated protein cross-links were formed at the periodate concentration of 2 mM.

Deformability and osmotic fragility of the modified erythrocytes were measured as physical alterations of the cells. Deformability of the cells, assessed by the rate

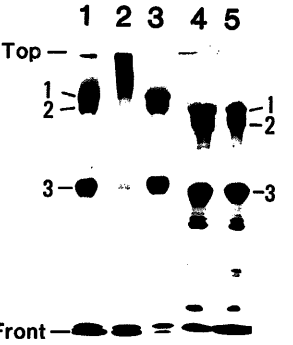

Fig. 1. SDS-polyacrylamide gel electrophoresis of the ghosts from the periodate-treated erythrocytes. Mouse erythrocytes were incubated with or without 2 mM $NaIO_4$ at 0 °C for 15 min, and the ghosts were isolated and electrophoresed. Lanes 1, 2 and 3: electrophoresis under nonreducing conditions of the ghosts from the erythrocytes incubated without $NaIO_4$, with 2 mM $NaIO_4$ and with 2 mM $NaIO_4$ followed by 10 mM dithiothreitol, respectively; lanes 4 and 5: electrophoresis of the same ghosts as lanes 1 and 2, respectively, under reducing conditions.

of the cells passing through a Nucleopore membrane [13], did not decrease at the periodate concentrations up to 10 mM, but drastically decreased at 20 mM (data not shown). Osmotic fragility of the cells modified at the periodate concentration of 2 mM only slightly increased. The fragility of the cells modified at 5 mM considerably increased and most cells modified at 10 mM lyzed even in an isotonic solution. However, the cells modified at 20 mM were highly resistant to the osmotic pressures since the maximal hemolysis in 0.2% NaCl decreased to 25% (data not shown). Hemoglobin released from the cells treated with 2–10 mM periodate by the hypotonic hemolysis was oxyhemoglobin, and that from the cells treated with 20 mM periodate was a mixture of oxy- and methemoglobin, as judged by the spectra of the solutions. Very little morphological change was observed for the erythrocytes treated with 2 mM periodate, but the cells treated with 5 and 20 mM periodate exhibited round and swollen morphology (Fig. 2). These observations indicate that alterations in physical properties and morphology of the erythrocytes treated with 2 mM periodate are minimal, while excessive alterations in physical properties take place when treated with 10 mM or higher concentrations of periodate.

TABLE I

Oxidation of arachidonic acid and sialyl residues of erythrocyte membrane by periodate

Mouse erythrocytes were incubated with the indicated concentrations of $NaIO_4$ at 0 °C for 15 min. Arachidonic acid content was determined by gas chromatography of the lipid extracts from the periodate-treated erythrocytes. Amount of unoxidized sialyl residues was estimated by the amount of sialic acid released by neuraminidase from the ghosts of the periodate-treated erythrocytes.

Reagent (mM)	Arachidonic acid (% of control)	Sialyl residues (% of control)
None	100 [a]	100 [b]
$NaIO_4$ 2	60	9
5	65	1
10	29	
20	21	

[a] Arachidonic acid content in the control ghosts was 62% of palmitic acid content.

[b] Sialic acid released by neuraminidase from the control ghosts was 11.3 μg per mg ghost protein.

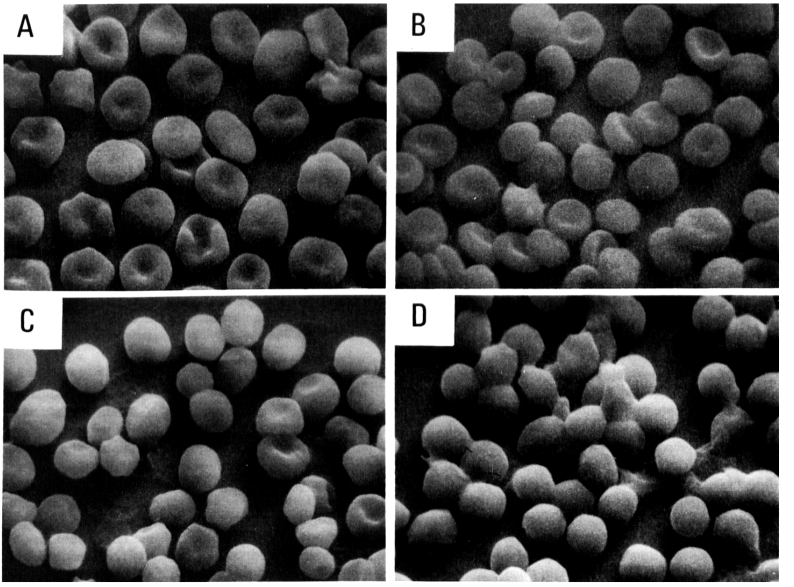

Fig. 2. Scanning electron micrographs of the periodate-treated erythocytes (magnification ×3500). (A) Control erythrocytes; (B, C and D) erythrocytes treated with 2, 5 and 20 mM $NaIO_4$ at 0°C for 15 min, respectively.

Recognition of the periodate-treated erythrocytes by mouse peritoneal macrophages

The periodate-treated erythrocytes were examined for recognition by macrophages (adhesion and phagocytosis). The treated erythrocytes were incubated with monolayers of resident or thioglycollate-elicited mouse peritoneal macrophages in the absence of serum at 37°C for 3 h, and the proportion of the macrophages binding or ingesting erythrocytes was determined.

There was no significant increase in adhesion and phagocytosis of erythrocytes by thioglycollate-elicited peritoneal macrophages upon treatment of erythrocytes with 2 mM periodate. Adherence and phagocytosis by these macrophages increased proportionately upon treatment of erythrocytes with higher concentrations of periodate (Table II). On the other hand, as many as 80% of resident peritoneal macrophages bound the erythrocytes treated with 2 mM periodate, although no significant increase in erythrophagocytosis was observed at this concentration of periodate (Table II). The

TABLE II

Adhesion and phagocytosis of periodate-treated mouse erythrocytes by thioglycollate-elicited and resident mouse peritoneal macrophages

Mouse erythrocytes incubated with the indicated concentrations of $NaIO_4$ at 0°C for 15 min were washed, resuspended in RPMI-Hepes medium, and assayed for adhesion and phagocytosis by the monolayers of thioglycollate-elicited and resident mouse peritoneal macrophages in the absence of serum. Results shown are the mean ± S.D. of triplicate assays.

Reagent for erythrocyte modification (mM)	Peritoneal macrophages	Macrophage recognition (%)	
		adhesion	phagocytosis
None	Thioglycollate-elicited	7±3	4±2
$NaIO_4$ 2		10±2	5±2
5		22±4	9±2
10		25±9	13±2
20		40±6	34±2
None	Resident	12±7	6±3
$NaIO_4$ 2		80±6	4±3

erythrocytes showed typical rosette attachment to the macrophage monolayers, which is consistent with the earlier observation of Rabinovitch [5]. The increased rosette attachment was observed at the periodate concentrations above 0.5 mM and maximized at 1–2 mM (data not shown). These results suggest that resident peritoneal macrophages can recognize the periodate-treated erythrocytes showing no excessive physical alterations, while thioglycollate-elicited peritoneal macrophages recognize only those cells with excessively altered physical properties.

Membrane alterations of erythrocytes involved in the recognition by macrophages

To clarify the alterations of the periodate-treated erythrocytes responsible for the macrophage recognition, we have focused our study on the attachment of the erythrocytes treated with 2 mM periodate, which show no excessive physical alterations, to the resident peritoneal macrophages. Effects of inhibition of lipid oxidation, reduction of aldehyde moieties of the oxidized sialyl residues, and reduction of the protein disulfides on the erythrocyte attachment to the macrophages were studied.

Mouse erythrocytes were treated with 2 mM $NaIO_4$ at 4°C for 15 min in the presence of butylated hydroxytoluene, a radical scavenger, and desferrioxamine [23], an iron chelator, both of which are known to be inhibitors of lipid oxidation [12,24,25]. Lipid oxidation was totally inhibited by these reagents as assessed by the arachidonic acid content of the resultant membrane lipids, while oxidation of the cell surface sialyl residues was not (Table III). Disulfide formation in the membrane proteins was not inhibited by butylated hydroxytoluene, and partially inhibited by desferrioxamine as judged by SDS-gel electrophoresis (data not shown). Frythrocytes treated with periodate in the presence of butylated hydroxytoluene were adherent to the macrophages to an extent comparable to those treated with periodate alone. The adhesion to the macrophages of erythrocytes treated in the presence of desferrioxamine was decreased to about 60% of that of erythrocytes treated with periodate alone (Table III). These results indicate that lipid oxidation of the erythrocyte membrane is not essential for the macrophage recognition of the periodate-treated erythrocytes. The effect of desferrioxamine also suggests that oxidation of sialic acid is not essential, but disulfide formation may be important.

TABLE III

Macrophage binding of erythrocytes treated with periodate in the presence of the inhibitors of lipid oxidation

Mouse erythrocytes were treated with 2 mM $NaIO_4$ in the absence or presence of butylated hydroxytoluene or desferrioxamine. Ghosts were isolated and analyzed for arachidonic acid content and amounts of unoxidized sialyl residues as described in Materials and Methods. Attachment of these cells to resident mouse peritoneal macrophages was measured, and the data are presented as the mean ± S.D. of triplicate assays.

Treatment	Arachidonic acid (% of control)	Sialyl residues (% of control)	Macrophage (%) adhesion
None	100	100	11 ± 2
$NaIO_4$	79	8	76 ± 2
$NaIO_4$ in the presence of butylated hydroxytoluene (0.05 mM)	100	5	81 ± 3
desferrioxamine (0.5 mM)	98	8	45 ± 4

To examine the effect of aldehyde formation on the erythrocyte surface saccharide chains on the macrophage recognition of the erythrocytes, mouse erythrocytes were treated with neuraminidase and galactose oxidase and examined for macrophage recognition. Treatment of erythrocytes with neuraminidase alone did not show any significant increase in macrophage binding, indicating that removal of sialic acid was of no effect (Table IV). Successive treatment of the neuraminidase-treated erythrocytes with galactose oxidase oxidizes exposed galactosyl or *N*-acetyl galactosaminyl residues of the saccharide chains to form aldehyde moieties [26,27]. Treatment of mouse erythrocytes with the two enzymes resulted in increased macrophage binding of the treated cells (Table IV). Borohydride reduces both aldehydes and Schiff bases between aldehyde moieties and amino groups, and cyanoborohydride reduces the Schiff bases selectively [28]. Reduction of the neuraminidase and galactose-oxidase-treated cells with borohydride or cyanoborohydride abrogated the effect of the enzyme treatment (Table IV). Thus,

TABLE IV

Macrophage binding of neuraminidase and galactose oxidase-treated erythrocytes and effect of reduction of the treated cells on the binding

Mouse erythrocytes were treated with neuraminidase (25 mU/ml) or neuraminidase (25 mU/ml) plus galactose oxidase (10 U/ml), then treated with or without 5 mM $NaBH_4$ or 30 mM $NaBH_3CN$, and assayed for recognition by resident mouse peritoneal macrophages. Values given are the mean ± S.D. of triplicate assays.

Treatment		Macrophage adhesion (%)
first	second	
None	none	17 ± 1
Neuraminidase [a]	none	20 ± 3
Neuraminidase plus galactose	none	30 ± 1
Neuraminidase plus galactose oxidase	$NaBH_4$	16 ± 6
Neuraminidase plus galactose oxidase	$NaBH_3CN$	21 ± 3

[a] Approx. 55% of the total sialic acids were removed.

TABLE V

Effect of reduction of periodate-treated erythrocytes by borohydride and cyanoborohydride, and desialylation of erythrocytes prior to periodate treatment on their recognition by macrophages

Mouse erythrocytes were treated with 2 mM $NaIO_4$ followed by reduction by 5 mM $NaBH_4$ or 30 mM $NaBH_3CN$, and assayed for recognition by resident mouse peritoneal macrophages. Mouse erythrocytes treated with neuraminidase (25 mU/ml) prior to the oxidation by 2 mM $NaIO_4$ were also assayed for the macrophage recognition. Values given are the mean ± S.D. of triplicate assays.

Treatment		Macrophage adhesion (%)
first	second	
None	none	12 ± 8
$NaIO_4$	none	81 ± 7
$NaIO_4$	$NaBH_4$	79 ± 4
$NaIO_4$	$NaBH_3CN$	86 ± 3
Neuraminidase [a]	none	11 ± 1
Neuraminidase [a]	$NaIO_4$	72 ± 7

[a] Approx. 55% of the total sialic acids were removed.

aldehyde moieties generated at the galactosyl or *N*-acetyl galactosaminyl residues on the erythrocyte surface saccharide chains induced macrophage recognition of the cells.

Effect of the aldehyde moieties generated on the cell surface sialyl residues by periodate on the macrophage recognition was investigated. Macrophage binding of the periodate-treated mouse erythrocytes did not decrease on reduction of the cells with borohydride or cyanoborohydride (Table V). Neither of these reagents reduced the disulfide cross-links of the membrane proteins as judged by SDS-gel electrophoresis (data not shown). Neuraminidase-treated erythrocytes, where 55% of the total sialic acids were removed, were oxidized by periodate. The oxidized erythrocytes exhibited the macrophage binding to an extent comparable to that of the periodate-treated erythrocytes (Table V). Thus, it is evident that aldehydes and other reducible structures, like Schiff bases, formed on the sialyl residues are not involved in the macrophage recognition of the periodate-treated erythrocytes.

The effect of reduction of the periodate-treated erythrocytes by dithiothreitol, a disulfide reducing agent, on the macrophage recognition of the erythrocytes was then examined. The periodate-treated erythrocytes were incubated with dithiothreitol (10 mM) at 25 °C for 2 h, and subjected to disulfide analysis and the macrophage recognition. As shown in Fig. 1 (lane 3), the disulfide-mediated cross-linking of the membrane proteins was completely reversed by the reduction of the cells by dithiothreitol. The macrophage binding of the periodate-treated erythrocytes was also completely abrogated by the reduction (% adhesion of macrophage: control erythrocytes, 17 ± 1%; periodate-treated, 87 ± 4%; periodate-treated followed by dithiothreitol, 21 ± 7%). Furthermore, as mentioned above, the presence of desferrioxamine during the periodate treatment of erythrocytes partially inhibited the disulfide formation of the membrane proteins and the macrophage recognition of the erythrocytes. These results indicate that SH oxidation to disulfides is essential for the induction of the membrane change of the periodate-treated erythrocytes which macrophages recognize.

Macrophage recognition of the erythrocytes treated with SH-specific oxidizing agents

To clarify whether SH oxidation of the erythrocytes membrane proteins without concurrent oxidation of other membrane components leads to macrophage recognition, we have investigated the effect of SH oxidation of erythrocytes on macrophage recognition using SH-specific oxidizing agents, diamide [29,30] and tetrathionate [30].

Mouse erythrocytes were treated with various concentrations of diamide at 37 °C for 1 h, and the free SH groups remaining in the membrane proteins and intracellular GSH were measured. Both levels were decreased with the increase in the diamide concentration (Fig. 3).

Gel electrophoresis of the ghosts from the diamide-treated cells under nonreduced conditions showed high-molecular-weight protein aggregates at the top of the gel, with concurrent diminution of the protein bands of 1, 2 and 3 originally present in the ghosts from the untreated cells (Fig. 4A, lanes 1–5). The protein aggregation was prominent for the cells treated with 1 and 2 mM diamide. When the electrophoresis was carried out under reducing conditions, the protein aggregates disappeared and the intensities of the diminished protein bands were restored to the level of the control ghosts

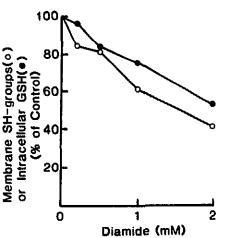

Fig. 3. Effects of diamide on membrane SH content and intracellular GSH concentration of erythrocytes. Mouse erythrocytes were incubated with the indicated concentrations of diamide at 37 C for 1 h, and free SH groups of the membrane and intracellular GSH concentration were measured as described in Materials and Methods. The amount of membrane SH groups and GSH concentration of the control erythrocytes were 119 nmol/mg protein and 12.6 nmol/mg hemoglobin, respectively.

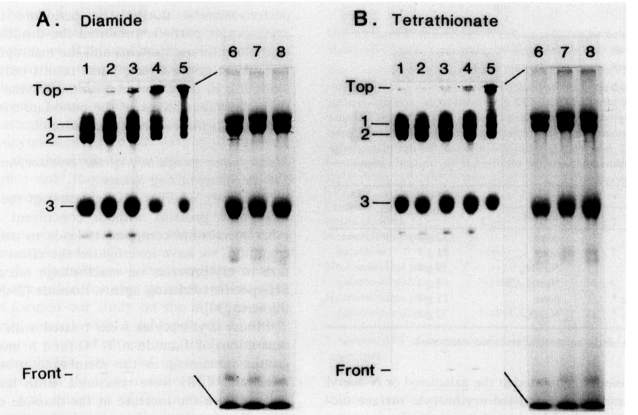

Fig. 4. SDS-polyacrylamide gel electrophoresis of the ghosts from the diamide(A)- and tetrathionate(B)-treated erythrocytes. Mouse erythrocytes were incubated with increasing concentrations of diamide (A) or sodium tetrathionate (B) at 37°C for 1 h, and the ghosts were isolated and electrophoresed. Lanes 1–5: electrophoresis under nonreducing conditions of the ghosts from the erythrocytes incubated without or with 0.2, 0.5, 1 and 2 mM reagents, respectively; lanes 6–8: electrophoresis of the same ghosts as lanes 1, 4 and 5, respectively, under reducing conditions. Absence of proteins below band 3 is due to low recovery during the ghost preparation.

(Fig. 4A, lanes 6–8). These results indicate that SH groups of the membrane proteins were oxidized by diamide to form disulfide-mediated intra and intermolecular protein cross-links.

The diamide-treated erythrocytes showed an increased attachment to the monolayers of resident mouse peritoneal macrophages in the absence of serum (Fig. 5A). Attachment to the macrophages increased remarkably at the diamide concentrations of 1 and 2 mM. The erythrocytes formed typical rosettes (Fig.6), similarly to the case of periodate-treated erythrocytes. An increase in erythrophagocytosis was also observed (Fig. 5A). When thioglycollate-elicited peritoneal macro-

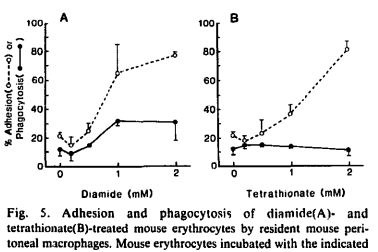

Fig. 5. Adhesion and phagocytosis of diamide(A)- and tetrathionate(B)-treated mouse erythrocytes by resident mouse peritoneal macrophages. Mouse erythrocytes incubated with the indicated concentrations of diamide or tetrathionate at 37°C for 1 h were washed, resuspended in RPMI-Hepes medium, and assayed for adhesion and phagocytosis by the monolayers of resident mouse peritoneal macrophages in the absence of serum. Results shown are the mean ± S.D. of triplicate assays.

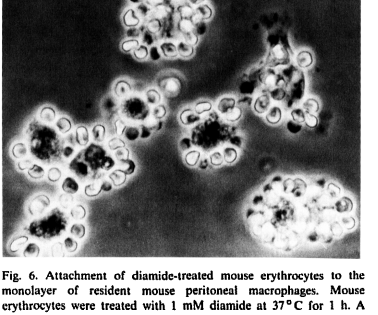

Fig. 6. Attachment of diamide-treated mouse erythrocytes to the monolayer of resident mouse peritoneal macrophages. Mouse erythrocytes were treated with 1 mM diamide at 37°C for 1 h. A typical field of a phase contrast micrograph is presented.

phages were used, no significant increase in erythrocyte attachment was observed (data not shown), which is consistent with the result obtained for the periodate-treated erythrocytes.

Reduction of the diamide(1 mM)-treated erythrocytes with dithiothreitol (10 mM) completely prevented the macrophage adhesion (% adhesion: control erythrocytes, $15 \pm 1\%$; diamide-treated, $60 \pm 4\%$; diamide-treated followed by dithiothreitol $3 \pm 1\%$).

Tetrathionate oxidized SH groups of mouse erythrocytes significantly at 1 and 2 mM, as assessed by disulfide-mediated membrane protein cross-linking (Fig. 4B), and the tetrathionate-treated erythrocytes also attached to the resident peritoneal macrophages better than the untreated control cells at the reagent concentrations of 1 and 2 mM, although no increase in erythrophagocytosis was observed (Fig. 5B).

These results indicate that oxidation of SH groups of erythrocytes, possibly those of the membrane proteins, into disulfides leads to the macrophage recognition of the erythrocytes.

Discussion

Attachment of the periodate-treated erythrocytes to macrophages in the absence of serum, which was first observed by Rabinovitch [5], may be regarded as recognition of the oxidatively damaged erythrocytes by the macrophages [5,31]. Previously, we have reported that periodate treatment of erythrocytes results in not only oxidation of the cell surface sialyl residues, but also oxidation of the membrane lipids and the SH groups of the membrane proteins [12]. In the present study, we have found that oxidation of the SH groups to disulfides is the critical damage which renders the periodate-treated erythrocytes susceptible to the macrophage recognition. The finding is of interest because SH oxidation can take place in vivo.

Inhibition of lipid oxidation by butylated hydroxytoluene did not prevent the attachment of the periodate-treated erythrocytes to the macrophages, and the results excluded the possibility that lipid oxidation induced by periodate is involved in the macrophage recognition. Recently, we have reported that erythrocytes, which underwent lipid oxidation induced by ADP/Fe^{3+}, were recognized by macrophages, and inhibition of the lipid oxidation prevented the subsequent macrophage recognition [13]. In contrast to the lipid oxidation induced by periodate, the lipid oxidation induced by ADP/Fe^{3+} was so mild that no detectable decrease in the unsaturated fatty acid contents of the membrane lipids was observed (unpublished results). Thus, excessive lipid oxidation induced in the periodate-treated erythrocytes may diminish the determinants produced by mild lipid oxidation which macrophages recognize.

Aldehyde formation on the sialyl residues of the cell surface glycoconjugates has been postulated to be involved in the activation of periodate-treated lymphocytes, leading to mitogenesis and cytotoxicity to tumor cells, since removal of sialic acid before the periodate-treatment or reduction of the periodate-treated cells with borohydride prevents the activation of the cells [6–8]. In the case of the macrophage recognition of the periodate-treated erythrocytes, the aldehyde formation did not appear to be involved since treatment of the erythrocytes with borohydride or cyanoborohydride did not prevent erythrocyte attachment to macrophages. However, aldehyde moieties produced on the cell surface saccharide chains by neuraminidase and galactose oxidase appeared to be effective in the erythrocyte attachment to macrophages. It is possible that aldehyde moieties produced on the sialosaccharide chains and those produced on the terminal galactosyl or *N*-acetyl galactosaminyl residues of the asialosaccharide chains have different effects on cell interactions because of difference in the negative charges of the sialyl residues.

Failure of the periodate-treated erythrocytes to attach to macrophages after reduction by dithiothreitol clearly indicates that disulfide formation, or loss of free SH groups, is responsible for the macrophage recognition of the periodate-treated erythrocytes. Although peroxidized lipids can oxidize SH groups into disulfides, their contribution to the disulfide formation in the periodate-treated erythrocytes seems to be minor since inhibition of periodate-induced lipid oxidation by butylated hydroxytoluene did not affect the disulfide-mediated membrane protein cross-linking. The partial inhibition of the disulfide-mediated protein cross-linking by desferrioxamine, which was consistent with the partial inhibition of the erythrocyte attachment to the macrophages, is probably due to direct reaction of periodate with desferrioxamine.

The observation that the erythrocytes treated with SH-specific oxidizing agents attached to the macrophages gave further evidence for the macrophage recognition of SH-oxidized erythrocytes. Although the major SH-containing components of erythrocytes are hemoglobin and GSH [32], it is likely that oxidation of SH groups of the membrane proteins causes a certain membrane change, which macrophages recognize. It is known that SH oxidation of erythrocyte membrane proteins occurs in vivo. For example, the free SH content of the erythrocyte membrane from patients with β-thalasemia major is lower than that of normal cells [33], and protein 4.1 of sickle erythrocyte membrane contains cysteic acid, while normal protein 4.1 does not [34]. The unusually adherent propensity of β-thalassemic cells [35] and sickle cells [36] to macrophages may be due, at least in part, to recognition by macrophages of the SH-oxidized membrane.

It is not known which changes of the SH-oxidized

erythrocyte membrane macrophages recognize. The membrane change responsible for the macrophage recognition is obviously reverted by reduction of disulfides to free SH groups. SH oxidation of erythrocytes has been known to induce a variety of changes of the membrane properties, including an increase in the permeability for hydrophilic nonelectrolytes and ions [14,22], transbilayer reorientation of phospholipids [37,39], decreases in the lateral mobility of band 3 glycoprotein [40] and cell deformability [41]. It is noteworthy that the transbilayer reorientation of phospholipids caused by SH oxidation of the membrane proteins results in exposure of phosphatidylserine, the only phospholipid that resides exclusively in the inner leaflet of the normal erythrocyte membrane [42,43], on the outer surface of the cells [37,38], because macrophages have been reported to recognize the erythrocytes displaying an exogenously supplied phosphatidylserine analog on the outer surface [44,45]. It is possible that phosphatidylserine, which may be exposed on the outer cell surface by SH oxidation of the membrane proteins, is the determinant of the macrophage recognition of the periodate-treated and diamide- or tetrathionate-treated erythrocytes.

In the present work, thioglycollate-elicited mouse peritoneal macrophages did not significantly recognize the mouse erythrocytes treated with a low concentration of periodate (2 mM) or with diamide, while more than 80% of the resident peritoneal macrophages did. Recently, Wali et al. [46] reported that diamide-treated erythrocytes are adherent to cultured endothelial cells, but scarcely to cultured fibroblasts and smooth muscle cells. Their observations and ours suggest that there exist specific interactions between the SH-oxidized erythrocytes and some cell types, like resident peritoneal macrophages and endothelial cells. There may be a receptor recognizing SH-oxidized erythrocytes on the surface of these cells.

Johnson et al. [47] reported that in vivo injection of diamide-treated dog erythrocytes resulted in rapid sequesteration of the cells into spleen and liver, suggesting that the SH-oxidized cells are recognized and phagocytized by the macrophages in these organs. In our vitro studies, recognition (binding) of the SH-oxidized erythrocytes by resident peritoneal macrophages was not always followed by phagocytosis. The lack of phagocytic response may be due, in part, to the conditions of the macrophages, since phagocytosis is an energy-dependent process which is affected by culture conditions [48]. It should be pointed out that factors potentiating the phagocytic activity of macrophages could exist in vivo. For instance, fibronectin and a specific lymphokine, respectively, have been reported to stimulate phagocytic process of the complement(C3)-coated erythrocytes bound to cultured human monocytes [49] and to resident mouse peritoneal macrophages [50]. Thus, it is conceivable that some types of macrophages are able to take up SH-oxidized erythrocytes in vivo.

Very recently, it was demonstrated that diamide-treated human erythrocytes are ingested by monocytes in the presence of autologous serum [51]. The serum components (opsonins) responsible for the observed phagocytosis were anti-band 3-autoantibodies and complement [52], indicating that macrophages recognize the SH-oxidized erythrocytes to which anti-band 3-antibodies and complement bound. The present results, however, indicate that macrophages have an ability to recognize directly the SH-oxidized erythrocytes, without participation of opsonins. Thus, there are two distinct mechanisms for the recognition of SH-oxidized erythrocytes by macrophages, i.e., opsonin-dependent and -independent. The opsonin-dependent mechanism [51,52] appears to recognize less extensively oxidized erythrocytes than the erythrocytes recognized by the opsonin-independent mechanism, since the concentration of diamide required for the modification of human erythrocytes to induce the opsonin-dependent recognition [51,52] is lower than that required for the modification of mouse cells to induce the opsonin-independent recognition. Further investigation is necessary for the evaluation of actual roles of these mechanisms in the in vivo clearance of oxidized erythrocytes.

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