SELECTIVE INHIBITION BY THE SULFHYDRYL REAGENT MALEIMIDE OF ZYMOSAN PARTICLE PHAGOCYTOSIS BY NEUTROPHILS

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1. Introduction

It has been suggested that sulfhydryl groups may play an important role in polymorphonuclear neutrophil functions on the basis of chemical modification with sulfhydryl binding reagents [1–7]. However, since the reagents used were rapidly penetrating agents such as *N*-ethylmaleimide (NEM), different SH groups could have been blocked simultaneously under those modification conditions. During studies on chemical modification of neutrophils with maleimide derivatives we have observed that separate modification of the cell functions would be possible by using a reagent with suitable lipophilicity; i.e., with an appropriate penetrating rate [2]. Therefore, we have tried to modify neutrophils using 3 maleimide derivatives with different degrees of hydrophilicity and lipophilicity. Phagocytosis of zymosan particles was selectively inhibited by the maleimide modification.

2. Materials and methods

Guinea-pig neutrophils were isolated from glycogen-induced peritoneal exudates by hypotonic lysis of erythrocytes and 2 subsequent washings with phosphate-buffered saline (PBS, pH 7.4) without divalent cations [PBS(−)] as in [8]. Such preparations contained >97% neutrophils.

Chemical modification of neutrophils was done by incubating 2×10^7 cells/ml with 75–100 μM maleimide derivatives at 0°C for a defined time in PBS. The reaction was stopped by the addition of a 2-fold molar excess of cysteine followed by washings with a large excess (~10-fold) of PBS. Control unmodified neutrophils were treated under the same conditions except that water was used instead of maleimide derivatives solution. Samples were used for assays of chemotaxis, adhesion, phagocytosis, superoxide anion generation, lactate production, myosin ATPase and polymerizable actin.

Chemotaxis was assayed by incubating a modified Boyden chamber with a Sartorius membrane filter (pore size 3 μm) containing 2 ml supernatant from a sonicated PBS suspension of *Escherichia coli* in the lower compartment and 1 ml neutrophil suspension (5×10^6 cells/ml in PBS) in the upper compartment at 37°C for 90 min and by measuring the distance from the top of the filter to the furthest 2 cells at the same focal plane as in [8]. The results were expressed as an average migration distance/10 random fields in duplicate at a magnification of 600× in separate experiments.

Cell adhesion was assayed by estimating cells adhering to glass tubes in terms of cell protein concentration as in [9]. Aliquots (0.4 ml) from a PBS suspension of cells (5×10^6/ml), supplemented with 20% of heated guinea-pig serum (56°C, 30 min; complement-inactivated serum) were placed on glass tube (13 × 120 mm). The cells became attached to glass by incubating for 1 h at 37°C establishing monolayers. The glass tubes were then rinsed 3 times with 1 ml PBS to remove non-adhering cells. The quantity of adherent cells remaining on each tube was measured on a 0.5 ml sample as in [10] after an addition of 2 ml PBS and subsequent sonication in ice for 1 min, using a Supersonic vibrator (Tominaga Works Ltd.) and expressed as μg protein/10^6 cells; 10^6 cells contained 74 ± 3.5 μg protein. A control without cells indicated that serum proteins hardly stuck to glass tube after 3 washings under our conditions.

Phagocytosis assay was carried out by incubating

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2×10^6 cells with serum-opsonized zymosan particles (cell/zymosan, 1:5) in PBS at 37°C for 30 min in 1.0 ml total vol. with constant shaking unless otherwise noted. Tubes were then placed in an ice-cold bath to stop the reaction. Aliquots (0.5 ml) of the mixture were fixed by addition of 0.05 ml 25% (w/v) glutaraldehyde and examined by phase-contrast microscopy as in [11]. Opsonized zymosan was obtained by the incubation of 10 mg zymosan particles with 1.25 ml fresh homologous serum at 37°C for 30 min with constant shaking. After washing by centrifugation at $1870 \times g$ for 5 min, opsonized zymosan was suspended in 1 ml PBS. Phagocytic index and attachment index were defined as % positive ingestion multiplied by av. no. ingested particles/cell, and % positive attachment multiplied by av. no. attached particles/cell, respectively.

Myosin ATPase activity of neutrophils was determined as in [12]. Modified neutrophils were washed with PBS, then with 0.34 M sucrose/10 mM Tris–maleate buffer (pH 7.4) and suspended at 10^8 cells/ml in sucrose/Tris–maleate buffer. The suspension was sonicated in ice for 30 s with intermittent pulses at 168 W (Supersonic vibrator, Tominaga Works Ltd.). Determination of EDTA-activated ATPase was carried out in 0.5 M KCl/20 mM histidine buffer (pH 7.6) in the presence of 5 mM EDTA at 37°C for 1.5 h, using 0.1 ml sonicated suspension, in 1.1 ml total vol. The reaction was stopped by an addition of 0.5 ml 20% trichloroacetic acid. After centrifugation at $1870 \times g$ for 5 min, 1.0 ml supernatant was used for measuring the amount of P_i released.

Superoxide anion formation during phagocytosis was measured by a spectrophotometric assay of superoxide dimutase-inhibitable cytochrome *c* reduction [13]. One assay medium contained 0.5 ml neutrophils (2×10^7/ml in PBS), 0.3 ml 400 μM cytochrome *c* in PBS, 0.09 ml 500 μg superoxide dismutase/ml PBS and 0.4 ml PBS, whereas the other assay medium contained PBS instead of superoxide dismutase solution. The reaction was initiated with an addition of 0.2 ml suspension of opsonized zymosan particles in PBS. After 20 min incubation at 37°C, the assay mixture was promptly cleared by centrifugation at $1870 \times g$ for 5 min at 4°C. After an addition of the same volume of water, the absorbance of the supernatant was determined spectrophotometrically and the concentration of cytochrome *c* reduced was determined using the equation $E_{550\ nm} = 2.1 \times 10^4\ M^{-1}\ cm^{-1}$ according to [14].

Lactate was assayed with the Biochemica test combination lactate–UV test as in [15]. Neutrophils (5×10^6) were incubated with opsonized zymosan (particle/cell, 1:1) at 37°C in 1.0 ml of PBS containing 10 μmol glucose. After addition of 1 ml ice-cold PBS in 1 h, the incubation mixtures were centrifuged at $1870 \times g$ for 5 min at 4°C and the supernatant fluid (0.5 ml) were used for the lactate assay.

The release of lysozyme from neutrophils was examined by incubating 2×10^7 cells with 10^8 opsonized zymosan particles for 60 min at 37°C in 1 ml total vol. PBS with shaking. After incubation, the tubes were placed in an ice bath and then centrifuged at $1870 \times g$ for 5 min. The enzyme activity in the supernatant was measured. The total enzyme activity was determined using the supernatant after an incubation of 2×10^7 neutrophils/ml PBS at 37°C for 60 min and then adding 0.02 ml 10% Triton X-100, followed by centrifugation. Lysozyme was assayed by the rate of the increase of transmittance at 540 nm for 5 min at 25°C. The reaction mixture contained 0.5 ml 0.15 M sodium phosphate buffer (pH 6.2), 0.5 ml 0.3 M NaCl, 1.5 ml 0.2 mg *Micrococcus lysodeikticus*/ml and 0.035 ml 10% Triton X-100. The reaction was started with 0.2 ml supernatant. The enzyme activity was determined according to the calibration curve in terms of μg egg white lysozyme. The total activity of lysozyme of neutrophils was 2.57 ± 0.16 μg/10^7 cells.

Polymerizable actin in neutrophils was measured essentially as in [16].

Maleimide and NEM were purchased from Nakarai Chemicals Ltd and Wako Pure Chemical Industries Ltd, respectively. Showdomycin was donated by the Shionogi Research Laboratory. Zymosan A (from *Saccharomyces cerevisiae* yeast), superoxide dismutase (type I from bovine blood), cytochrome *c* (type VI from horse heart), *M. lysodeikticus* (dried cells), lysozyme (grade I from chicken egg white) and cytochalasin B were obtained from Sigma Chemical Co. (St Louis MO). Polystyrene latex (0.81 μm diam.) was obtained from Difco Lab., (Detroit MI). Biochemica test combination lactate–UV test was obtained from Boehringer GmbH (Mannheim). Sartorius membranfilter (type SM 11302) was obtained from Sartorius-Membranfilter GmbH (Göttingen).

3. Results and discussion

As seen in table 1, NEM, the most lipophilic (i.e.,

Table 1
Effect of maleimide derivatives on chemotaxis, adhesion and phagocytosis of neutrophils

Reagents	Modification time (min)	Chemotaxis (μm)	Adhesion (μg)	Phagocytic index
N-ethylmaleimide	0	52 ± 3	67 ± 3	123 ± 11
75 μM	0.5	31 ± 4	32 ± 6	42 ± 3
(n = 6)	1	24 ± 2	15 ± 3	27 ± 4
	2	21 ± 2	13 ± 1	20 ± 2
	3	17 ± 1	12 ± 1	18 ± 3
Maleimide	0	48 ± 2	69 ± 6	141 ± 9
75 μM	2	41 ± 4	–	105 ± 10
(n = 6–8)	3	43 ± 3	68 ± 4	86 ± 9
	4	41 ± 2	70 ± 6	70 ± 7
	5	40 ± 3	69 ± 5	55 ± 6
	6	38 ± 2	69 ± 7	36 ± 5
Showdomycin	0	56 ± 3	65 ± 6	93 ± 10
100 μM	3	53 ± 3	63 ± 4	80 ± 3
(n = 3)	30	49 ± 4	58 ± 5	81 ± 4

Polymorphonuclear neutrophils were chemically modified with maleimide derivatives at an indicated concentration at 0°C for the given times. Data are the mean ± SE from the number of separate experiments given in parentheses

the most rapidly penetrating [2]) among reagents examined, inhibited markedly chemotaxis, adhesion and phagocytosis during the very short modification times (30 s), suggesting the random blocking of sulfhydryl groups involved in these functions. On the other hand, showdomycin, the most hydrophilic (i.e., the very slowly penetrating [2]) did not inhibit these functions at all even by 30 min modification, suggesting that sulfhydryl groups involved in these functions were not exposed on the cell surface. Maleimide which has the intermediate lipophilicity between 2 reagents [2], inhibited only phagocytosis without essential inhibition of chemotaxis and adhesion. The involvement of contractile elements in phagocytosis by phagocytes has been reported [17–22]. Sulfhydryl reagents adversely affect not only myosin ATPase [23–27] but also actin polymerizability of skeletal muscle [28,29]. Then it was examined whether the inhibition of phagocytosis was based on the impairment of these contractile proteins by maleimide modification. The EDTA-ATPase activity in neutrophil homogenate was determined as an enzymatic marker for myosin as in [12]. Polymerizability of actin was examined by analyzing the amount of actin sedimented after the attempt at polymerization with KCl and/or skeletal myosin using acrylamide gel electrophoresis. Actin polymerizability was resistant to maleimide derivative modification and not inhibited even by 60 min modification of 1 mM NEM where EDTA–ATPase and other above functions were completely inhibited. This result would be explained by the observations that NEM reacts only with 2 fast-reacting SH groups/mol actin whose modification neither prevents polymerization of G-actin nor causes depolymerization of F-actin, but not with 4 remaining SH groups whose modification do affect the polymerization and depolymerization of muscle actin [28,29]. Maleimide did not inhibit the EDTA–ATPase activity at least during 6 min modification (table 2), suggesting that the inhibition of phagocytosis by maleimide is not due to the modification of contractile proteins in the cytoplasm. Phagocytosis or exposure to an appropriate soluble or particulate stimulus, stimulates glycolysis [30] and superoxide production [31,32] of neutrophils and causes the extracellular release of granule enzymes from neutrophils [33]. The source of energy for phagocytosis is thought to be ATP generated by glycolysis and glycolytic inhibitors, such as iodoacetate, are thought to inhibit phagocytosis [30]. The inhibition of superoxide formation by sulfhydryl reagent such as NEM has been reported [4–7]. Therefore, the effect of maleimide modification on these

Table 2
Effect of maleimide modification on contractile protein, stimulated O_2^- generation, lactate production and granule enzyme release

Modification time (min)	EDTA-activated ATPase activity (nmol P_i . min^{-1} . 10^9 $cells^{-1}$) (n = 4)	Stimulated O_2^- generation (nmol cytochrome c reduced . 20 min^{-1} . 10^7 $cells^{-1}$) (n = 4)	Lactate produced (μmol . h^{-1} . 5 X 10^7 $cells^{-1}$) (n = 3)	Lysozyme released (μg) (n = 2)
0	436 ± 34	74 ± 4	4.2 ± 0.3	1.26 ± 0
3	418 ± 39	73 ± 2	4.0 ± 0.3	–
4	432 ± 45	74 ± 2	4.0 ± 0.3	–
5	378 ± 39	75 ± 2	4.1 ± 0.1	1.19 ± 0
6	413 ± 39	75 ± 4	4.0 ± 0.4	1.23 ± 0.05

Neutrophils were modified with 75 μM maleimide at 0°C for indicated times. Values indicate the mean ± SE or average deviation

Table 3
Effect of maleimide modification on phagocytosis and attachment of particles by and to neutrophils

Particles	Cell/ particle ratio	Cytochalasin B	Modification time (min)	Phagocytosis of particles by neutrophils			Attachment of particles to neutrophils		
				%	No./ neutrophil	Index	%	No./ neutrophil	Index
Opsonized zymosan									
n = 4	1:5	–	0	66 ± 3	1.8 ± 0.2	122 ± 17	12 ± 2	0.2 ± 0.3	2 ± 0.6
			6	34 ± 5	0.9 ± 0.2	31 ± 11	15 ± 2	0.3 ± 0.03	4 ± 1
n = 4	1:5	+	0	34 ± 2	0.5 ± 0.03	18 ± 2	41 ± 1	0.8 ± 0.04	32 ± 1
			6	20 ± 4	0.3 ± 0.08	6 ± 3	40 ± 3	0.8 ± 0.07	30 ± 6
n = 4	1:50	–	0	86 ± 1	5.6 ± 0.3	478 ± 27	79 ± 4	3 ± 0.2	244 ± 30
			6	64 ± 2	3.4 ± 0.3	221 ± 22	79 ± 3	3.9 ± 0.3	305 ± 40
Nonopsonized zymosan									
n = 4	1:25	–	0	82 ± 1	3.1 ± 0.2	253 ± 13			
			6	50 ± 2	1.4 ± 0.1	70 ± 6			
Latex									
n = 2	1:50	–	0	90 ± 9	–	–			
			6	90 ± 8	–	–			

Data represent the mean ± SE or average deviation

Neutrophils were modified by incubating 2 X 10^7/ml neutrophils with 75 μM maleimide in PBS for 6 min at 0°C. The reaction was terminated on addition of 150 μM cysteine. Modified neutrophils were suspended in PBS, at 2 X 10^7/ml, after washing with a large excess of PBS. Control neutrophils (0 min) were treated under the same conditions except that water was used instead of maleimide solution. Phagocytosis of opsonized zymosan was carried out by an incubation of 2 X 10^6 cells/ml with serum-opsonized zymosan in an indicated cell to particle ratio. Phagocytosis in the presence of cytochalasin B was performed after preincubation with 6 μg cytochalasin B/ml at 37°C for 10 min. Phagocytosis of non-opsonized zymosan by neutrophils was assayed by incubating cells in a pellet with zymosan particles at 37°C for 30 min without agitation after centrifugation at 400 X g for 5 min at room temperature of an incubation mixture containing 2 X 10^6 neutrophils and 5 X 10^7 non-opsonized particles in a total volume of 1 ml PBS. Phagocytosis of latex particles was determined by an incubation of 2 X 10^6 neutrophils with 10^8 latex particles (counted microscopically) at 37°C for 30 min in 1 ml total vol. PBS

functions was studied. Neither aerobic lactate production, O_2^- production nor extracellular lysozyme release was affected by a chemical modification (table 2), suggesting no impairment of glycolytic system in the cytoplasm, O_2^--generating system in plasmalemma and degranulation process under this condition. These findings indicate the possibly selective inhibition by maleimide of phagocytosis by neutrophils. Then, the effect of maleimide modification on natural phagocytosis, a phagocytosis by neutrophils of latex beads, was examined. These do not bind to physiologically relevant receptors but to surface membrane through non-specific hydrophobic interaction and to be readily internalized [11]. Maleimide modification resulted in no inhibition of phagocytosis of polystyrene latex (table 3), suggesting that maleimide modification is not random but specific to zymosan phagocytosis. Next, whether an attachment or ingestion step in phagocytosis of opsonized zymosan is inhibited, was examined. When neutrophils were exposed to opsonized zymosan particles in the presence of cytochalasin B, which interferes with phagocytosis but not attachment of particles to the membrane receptors [17,34], particles were hardly ingested but remained adherent to the cell surface in modified cells as well as in intact cells. The incubation of neutrophils with 50-fold excess of opsonized zymosan as in a natural phagocytosis also showed the intactness of particle attachment in spite of a marked inhibition of ingestion. In [35], human neutrophils ingested not only C_3-coated zymosan but also non-opsonized zymosan if forced into close contact with these particles by physical means, such as centrifugation and suggested that zymosan receptor on neutrophils mediate phagocytosis and probably, C_3 receptors only mediate binding of particles to neutrophils, a prerequisite for the interaction with the zymosan receptors. When centrifuged with cells in a pellet, non-opsonized zymosan was considerably ingested in intact neutrophils. But this phagocytosis was also inhibited by a maleimide modification. Therefore, these results suggest the specific modification of 'zymosan-receptor-mediated' phagocytosis sites by maleimide without affecting other neutrophil functions.

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