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Recognition of oxidatively damaged erythrocytes by a macrophage receptor with specificity for oxidized low density lipoprotein

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ABSTRACT **Macrophages specifically bind and internalize oxidatively modified low density lipoprotein (LDL) via the acetyl-LDL receptor and possibly one or more additional receptors jointly designated here as scavenger receptors. It is well accepted that these receptors are intimately involved in the formation of foam cells during atherogenesis. However, the normal physiological or pathophysiological role for these receptors has not been established. Oxidation of plasma membranes is a common accompaniment of cell damage and senescence. In particular, aged erythrocytes demonstrate peroxidation of their cell membrane lipids. In the present studies we show that oxidized human erythrocytes (treated with copper plus ascorbate or hydrogen peroxide) are bound and phagocytosed by mouse peritoneal macrophages in the absence of opsonizing antibodies. There was little or no binding of untreated erythrocytes. Oxidized LDL, but not acetylated or native LDL, inhibited this binding and uptake of oxidized erythrocytes. Inhibitors of scavenger receptor binding, including polyinosinic acid and fucoidin, also prevented binding of the oxidized red blood cells. We suggest that oxidative damage of erythrocytes results in the formation of lipid–protein conjugate(s) closely related to some of the conjugates found in oxidized LDL, making the oxidized erythrocyte a ligand for the macrophage scavenger receptors, apparently at a site distinct from that responsible for the binding of acetylated LDL. Oxidative modification of plasma membranes may represent a general mechanism that marks damaged cells for phagocytosis by macrophages.**

The formation of macrophage-derived, lipid-laden foam cells in the arterial intima, particularly that mediated by low density lipoprotein (LDL), is a key process contributing to the development of atherosclerosis and subsequent heart disease (1). Paradoxically, LDL in its native form lacks the capacity to generate foam cells from either macrophages or smooth muscle cells, implying that the receptor for native LDL is not required for foam-cell formation (2). This is most evident in patients with familial hypercholesterolemia who lack functional LDL receptors yet develop foam cells and atherosclerotic lesions similar to those in patients with normal LDL receptors. Chemical *in vitro* modification of LDL, including acetylation (2) and oxidation (3), results in avid uptake of the lipoprotein by macrophages. Oxidatively modified LDL (OxLDL) occurs *in vivo* (4), and several lines of evidence suggest that macrophage recognition of OxLDL via specific receptors is likely to be an important factor in fatty streak formation (5). Most important, inhibition of LDL oxidation can slow the progression of experimental atherosclerosis (6, 7).

A large part of the uptake and degradation of OxLDL by macrophages occurs via the acetyl-LDL (AcLDL) receptor (8–10). That receptor has been cloned and fully characterized (11, 12). However, as much as 40–60% of OxLDL uptake in mouse peritoneal macrophages is not inhibited by AcLDL and appears to be due to an OxLDL receptor(s), not yet characterized in detail (9, 10).

It is clear *a priori* that the evolutionary persistence of the scavenger receptors and related proteins must relate to functions other than those that account for their participation in atherosclerosis. Atherosclerosis exerts no genetic pressure, either positive or negative, since it occurs to a significant extent only in humans and then only after the childbearing years. It was suggested elsewhere that the "normal" function of the scavenger receptor(s) might be to protect against the cytotoxic effects of OxLDL by taking it up and removing it from the extravascular space (13). Additionally, these receptors may recognize *any* damaged cell by virtue of its oxidized cell membranes, which might contain domains analogous to those found in OxLDL. In a global sense, scavenger receptors might represent a means of removing oxidatively damaged components or "senescent self" which could otherwise injure surrounding tissue (13).

Krieger *et al.* (12) have recently reviewed the structure and binding properties of macrophage scavenger receptors, stressing their very broad but by no means totally indiscriminate binding specificity. In addition to OxLDL and AcLDL, the receptors have been shown to bind polyribonucleotides, polysaccharides, anionic phospholipids, and bacterial lipopolysaccharide. Nevertheless, there is a degree of specificity, most strikingly illustrated by the fact that polyinosinic acid [poly(I)] binds very effectively, but that polycytidylic acid [poly(C)] and polyadenylic acid [poly(A)] do not.

The present studies were initiated to test the hypothesis that macrophage scavenger receptors can recognize and internalize damaged cells by recognizing oxidized domains in their membranes. The red blood cell (RBC) was chosen as the model cell for study in part because of its simplicity and also because it has been so widely studied. There is already considerable evidence that RBCs do undergo lipid peroxidation *in vivo*, that older cells are more likely to be oxidized (14), and that oxidized RBCs are more rapidly cleared from the circulation (15). However, the mechanism by which macrophages recognize oxidatively damaged or senescent RBCs remains uncertain. Most investigators believe that removal is contingent on the binding of antibodies that recognize damaged cell membrane domains and that the phagocytosis depends upon recognition of the antibody–RBC complex by the Fc receptor (16, 17). Yet, oxidatively damaged RBCs under some conditions are known to bind to macrophages in the absence of opsonizing antibodies (18). We show here that oxidatively damaged RBCs are recognized in a direct, antibody-independent fashion by a macrophage

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Abbreviations: LDL, low density lipoprotein; OxLDL, oxidized LDL; AcLDL, acetyl-LDL; RBC, red blood cell; BSA, bovine serum albumin; HSA, human serum albumin; $Fc_\gamma RII$, Fc_γ receptor type II.

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scavenger receptor which results in their binding and phagocytosis. Furthermore, OxLDL, but not AcLDL, competitively inhibits such binding and phagocytosis.

MATERIALS AND METHODS

Materials. $CuSO_4$, $FeCl_3$, ascorbate, 30% hydrogen peroxide, poly(I), poly(C), fucoidin, tetramethoxypropane, and fatty acid-free bovine serum albumin (BSA) and human serum albumin (HSA) were obtained from Sigma; acetic anhydride was from Aldrich; 25% glutaraldehyde in water was from Fisher Scientific; mouse monoclonal IgG1 anti-human glycophorin A (10F7) was a generous gift of Ann Rearden (University of California at San Diego); and rat anti-mouse Fc_γ receptor type II ($Fc_\gamma RII$) monoclonal antibody was from PharMingen.

Lipoproteins. Human LDL (1.019–1.063 g/cm^3) was isolated in EDTA (1 mg/ml) from fresh plasma by preparative ultracentrifugation (19). Concentration was based on protein content determined by the method of Lowry *et al.* (20). LDL (100 μg/ml) was oxidized by incubation overnight in phosphate-buffered saline (PBS) in the presence of 5 μM Cu^{2+}. The lysophosphatidylcholine accumulating during oxidation was removed by incubation with fatty acid-free BSA (10 mg/ml) for 24 hr and reisolation of the LDL by preparative ultracentrifugation (density <1.210 g/cm^3). Acetylation of LDL with acetic anhydride was as described (21).

Cells. Erythrocytes from fresh human blood were washed three times with PBS and resuspended at 20% hematocrit in PBS containing 0.1% glucose. This preparation was stored at 4°C and used as native RBCs. Oxidized RBCs (4% hematocrit in PBS) were prepared by incubation at 37°C for 90 min in the presence of 0.2 mM $CuSO_4$ plus 5 mM ascorbate or for 150 min in the presence of 0.2 mM $CuSO_4$ plus 10 mM H_2O_2 or $FeCl_3$ plus 10 mM H_2O_2. The oxidized RBCs were subsequently washed twice with PBS containing 0.2% EDTA and twice with PBS. Aldehyde modification of 4% hematocrit RBC suspension was accomplished with 1 mM glutaraldehyde or 10 mM malondialdehyde in PBS for 1 hr at 37°C. Unreacted aldehyde was removed by washing the cells three times with PBS. "*In vitro* aged RBCs" were made by incubating cells (20% hematocrit) in PBS at 37°C for 48 hr.

Resident mouse peritoneal macrophages were isolated (9) and plated in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics. After 4 hr, nonadherent cells were removed by washing three times with PBS. The adherent macrophages were kept in Dulbecco's modified Eagle's medium for binding and phagocytosis experiments.

Binding and Phagocytosis. RBCs (hematocrit 1%) were incubated with macrophages at 37°C for 1 hr. After washing to remove unbound RBCs, the percentage of macrophages binding one or more RBCs was determined. Conditions were tested in duplicate wells and each well was counted twice (100 macrophages were counted each time). Macrophage-bound RBCs were removed by hypotonic lysis with 5 mM phosphate buffer and macrophages were fixed with methanol prior to determination of RBC phagocytosis. Phagocytosis was also measured as the percentage of macrophages ingesting one or more RBCs.

Other Methods. SDS/PAGE of RBC membranes was performed following hypotonic lysis of cells in 5 mM phosphate buffer and isolation of membranes by centrifugation at 11,000 × *g*. RBCs were opsonized by incubation with a mouse monoclonal IgG1 anti-human glycophorin A antibody at 37°C

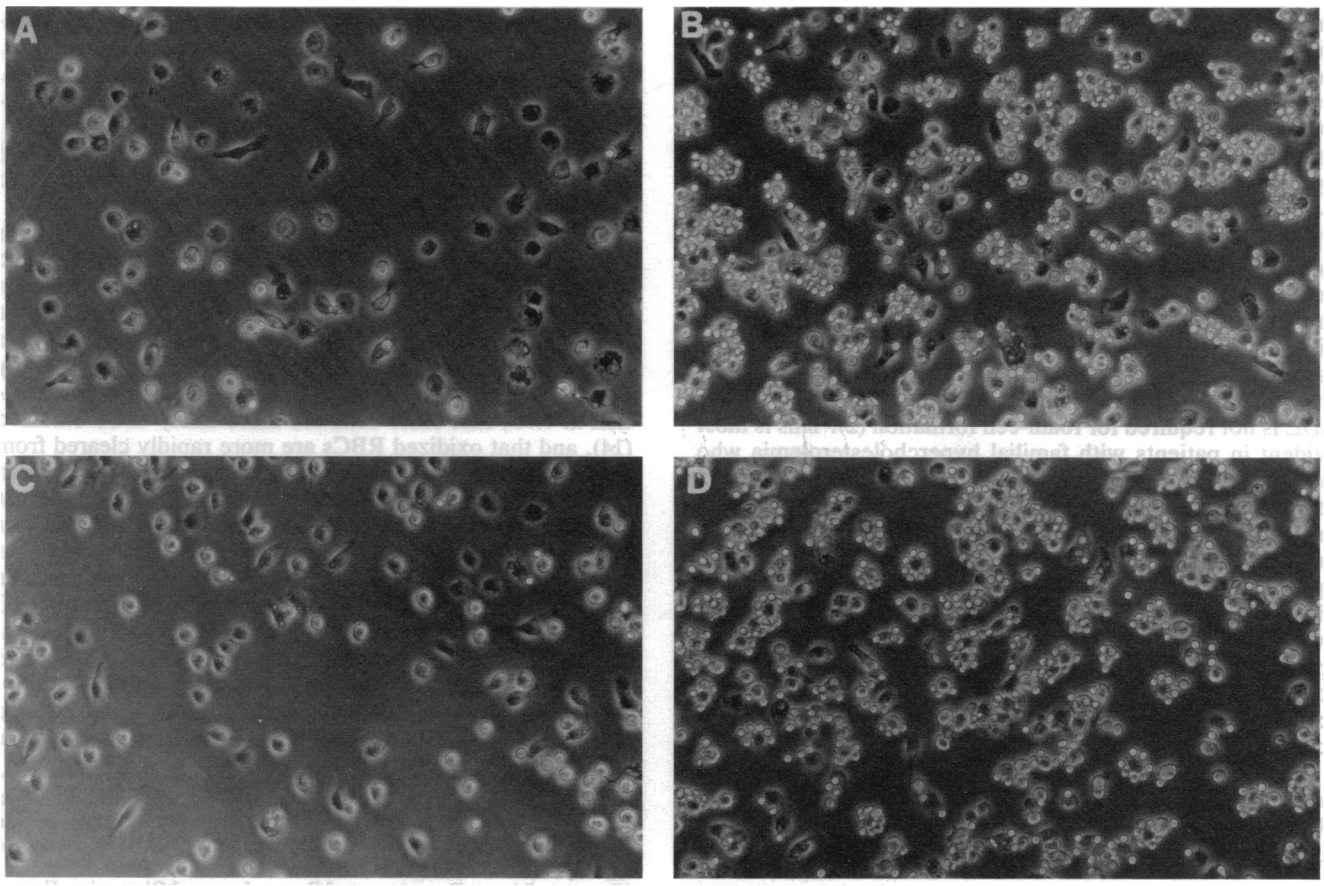

FIG. 1. Rosetting of RBCs on mouse peritoneal macrophages. Macrophages were plated 4 hr prior to testing and photographs were taken 30 min after addition of RBCs. (*A*) Native RBCs. (*B*) Oxidized RBCs (Cu^{2+}/ascorbate). (*C*) Oxidized RBCs plus OxLDL (100 μg/ml). (*D*) Oxidized RBCs plus AcLDL (100 μg/ml).

for 1 hr. Malondialdehyde was prepared by acid hydrolysis of tetramethoxypropane; 2.5 ml of 4 M HCl was added to 1 ml of tetramethoxypropane and the mixture was incubated for 90 min at 37°C. The pH was then adjusted to 6.9 with 10 M NaOH and the volume was brought to 5 ml (1.2 M) with distilled water. To prepare malondialdehyde-modified albumin, 5 mg of protein was mixed with 1 ml of 1.2 M malondialdehyde and incubated at 37°C for 2 hr. The protein was subsequently dialyzed against PBS with several changes to remove unreacted malondialdehyde.

RESULTS

In preliminary studies, mouse peritoneal macrophages were studied 16–24 hr after plating. Native RBCs bound very avidly to these macrophages, making the assessment of any increase in binding of oxidized RBCs difficult. This binding, however, did not lead to internalization and was inhibited by neuraminidase treatment of RBCs (data not shown). Previous studies have shown that the expression of a macrophage receptor that recognizes sialic acid residues (sialoadhesin; sheep erythrocyte receptor) (22) increases with time in culture (maximal expression at 72 hr) (23). Expression of this receptor in freshly isolated peritoneal macrophages is low or undetectable (23). When mouse peritoneal macrophages were plated and used within 4 hr, only about 5% of them bound untreated native RBCs (Fig. 1*A*; Fig. 2). In all subsequent studies macrophages were used within 4 hr of plating.

Oxidized RBCs showed extensive direct, antibody-independent binding to mouse peritoneal macrophages (Fig. 1*B*; Fig. 2). Cu^{2+}-catalyzed oxidation was clearly more effective than Fe^{3+}-catalyzed oxidation (Fig. 2). Moreover, binding of copper-treated RBCs was accompanied by their subsequent internalization, whereas iron-treated RBCs became bound but with almost no subsequent phagocytosis.

Binding and phagocytosis of Cu^{2+}/ascorbate-oxidized RBCs was greatly inhibited by the addition of OxLDL (Fig. 1*C*; Fig. 3), but almost not at all by equal concentrations of AcLDL or native LDL (Fig. 1*D*; Fig. 3). Similar results were obtained for Cu^{2+}/H_2O_2-oxidized RBCs (data not shown). Inhibition of Cu^{2+}/ascorbate-oxidized RBC binding and phagocytosis by OxLDL was found to be concentration

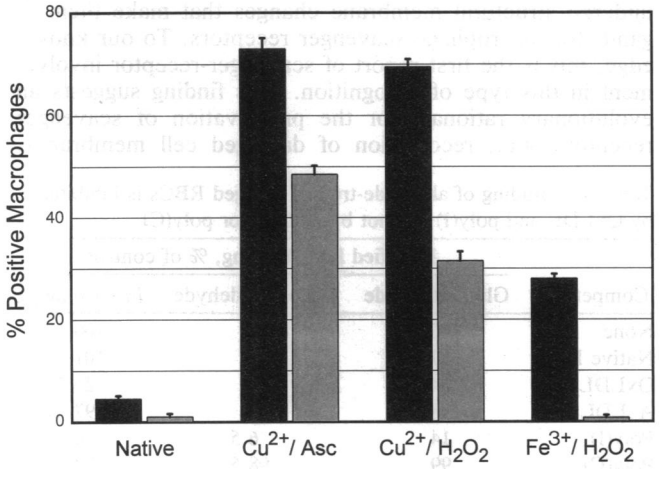

FIG. 2. Binding (black bars) and phagocytosis (gray bars) by mouse peritoneal macrophages of human native RBCs and RBCs oxidized by Cu^{2+}/ascorbate (Cu^{2+}/Asc), Cu^{2+}/H_2O_2, or Fe^{3+}/H_2O_2 (see *Materials and Methods*). Native RBCs or oxidized RBCs were added to macrophages (1% final hematocrit) for 1 hr at 37°C. Values represent the percentage of macrophages having bound or phagocytosed one or more RBCs and are expressed as a mean ± SE of four determinations.

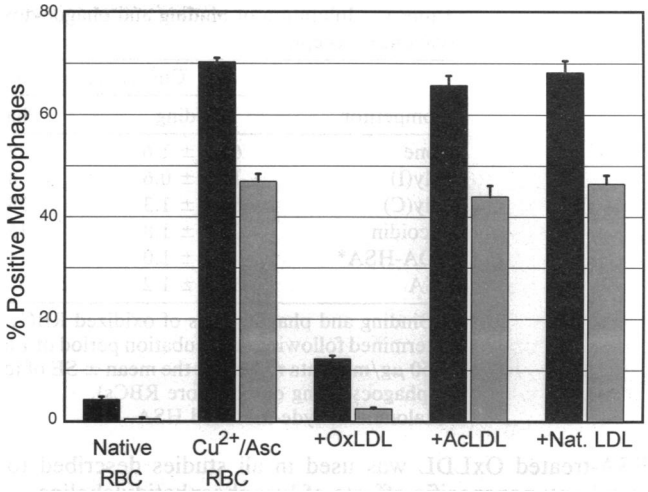

FIG. 3. Competitive inhibition of macrophage binding (black bars) and phagocytosis (gray bars) of oxidized RBCs (Cu^{2+}/Asc) by OxLDL, AcLDL, and native LDL. The study was performed using a 1% final hematocrit and 100 μg/ml concentration of competitor. Binding and phagocytosis were assessed as described in the legend to Fig. 2.

dependent (Fig. 4). Up to 90% inhibition was obtained with OxLDL at 100 μg/ml, whereas AcLDL at up to 200 μg/ml failed to inhibit binding or phagocytosis.

Oxidation of LDL produces a significant amount of lysophosphatidylcholine, which is known to be a hemolytic agent (3). To test whether lysophosphatidylcholine played a role in the inhibition of RBC binding by OxLDL, OxLDL was pretreated with fatty acid-free BSA (10 mg/ml) to remove the lysophosphatidylcholine and reisolated by preparative ultracentrifugation. Removal of lysophosphatidylcholine had no effect on competition. Neither OxLDL nor BSA-treated OxLDL induced hemolysis of RBCs. However, incubation of RBCs with OxLDL resulted in crenation and this effect was eliminated by prior treatment of the OxLDL with BSA.

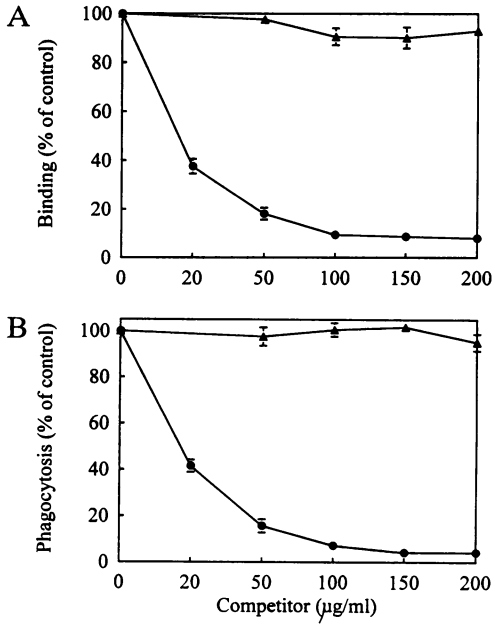

FIG. 4. Concentration-dependent competition of macrophage binding (*A*) and phagocytosis (*B*) of oxidized RBCs (Cu^{2+}/ascorbate) by OxLDL (●) and AcLDL (▲). Competition studies were performed using a 1% final hematocrit and indicated concentrations of competitor. Binding and phagocytosis were assessed as described in the legend to Fig. 2.

Table 1. Inhibition of binding and phagocytosis of oxidized RBCs by known ligands for scavenger receptor

Competitor	Cu^{2+}/ascorbate RBCs		Cu^{2+}/H_2O_2 RBCs	
	Binding	Phagocytosis	Binding	Phagocytosis
None	66.5 ± 1.6	38.8 ± 1.6	52.8 ± 2.6	29.8 ± 1.0
Poly(I)	15.8 ± 0.6	8.0 ± 0.4	11.3 ± 0.9	6.3 ± 0.6
Poly(C)	65.3 ± 1.3	38.3 ± 1.5	56.8 ± 1.2	27.8 ± 2.3
Fucoidin	24.8 ± 1.8	15.5 ± 1.7	22.8 ± 1.9	9.8 ± 1.4
MDA-HSA*	9.5 ± 1.0	4.8 ± 0.5	13.3 ± 1.9	2.5 ± 0.7
HSA	65.5 ± 1.2	35.3 ± 0.3	55.5 ± 1.9	24.3 ± 2.1

Binding and phagocytosis of oxidized RBCs (Cu^{2+}/ascorbate or Cu^{2+}/H_2O_2) to macrophages was determined following an incubation period of 1 hr at 37°C. All competitors were used at a concentration of 50 μg/ml. Data represent the mean ± SE of four determinations (percentage of macrophages binding or phagocytosing one or more RBCs).
*Malondialdehyde-modified HSA.

BSA-treated OxLDL was used in all studies described to avoid any nonspecific effects of lysophosphatidylcholine.

Various inhibitors of scavenger-receptor binding of AcLDL or OxLDL were tested for their ability to inhibit macrophage binding and phagocytosis of oxidized RBCs (Table 1). Poly(I), fucoidin, and malondialdehyde-modified HSA, all at a concentration of 25 μg/ml, significantly reduced the binding and phagocytosis of oxidized RBCs. Poly(C), which does not compete with AcLDL or OxLDL for binding to macrophages, was without effect.

Because recent studies have implicated $Fc_\gamma RII$ as a receptor for oxidized LDL (24), the binding of oxidized RBCs to this receptor was examined. Native RBCs were opsonized with a monoclonal IgG1 against human glycophorin A. As expected, these opsonized RBCs were avidly bound (Table 2). A monoclonal antibody directed against $Fc_\gamma RII$ blocked this binding by ≈94%, whereas OxLDL and poly(I) had no effect. Conversely, the anti-$Fc_\gamma RII$ antibody had no effect on the binding or phagocytosis of oxidized RBCs. These data suggest that $Fc_\gamma RII$ is not involved in recognition of oxidized RBCs under the conditions of these experiments.

Oxidization of lipid–protein complexes results in the production of a wide array of reactive aldehydes (derived from polyunsaturated fatty acids) which can form Schiff bases with lysine ε-amino groups (25) and lead to crosslinking of membrane proteins. Membrane proteins of RBCs oxidized with Cu^{2+}/ascorbate or with Cu^{2+}/H_2O_2 revealed extensive protein crosslinking, but cells oxidized with Fe^{2+}/H_2O_2 did not (data not shown). To test the hypothesis that recognition of oxidized RBCs might be mediated by aldehyde modification, native RBCs were treated with various concentrations of glutaraldehyde or malondialdehyde. Such treatment resulted in binding and phagocytosis by macrophages, as observed previously by others (26, 27). Treatment of RBCs with 1 mM glutaraldehyde was sufficient to achieve binding and phagocytosis of a degree similar to that achieved with Cu^{2+}/ascorbate treatment. A 10 mM final concentration was necessary to obtain similar results with malondialdehyde. Modification of RBCs with these aldehydes resulted in major crosslinking of membrane proteins as shown by SDS/PAGE. Recognition of the aldehyde-modified RBCs was inhibited by OxLDL and poly(I), but not AcLDL, poly(C), or native LDL (Table 3).

A ligand similar to that produced by Cu^{2+}/ascorbate was also created when RBCs were simply allowed to age *in vitro* for 48 hr at 37°C. The aged RBCs contained membrane proteins that became crosslinked and these cells were recognized and internalized by macrophages. The recognition of these oxidized RBCs was also strongly inhibited by OxLDL and poly(I), but not by AcLDL, poly(C), or native LDL (Table 3).

Table 2. Binding of opsonized or oxidized RBCs to macrophages: Inhibition by a blocking antibody (Ab) against $Fc_\gamma RII$

RBC preparation	% positive macrophages
Opsonized RBCs	61.5 ± 2.3
+ $Fc_\gamma RII$ blocking Ab	3.5 ± 0.7
+ OxLDL	60.3 ± 1.9
+ Poly(I)	62.8 ± 1.7
Cu^{2+}/ascorbate RBCs	40.3 ± 2.2
+ $Fc_\gamma RII$ blocking Ab	40.3 ± 3.4
Cu^{2+}/H_2O_2 RBCs	51.8 ± 3.4
+ $Fc_\gamma RII$ blocking Ab	51.8 ± 2.7

Oxidized RBCs or RBC opsonized with a mouse anti-human glycophorin A antibody were introduced to adhered macrophages for 1 hr at 37°C with or without a mouse-specific $Fc_\gamma RII$ blocking antibody (4 μg/ml). OxLDL (100 μg/ml) and poly(I) (50 μg/ml) were added as competitors to test their effect on Fc receptor binding. Data represent percentage of macrophages binding one or more RBCs, and are expressed as the mean ± SE of four determinations.

DISCUSSION

Various receptors have been implicated in the recognition of damaged or dying cells, including an advanced glycosylation endproducts (AGE) receptor (28), a vitronectin receptor, a phosphatidylserine receptor (29), and a lectin-like receptor (30). These studies show that Cu^{2+}-oxidized human RBCs undergo structural membrane changes that make them ligands for macrophage scavenger receptors. To our knowledge, this is the first report of scavenger-receptor involvement in this type of recognition. This finding suggests an evolutionary rationale for the preservation of scavenger receptors—i.e., recognition of damaged cell membranes.

Table 3. Binding of aldehyde-treated or aged RBCs is inhibited by OxLDL and poly(I) but not by AcLDL or poly(C)

Competitor	Modified RBC binding, % of control		
	Glutaraldehyde	Malondialdehyde	*In vitro* aging
None	100	100	100
Native LDL	97.5	99	101
OxLDL	24.5	17	20.5
AcLDL	98.5	98	97
Poly(I)	14	6.5	4.5
Poly(C)	99	98.5	97.5

RBCs at a concentration of 4% were treated with 1 mM glutaraldehyde or 10 mM malondialdehyde in PBS for 1 hr at 37°C. *In vitro* aged RBCs were obtained by incubating a 20% cell suspension in PBS at 37°C for 48 hr. Binding of RBCs to macrophages was determined in the presence or absence of indicated competitors. Native LDL, OxLDL, and AcLDL were used at 100 μg/ml, and poly(I) and poly(C) were used at 50 μg/ml. Data represent the mean percent of the control value obtained from duplicate experiments.

Numerous hypotheses have been proposed for the mechanism of senescent RBC clearance (31), and the involvement of antibodies in this process has been well documented (16, 17). However, binding and phagocytosis of oxidized RBCs do not require opsonization and are not inhibited by a blocking antibody against the Fc receptor. *In vivo*, both antibody-dependent and antibody-independent uptake may play a role in the removal of senescent RBCs.

The findings imply that oxidation induces common structural alterations in OxLDL and in oxidized RBCs, resulting in their uptake by a common receptor or receptors. Exactly which structural changes are critically important remains to be established. We suggest that there may be sufficient commonality among plasma membranes such that oxidative damage can produce the same or very similar lipid–protein complexes in them. Both in oxidation of LDL (32) and in oxidation of RBCs (14), generation of active aldehydes appears to be critical for ligand formation. Aldehyde modification of LDL has already been shown to generate a ligand recognized by scavenger receptors (33). In the present studies, we show that recognition of aldehyde-modified RBCs is mediated by receptor(s) closely related or identical to those that recognize OxLDL and oxidized RBCs.

Oxidatively modified LDL, but not native or acetylated LDL, inhibited the binding and phagocytosis of oxidized RBCs, indicating that the binding site is not identical with that for AcLDL. However, some of the binding could nevertheless involve the AcLDL receptor, which binds both AcLDL and OxLDL, the latter with a much lower affinity (11, 12, 34). In intact mouse peritoneal macrophages, OxLDL binds with an affinity comparable to that of AcLDL and is only partially blocked by AcLDL (9, 10), implying a role for a separate OxLDL receptor(s).

Using expression cloning based on binding of OxLDL, Stanton *et al.* (24) isolated the Fc_{γ}RII-B2 receptor and suggested it as a receptor for OxLDL. However, it does not appear to play an important functional role in the uptake of OxLDL by mouse peritoneal macrophages. Studies by Endemann *et al.* (35) and studies by Elke Ottnad in this laboratory (unpublished data) failed to show any inhibition of OxLDL uptake into mouse macrophages by a blocking antibody against the Fc_{γ}RII-B2 receptor. Furthermore, in the present studies, OxLDL failed to inhibit binding of opsonized RBCs and the blocking antibody against the Fc_{γ}RII-B2 receptor did not affect binding of OxRBC (Table 2).

Another membrane protein that binds OxLDL has been identified, again through expression cloning—namely, CD36 (35). Support for a functional role for CD36 came from studies demonstrating that a monoclonal antibody directed against it partially inhibited the binding and uptake of OxLDL by human macrophage-like THP-1 cells (35). Further studies will be needed to determine the extent to which binding and uptake of oxidatively damaged RBCs are attributable to each of the several candidate receptors, and *in vivo* studies will be needed to assess the quantitative importance of clearance of damaged RBCs by this newly described direct, antibody-independent uptake mechanism.

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