Opsonin-Independent Phagocytosis of Periodate-Treated Sheep Red Blood Cells by Macrophages

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The opsonin-independent recognition of periodate-treated sheep red blood cells (P-SRBC) by macrophages was studied by observation of phagocytosis and the mechanism of the recognition was compared with those for other particles. Thioglycollate-elicited guinea pig peritoneal macrophages time-dependently ingested sheep red blood cells (SRBC) treated with periodate, as well as immunoglobulin G-coated sheep red blood cells (IgG-SRBC), zymosan (Z), serum-treated zymosan (STZ) and latex beads. Trypsinization of macrophages decreased the ingestion of P-SRBC to under 50% of the value of untreated cells and virtually abolished the ingestion of Z and STZ at a concentration that did not inhibit the phagocytosis of IgG-SRBC and latex beads. The decreased activities for P-SRBC recovered to 80% of the control value on incubation of the macrophages for 5 h at 37 °C. The restoration of the ability to ingest P-SRBC following trypsin digestion was inhibited by cycloheximide and tunicamycin. Pretreatment of macrophages with ConA dose-dependently decreased ingestion of P-SRBC to under 50% of the original level, but did not decrease the internalization of IgG-SRBC and STZ. Rabbit anti-guinea pig peritoneal macrophage IgG abolished the ingestion of Z and caused marked and slight decreases of phagocytosis of IgG-SRBC and P-SRBC, respectively.

These results indicated that the site of recognition of P-SRBC on the macrophage cell membrane could be composed of glycoproteins and is distinct from the receptors for C3b and Z. The role of plasma membrane components on macrophages in the action of opsonin-independent recognition is discussed in relation to the opsonin-mediated recognitions.

Keywords—macrophage; phagocytosis; opsonin-independent recognition; periodate-treated red blood cell; trypsin digestion; anti-macrophage IgG; zymosan; concanavalin A

Introduction

It is known that macrophages possess membrane receptors for Fc portion of immunoglobulin G (IgG) and C3 component of the complement system, which enable the cells to recognize and ingest foreign particles.1) Without these receptors, phagocytic recognition can still take place by direct binding of foreign substances such as bacteria, latex beads, zymosan (Z) and chemically altered red blood cells to the membrane of phagocytic cells.2) It is important to know the chemical principles of opsonin-free recognition by phagocytes in order to understand the self-defense mechanisms against invasion by foreign substances, especially before initiation of the immunological responses. Previously, Rabinovitch3) reported that red blood cells modified by exposure to periodate exhibit typical rosette formation at the macrophage cell surface and recently, Capo et al.4) described the opsonin-independent phagocytosis of aldehyde-treated erythrocytes by macrophages, though the chemical properties of the binding site were not elucidated clearly.

The present studies were performed to clarify the mechanisms which govern the discriminatory qualities of macrophages in opsonin-independent recognition. For this
purpose, we have examined the effect of periodate treatment of sheep red blood cells (SRBC) on the phagocytosis by and binding to guinea pig peritoneal macrophages, and the properties of the recognition site for periodate-treated SRBC (P-SRBC) were studied and compared with those of the sites for other particles such as IgG-coated SRBC (IgG-SRBC), Z, serum-treated zymosan (STZ) and latex beads. It was indicated that P-SRBC could be ingested by guinea pig macrophages, as could the other particles tested, and the site for recognition of P-SRBC was distinct from the sites for other particles in terms of sensitivity to trypsin digestion, and responses to treatment with concanavalin A (ConA) and sensitization with anti-macrophage rabbit IgG antibodies. The chemical properties of the recognition site for P-SRBC are discussed in relation to those of the receptors for IgG, C3b and Z.

Materials and Methods

Macrophages—Thioglycollate-elicited peritoneal macrophages were obtained as described by Griffin et al.5) Male or female Hartley guinea pigs weighing about 300 g were injected intraperitoneally with 20 ml of Brewer’s thioglycollate medium and 4 d later, peritoneal exudate cells (PEC) were harvested with Dulbecco’s phosphate buffer. After disruption of contaminating erythrocytes with hypotonic buffer, cells were washed with Dulbecco’s phosphate buffer and suspended in Eagle’s minimum essential medium (MEM) to a concentration of $1 \times 10^8$ PEC/ml. Then 50 µl of the PEC cell suspension was placed in 10 mm flat-bottomed Linbro tissue culture wells in a final volume of 0.5 ml and the plate was incubated for 2 h at 37°C in a humid chamber under an atmosphere of 5% CO₂ to permit adherence of macrophages. After elimination of non-adherent cells, 0.5 ml of MEM was added and phagocytosis and rosette formation were examined. Over 95% of cells remained viable during the experiment as assessed by exclusion of 0.5% trypan blue.

Assay of Phagocytosis—Macrophage monolayers were incubated with $5 \times 10^8$ IgG-SRBC, P-SRBC, latex beads or Z or the same number of Z treated with guinea pig serum (STZ) in 0.5 ml of MEM, usually for 60 min at 37°C in a humid chamber under an atmosphere of 5% CO₂. After being rinsed four times with warmed MEM to remove free particles, the cells that had ingested particles were counted under a microscope. Phagocytic activities were determined by scoring macrophages incorporating one or more particles. At least 100 macrophages were examined, and the results were expressed as the percentage of particle-carrying cells.

Assay of Attachment—Suspensions of target particles, 0.05 ml were added to macrophage monolayers as mentioned above and incubated for 15 min at 37°C. After being washed with MEM, monolayers were examined for binding of particles under a microscope. Binding activities were calculated by scoring cells carrying one or more particles. At least 100 macrophages were examined.

IgG-SRBC—A 0.5% SRBC suspension in phosphate-buffered saline (PBS) 2 ml was sensitized with 0.22 mg (a subagglutinating dose) of rabbit IgG antibodies for 20 min at 37°C and after being washed with PBS, the resulting IgG-SRBC were resuspended in MEM at $1 \times 10^8$ cells/ml.

P-SRBC—SRBC were usually treated with 4 mm sodium metaperiodate in saline containing 10 mm acetate buffer, pH 5.5 for 20 min at 37°C in the dark. After thorough washing with saline, the P-SRBC were suspended in PBS at 10^9/ml and stored at 4°C. They were used within a week.

Z and STZ—Z suspended in PBS at 10 mg dry weight/ml was homogenized in a Teflon homogenizer, incubated for 60 min in boiling water, suspended in saline and stored at $-80°C$. For each experiment, the particles were washed with PBS three times by centrifugation and suspended at 5 mg/ml in MEM. STZ was prepared as follows; one ml of Z suspension (5 mg/ml saline) was incubated with 0.2 ml of fresh guinea pig serum in gelatin veronal-buffered saline for 30 min at 37°C. After being washed with PBS three times, STZ thus obtained was suspended in 1 ml of MEM.

Anti-SRBC Rabbit IgG Antibodies—Rabbits were immunized intravenously five times with 5 ml of 10% SRBC at intervals of 3 d and were bled 5 d after the last injection. The IgG fraction of antisera was prepared by precipitation with 50% saturation of ammonium sulfate followed by chromatography on a diethylaminoethyl (DEAE)-cellulose column equilibrated with 0.01 M phosphate buffer, pH 7.4. The unadsorbed fraction was collected and used as anti-SRBC IgG antibodies for sensitization.

Rabbit IgG-Antibodies against Guinea Pig Peritoneal Macrophages—Guinea pig peritoneal exudate cells were cultured for 2 h on a 100 mm tissue culture dish (Falcon no. 3003) and after removal of non-adherent cells the adherent cells were harvested with a rubber policeman and suspended in PBS. The cell suspensions were mixed with an equal volume of complete Freund’s adjuvant and used as an immunogen. A rabbit was immunized with four weekly injections of 3.75 mg each of the macrophage protein. The serum was obtained 10 d after the last injection and the IgG fraction of the antisera was isolated by precipitation with ammonium sulfate followed by chromatography on a DEAE-cellulose column. The IgG antibodies obtained were absorbed with SRBC three times to remove antibodies against SRBC.
Determination of Protein—Proteins were determined according to the method of Lowry et al. using bovine serum albumin as a standard.6)

Reagents—Cycloheximide, a product of Tanabe Pharmaceutical Industry, was dissolved in saline at 1 mg/ml and kept for up to a week at 4 °C. Tunicamycin and ConA were obtained from Sigma Chemicals and dissolved in 5 mm NaOH to 1 mg/ml and in saline to 10 μg/ml, respectively. The solutions were kept for up to a week at 4 °C. Phospholipase C was purchased from Sigma Chemicals and dissolved in saline at 10 units/ml for each experiment. Latex beads, 3.5 μm in diameter, were purchased from Dow, Indianapolis, U.S.A., and after being washed with PBS three times, the particles were suspended in PBS at 10⁹/ml.

Results

Phagocytosis of P-SRBC

Rabinovitch reported that red blood cells modified by exposure to periodate were bound to the macrophage cell surface in the absence of opsonin.3) First, we examined the effect of periodate treatment of SRBC on the phagocytosis by macrophages under various conditions. SRBC were treated with various concentrations of sodium metaperiodate at 37 °C in saline containing 10 mm acetate buffer, pH 5.5, for 20 min and after being washed with saline, the P-SRBC obtained were added to macrophage monolayers in a ratio of 10:1. After 60 min of incubation at 37 °C in MEM, monolayers were washed thoroughly to remove free particles, and phagocytosis was examined under a microscope. As can be seen in Fig. 1, guinea pig peritoneal macrophages ingested P-SRBC; over 90% of the cells ingested one or more particles when SRBC were treated with 10 mm periodate. The effect of the ratio of the number of target particles to that of phagocytes was estimated and it was found that at the ratio of 10:1, about 90% of macrophages ingest one or more P-SRBC.

Then, the time course of ingestion of P-SRBC was followed in comparison with that mediated by Fc receptors and that by C3b, assessed in terms of ingestion of STZ as described previously.7) SRBC treated with 5 mm periodate were ingested avidly by macrophages, as were

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Fig. 1. Ingestion of Periodate-Treated Sheep Red Blood Cells (P-SRBC) by Macrophages
SRBC (4 × 10⁹) were treated with various concentrations of periodate in 4 ml of 20 mm acetate-buffered saline, pH 5.5 for 20 min at 37 °C in the dark. After thorough washing with PBS, the P-SRBC were suspended in MEM at 10⁶ cells/ml. P-SRBC (5 × 10⁶) cells were added to macrophage monolayers and incubated for 60 min in a CO₂ incubator as described in Materials and Methods. After washing away of free particles, phagocytosis was estimated under a microscope. Results are expressed as percentage of cells carrying one or more particles. Values are mean ± S.D. of triplicate determinants.

Fig. 2. Phagocytic Activities of Macrophages towards Various Particles
Macrophage monolayers were incubated with 5 × 10⁶ IgG-SRBC, P-SRBC, latex beads, or Z in 0.5 ml of MEM for various intervals at 37 °C in a 5% CO₂ atmosphere. After elimination of free particles, phagocytic activities were estimated. Results are expressed as described in the legend to Fig. 1. Values are mean ± S.D. of three to five determinations. IgG-SRBC (n = 3), ○; P-SRBC (n = 5), □; Z (n = 4), △; latex beads (n = 3), ●.
the other particles, IgG-SRBC, Z, STZ and latex beads (Fig. 2).

**Effect of Trypsin Digestion on the Phagocytic Activities**

To identify the chemical principle of opsonin-independent recognition of P-SRBC, macrophage monolayers were treated with trypsin at 37 °C, then washed with PBS, and the phagocytic activities were estimated. When the monolayers were treated with 1 mg/ml of trypsin, phagocytosis of P-SRBC decreased time-dependently to under 50% of the original value, while ingestion of IgG-SRBC was not affected significantly until 90 min after the start of the treatment (data not shown). Next, macrophage monolayers were treated with various concentrations of trypsin for 60 min and washed with PBS, and the phagocytic activities for P-SRBC, IgG-SRBC, STZ and Z were estimated. As can be seen in Fig. 3, ingestion of P-SRBC decreased dose-dependently on trypsinization of the cells, and it was found that the binding sites for Z and STZ were more sensitive to trypsin digestion, as the activities towards these particles were abolished by treatment with 0.1 and 0.5 mg/ml of trypsin, respectively. On the other hand, ingestions of IgG-SRBC and latex beads were not affected significantly even after treatment with up to 2.0 mg/ml of trypsin. These results suggested that the recognition site for P-SRBC is distinct from those for other particles. The phenomenon of phagocytosis can be divided into two phases, attachment and subsequent internalization. Thus, to determine whether trypsinization of macrophage monolayers affects the adherence of P-SRBC or the subsequent internalization, adherence of the particles was examined. As can be seen in Fig. 4, after trypsinization, attachment of P-SRBC to macrophages decreased to under 50% of the original value. Ögmundsdottir et al. reported that *C. parvum* binds to mouse peritoneal exudate cells in the absence of opsonin, and trypsin digestion of the phagocytes caused a decreased binding of the bacteria, while the activity was restored to the normal value after
incubation at 37 °C following trypsin digestion.8) Thus, to elucidate the chemical properties of the binding site for P-SRBC, trypsinized macrophage monolayers were washed with PBS and incubated at 37 °C in MEM, and phagocytic activity was followed. As shown in Fig. 5A, the activity of trypsinized macrophages for P-SRBC was restored to 80% of the original value by incubation for 5 h and the restoration of the activity was inhibited by the addition of cycloheximide or tunicamycin. When macrophage monolayers were pretreated with phospholipase c and then treated with trypsin, ingestion of P-SRBC decreased to under 30% of the original value and no restoration was observed during 5 h of incubation. These results suggested that the recognition site for P-SRBC on macrophage cell membrane is composed of proteins, glycosides and phospholipids. Phagocytic activities of trypsinized macrophages for STZ recovered similarly to about 50% of the original value after 5 h of incubation and the recovery was also inhibited by cycloheximide and tunicamycin (Fig. 5B). On the other hand, after trypsinization, the phagocytic activity for Z increased only slightly, and the recovery was inhibited by cycloheximide and tunicamycin (Fig. 5C). Untreated macrophage maintained constant values of phagocytic activities for these targets during 6 h of incubation, even in the presence of cycloheximide or tunicamycin (data not shown).

Effects of ConA

As mentioned above, the macrophage binding site for P-SRBC could be composed of glycoproteins. To examine whether glycoproteins participate in the recognition of P-SRBC, the effect of ConA on the phagocytosis was examined. The macrophage monolayers were treated with 10 μg/ml of ConA for various intervals then washed with PBS, and the phagocytic activities were determined. Treatment of macrophages with ConA decreased the ingestion of P-SRBC time dependently whereas phagocytosis of IgG-SRBC was not changed until 90 min after the start of treatment (data not shown). Next, macrophage monolayers were treated with various concentrations of ConA in MEM for 30 min at 37 °C and then washed with PBS, and the phagocytic activities for various particles were determined. Ingestion of P-
SRBC was decreased markedly by pretreatment of macrophages with increasing amounts of ConA, while Fc- and C3-mediated phagocytosis were not affected significantly (Fig. 6). In contrast, increased ingestion of Z was observed in the region of low concentration of ConA.

**Effect of Anti-macrophage IgG-Antibodies**

To examine whether the site for recognition of P-SRBC could be discriminated immunologically from the sites for other particles, macrophage monolayers were treated with rabbit IgG-antibodies against guinea pig peritoneal macrophages and the phagocytic activity for P-SRBC was determined and compared with the activities for various other particles. As can be seen in Fig. 7, after sensitization with the antibodies, the phagocytic activity for Z decreased dependently on the amount of antibodies and was abolished at the highest concentration of antibodies tested. The activities for IgG-SRBC and STZ decreased to under 20% and 60% of the original values, respectively, with the same amount of antibodies. In contrast, phagocytosis of P-SRBC decreased only slightly even at the highest concentration of IgG used in this experiment.

**Discussion**

The opsonin-independent recognition and ingestion of particles by phagocytes are essential for elimination of senescent self-materials and foreign substances, especially before the induction of immune responses. Rabinovitch has already described the attachment of P-SRBC to macrophages, but the experimental conditions for treatment of SRBC with periodate and the properties of the binding site on phagocytes were not studied in detail.3) In this paper, we have demonstrated that when SRBC were treated with periodate in acetate buffer, the resultant P-SRBC could be ingested by macrophages in proportion to the
concentration of periodate used for modification. It was found that the binding sites on macrophage cells were sensitive to trypsin digestion and that after elimination of the trypsin, phagocytic activity was restored to 80% of the original value by incubation for 5 h at 37°C. Furthermore, the restoration of the activity was inhibited by cycloheximide and tunicamycin. These results imply that the binding sites for P-SRBC on macrophage cells involve glycoproteins. This conclusion was supported by the observation that ingestion of P-SRBC was inhibited by pretreatment of macrophages with ConA. Pretreatment of macrophages with phospholipase C before trypsinization decreased the activities of the cells markedly, but the decrease was not observed after treatment with the enzyme alone. It is possible that phospholipid is essential for assembly of the binding site on macrophage cell surface.

The binding site for P-SRBC is distinct from the sites for other particles in several respects. Namely, IgG receptors are insensitive to trypsin digestion and ingestion of latex beads is not affected by trypsin digestion or anti-macrophage IgG. On the other hand, ingestion of Z was decreased markedly by treatment with a low concentration of trypsin and the activity was not restored significantly by incubation at 37°C. In contrast, C3b receptors were inactivated by trypsin digestion and restored by subsequent incubation at 37°C as binding sites for P-SRBC, but were distinct from the sites for P-SRBC in that preincubation of macrophages with increasing concentrations of ConA decreased the ingestion of P-SRBC markedly while ingestion of STZ was not affected significantly by the same treatment. Concerning the effect of pretreatment of macrophages with phytohemagglutinins, wheat germ agglutinin (WGA) and ricinus communis agglutinin-1 (RCA-1) decreased the ingestion of P-SRBC as did ConA, while peanut agglutinin (PNA), soybean agglutinin (SBA), maclura pomifera agglutinin (MPA), dolichos biflorus agglutinin (DBA) and bandeiraea simplicifolia agglutinin-1 (BSA-1) has no effect on the ingestion of P-SRBC (data not shown). These results further support the conclusion that glycoproteins participate in the ingestion of P-SRBC by macrophages. After treatment of macrophages with these lectins, attachment to or ingestion by macrophages of native SRBC was not observed.

Concerning opsonin-independent phagocytosis, Capo et al. reported that treatment of red blood cells with aldehydes allowed the cells to be ingested by rat macrophages, and they suggested that aldehyde-mediated phagocytosis of SRBC was due to both increased local rigidity and modified hydrophobicity of the cell surface induced by cross-linking of the cell membrane structures. In 1978, Czop et al. reported that human monocytes ingest Z and heterologous red blood cells without exogenous opsonin and demonstrated that these recognition sites were sensitive to trypsin digestion. In the present work the binding site for P-SRBC was discriminated clearly from the site for Z as follows. First, after treatment of macrophages with ConA, phagocytosis of P-SRBC but not of Z decreased dose-dependently; second, sensitization of macrophages with antibodies caused marked and slight decreases of the ingestion of Z and P-SRBC, respectively; third, the site for Z was destroyed by treatment with a low concentration of trypsin, in contrast to the site for P-SRBC, and the activity was not restored during 5 h of incubation. Treatment of macrophages with increasing concentrations of trypsin reduced the phagocytosis of P-SRBC to one-half of the original value while the activity for Z was lost entirely (Fig. 3). It is possible that macrophages possess two kinds of binding site for P-SRBC, and one of which is insensitive to trypsin digestion. Treatment of SRBC with periodate could induce chemical modification of glycoside chains, lipids and proteins on the cell membranes, resulting in recognition by macrophages, though the nature of the modifications is not known chemically. The relationship of phagocytosis to the stimulation of biological responses such as superoxide anion release and lysosomal enzyme release needs to be examined. The mechanisms of the opsonin-independent recognition of particles by macrophages, especially the protein chemical properties of the binding sites for P-SRBC and Z on the phagocytes, are under investigation.
References


