THE REQUIREMENT FOR MEMBRANE SIALIC ACID IN THE STIMULATION OF SUPEROXIDE PRODUCTION DURING PHAGOCYTOSIS BY HUMAN POLYMORPHONUCLEAR LEUKOCYTES*

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Phagocytosis of particles by polymorphonuclear leukocytes (PMN)[1] is accompanied by a burst of oxidative metabolic changes. These include increased oxygen consumption (1), hexose monophosphate shunt (HMS) activity (1), and hydrogen peroxide (H_2O_2) (2) and superoxide (O_2^-) (3) production. Despite intensive investigation the mechanism by which this oxidative metabolism is stimulated during phagocytosis is still unclear. HMS activity and H_2O_2 production in resting phagocytes also can be stimulated by a variety of surface-active agents (4, 5), endotoxin (4), phospholipase C (6), concanavalin A (Con A) (7), or leukocyte antibodies in the absence of complement (8). Under these conditions no phagocytosis is necessary, suggesting that mere perturbation of plasma membrane is sufficient for the stimulation of HMS activity and H_2O_2 production. Recently Goldstein and co-workers provided evidence that the O_2^- generating system is associated with the plasma membrane of PMN (9).

Membrane sialic acid has been shown to play a significant role in cell physiology. It is apparently involved in the cell deformability (10). It also plays a dual role in the metabolism of circulating glycoproteins (11). Its presence on liver cell membrane is essential for the binding and transport of serum glycoproteins into the liver cell. In order for binding to occur, however, the glycoprotein must first be desialylated (11). In PMN its presence is necessary for the maximal stimulation of phagocytosis by the natural tetrapeptide, tuftsin (12). In this study, we report the requirement of membrane sialic acid for the stimulation of superoxide production during phagocytosis in human PMN.

Materials and Methods

Chemicals. [1-^{14}C] glucose and [6-^{14}C]glucose were obtained from Amersham/Searle Corp., Arlington Heights, Ill. Ferricytochrome C (horse heart, Type V1), nitroblue tetrazolium (NBT), Con A, α-methylglucose, superoxide dismutase[2] (SOD, bovine blood, E.C.1.15.1.1), trypsin (bovine

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[1] *Abbreviations used in this paper:* CGD, chronic granulomatous disease; Con A, concanavalin A; HMS, hexose monophosphate shunt; NBT, nitroblue tetrazolium; PMN, polymorphonuclear leukocytes; SOD, superoxide dismutase.

[2] Superoxide dismutase, 2,810 U/mg. Assayed per method of McCord and Fridovich. Trypsin, 10,000 BAEE U/mg. One BAEE unit = Δ OD_{253} of 0.001/min with *N*-benzoyl-L-arginine ethyl ester

pancreas, Type III), and neuraminidase (from Cl perfringens, Type V, E.C.3.2.1.18) were obtained from Sigma Chemical Co., St. Louis, Mo. Ficoll was obtained from Pharmacia Fine Chemicals, Inc., Uppsala, Sweden and sodium diatriazoate (Hypaque) from Winthrop Laboratories, New York. Polyvinyl toluene latex (2.02 μm in diameter) and polystyrene latex (0.79 μm in diameter) were obtained from the Dow Chemical Co., Indianapolis, Ind.

Isolation of Human PMN. Isolation of human PMN was performed as described previously (13, 14). Briefly, venous blood was obtained from normal individuals; leukocytes were isolated by dextran sedimentation of red blood cells, differential centrifugation, and NH_4Cl lysis of contaminating red cells. The leukocytes then were suspended in 10 ml of modified Hanks' solution (5 mM glucose) (15), and placed on top of a 10 ml Ficoll-Hypaque mixture and centrifuged at 400 *g* for 40 min at 20°C according to Böyum (16). The pellet which consisted of 97–99% PMN by differential counting was washed twice with modified Hanks' solution (5 mM glucose) and PMN were diluted to a final concentration of 1×10^7 PMN/ml unless otherwise indicated.

Quantification of Phagocytosis. Quantitative measurement of phagocytosis of latex particles was performed as described previously (13, 17) except that PMN monolayers were preincubated for 20 min with aliquots of approximately 0.4 ml of modified Hanks' solution (5 mM glucose) in the presence and absence of neuraminidase or trypsin.

Measurement of Sialic Acid. The effect of neuraminidase on the removal of sialic acid from human PMN was performed by measuring the release of sialic acid into the medium. The determination was done in both monolayers and cell suspensions. After incubation of the cells with neuraminidase at 37°C for 20 min, the supernates were pooled and evaporated to dryness using Rotavapor-R (Slawil, Switzerland). The residues were then dissolved in an appropriate volume of distilled water and 0.2-ml aliquots were assayed for free sialic acid using the thiobarbituric acid method of Warren (18). The amount of sialic acid released by neuraminidase was compared with the amount of sialic acid released from PMN by total acid hydrolysis (0.1 N H_2SO_4 for 1 h at 80°C) (19).

Measurement of Ferricytochrome C Reduction by Human PMN. Superoxide production by PMN was measured by SOD inhibitable reduction of ferricytochrome C according to Babior et. al. (3). 3 ml of PMN (1×10^7/ml) were preincubated with and without neuraminidase for 20 min at 37°C and then 50 nmol ferricytochrome C was added in a final vol of 4 ml of modified Hanks' solution (5 mM glucose). Experimental additions yield the following concentrations: 0.79 μm polystyrene latex particles, 0.17%; Con A, 100 μg/ml; and SOD, 200 U/ml. The reaction mixtures were stored on ice. 2 ml of each reaction mixture was kept at 0°C for use as a blank; the remainder was incubated for 30 min at 37°C. The reaction was terminated by placing the flasks in the ice. After centrifugation of blanks and incubated mixtures at 20,000 *g* for 10 min at 4°C, the supernate was passed through a 0.22 μm Millipore filter to remove all the remaining particles and cells. Ferricytochrome C reduction was determined by measuring the absorbance of the incubated supernate at 550 nm with a Beckman spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.), using the nonincubated blank as reference. The difference of absorbance at 550 nm in the absence and presence of SOD was taken as a measurement of superoxide. In some experiments neuraminidase was inactivated by heating in a water bath at 80°C for 30 min.

Measurement of NBT Reduction by Human PMN. Quantitative measurement of NBT reduction was performed as described by Baehner and Nathan (20). To 15 ml conical plastic centrifuge tubes the following was added: 0.4 ml (0.35 ml in tubes containing latex particles) modified Hanks' solution (5 mM glucose, 4 mM Mg^{++}), 0.1 ml of 10 mM KCN, 0.4 ml of 1% NBT in normal saline, and 0.05 ml of 3.3% polystyrene latex particles (0.79 μm) in tubes designated for phagocytosis. The mixture was preincubated in a shaking water bath at 37°C for 15 min. Then 0.1 ml of PMN (2.5×10^7/ml) preincubated with and without neuraminidase for 20 min at 37°C was added to each tube. The reaction was allowed to proceed for 15 min and then it was stopped by the addition of 10 ml of 0.5 N hydrochloric acid. The tubes were then centrifuged at 1,000 *g* at 4°C for 15 min. The supernate was aspirated, and the visible purple granule button was extracted for 10 min with 2 ml of pyridine in a boiling water bath under an exhaust hood. The tubes were centrifuged at 1,000 *g* for 10 min and a second extraction with 2 ml of pyridine was repeated. The extracts were combined

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as substrate at pH 7.6, 25°C. Neuraminidase, 0.5 U/mg protein. One unit liberates 1.0 μmol of *N*-acetyl-neuraminic acid per minute at pH 5.0, 37°C using *N*-acetyl neuramin-lactose as substrate.

and the optical density of the pyridine extract of a mixture of cells and NBT which had been incubated for only 10 s was determined and was used as a reagent blank. The results were expressed as OD changes/15 min/2.5 × 10^6 PMN.

Measurement of [^{14}C]Glucose Oxidation. Measurement of [^{14}]CO_2 production by human PMN was done as described previously (13). 1 × 10^7 cells were preincubated with and without neuraminidase for 20 min at 37°C. Radioactive substrate and particles were then added to stimulate phagocytosis in a final vol of 2 ml of modified Hanks' solution. The final concentration of particles was 0.17% by weight. For the study of [1-^{14}C]glucose oxidation, 0.1 μCi was added to each flask with a final glucose concentration of 5 mM. For the study of [6-^{14}C]glucose oxidation, 0.2 μCi and a final glucose concentration of 0.5 mM were used. When PMN were not present in the incubation medium, the radioactivity recovered from glucose was negligible. Nevertheless, this was done each time to insure that the [^{14}]CO_2 measured had been produced by PMN. In each experiment, duplication samples were measured and the results were averaged.

Statistical Significance. The statistical significance of each experiment was determined by Student's *t* test (21).

Results

Effect of Neuraminidase on Phagocytosis of Latex Particles. As shown in Table I, preincubation with neuraminidase (0.02–0.66 U/ml) had no effect on phagocytosis of latex particles by human PMN. In contrast, after preincubation with trypsin (1 mg/ml), phagocytosis of latex particles was inhibited 50%. Direct counting of cells on monolayers with a phase-contrast microscope revealed that there was no loss of PMN from the monolayers after preincubation with neuraminidase or trypsin. There was also no significant loss of cell viability after preincubation with these two enzymes as determined by trypan blue dye exclusion (about 95% viable). Our observation that neuraminidase had no effect on phagocytosis by human PMN is in agreement with that of Noseworthy et al. (19) and Constantopoulos and Najjar (12).

Release of Sialic Acid from Human PMN by Neuraminidase. Bacterial neuraminidase cleaves sialic acid at both 2–3′ and 2–6′ glycosidic bonds (12). It does not penetrate the cell membrane and releases sialic acid only from the surface membrane. As shown in Table II, neuraminidase at a concentration of 0.11 U/ml removed about 20% of total PMN sialic acid. It should be noted that

TABLE I
Effect of Neuraminidase and Trypsin on Phagocytosis of Latex Particles by Human PMN

	OD absorbance at 274 nm*	*P* value (vs. control)
Control	0.102 ± 0.008 (8)	
Neuraminidase		
0.02 U/ml	0.097 ± 0.006 (2)	>0.8
0.11 U/ml	0.103 ± 0.009 (8)	>0.9
0.66 U/ml	0.096 ± 0.001 (2)	>0.8
Trypsin		
0.1 mg/ml	0.074 ± 0.005 (6)	<0.025
1.0 mg/ml	0.046 ± 0.006 (6)	<0.001

* The results are expressed as mean ± standard error of the mean (OD absorbance at 274 nm) of the dioxane-extracted polyvinyl toluene latex particles. Number in parentheses indicates number of experiments.

TABLE II
Effect of Neuraminidase and Trypsin on the Release of Sialic Acid from Human PMN

	nmol/1 × 10^7 PMN*
Total cellular sialic acid‡	10.43 ± 1.20 (3)
Sialic acid release after:§	
Neuraminidase (0.11 U/ml)	
Cell suspensions	2.52 ± 0.51 (3)
Cell monolayers	2.36 ± 0.25 (3)
Trypsin (0.1 mg/ml)	
Cell suspensions	0 ± 0.00 (3)
Cell monolayers	0 ± 0.00 (3)

* Number in parentheses indicates number of experiments. The results are expressed as mean ± standard error of the mean.
‡ Acid hydrolysis in 0.1 N H_2SO_4 for 1 h at 80°C.
§ Incubation for 20 min at 37°C.

the amount of sialic acid removed by neuraminidase was the same whether the experiments were done with monolayers or with cell suspensions. On the other hand, trypsin at a concentration of 0.1 mg/ml did not remove any sialic acid from PMN. Heat inactivation of neuraminidase (80°C for 30 min) eliminated its enzymatic ability to remove sialic acid. Karnovsky and co-workers (19, 22) have demonstrated that only about one-third to one-half of the total cellular sialic acid may be removed from intact PMN by neuraminidase. Thus, in our experiments with 0.11 U/ml of neuraminidase, we removed 40–60% of the total neuraminidase-accessible sialic acid from human PMN.

Effect of Desialylation on SOD-Inhibitable Ferricytochrome C Reduction by Human PMN. SOD-inhibitable ferricytochrome C reduction has been used for the measurement of superoxide production (3). In this reaction, O_2^- serves as an electron donor for the reduction of ferricytochrome C. During phagocytosis, there is a burst of stimulation of O_2^- production by phagocytes (3). The effect of desialylation (removal of sialic acid from cell membrane by neuraminidase) on superoxide production by resting and phagocytosing human PMN was investigated. As shown in Table III, desialylation had no effect on O_2^- production by resting PMN. In contrast, the stimulation of O_2^- production by phagocytosing PMN was markedly inhibited (90%) by preincubation with neuraminidase. Heat inactivation of neuraminidase eliminated this effect.

Effect of Desialylation on NBT Reduction by Human PMN. During phagocytosis, NBT reduction is also stimulated. This reduction of NBT during phagocytosis is diminished 60% by SOD, implying that the majority of NBT reduction by PMN is due to superoxide (23). The effect of desialylation on NBT reduction by resting and phagocytosing human PMN was studied. As shown in Table IV, desialylation had no effect on NBT reduction by resting human PMN, while it inhibited the stimulation of NBT reduction by phagocytosing PMN. Again, heat inactivation of neuraminidase eliminated this effect.

Effect of Desialylation on the Con A-Stimulated Superoxide Production by Human PMN. Our results indicated that removal of membrane sialic acid by neuraminidase had no effect on phagocytosis of latex particles by human PMN,

TABLE III

*Effect of Neuraminidase on SOD-Inhibitable Ferricytochrome C Reduction by Human PMN**

	Resting cells		Phagocytosing cells		
	Control	Neuraminidase	Control	Neuraminidase	Heat-inactivated neuraminidase
OD absorbance at 550 nm	0.030 ± 0.009 (7)	0.034 ± 0.013 (7)	0.093 ± 0.10 (7)	0.041 ± 0.013 (7)	0.096 ± 0.011 (2)
P value (vs. control)		>0.8		<0.01	<0.8

* PMN were preincubated with and without 0.11 U/ml neuraminidase for 20 min at 37°C, then phagocytosis was induced with latex particles. SOD-inhibitable ferricytochrome C reduction was determined after 30 min. The results are expressed as mean ± standard error of the mean (OD absorbance at 550 nm/30 min/1.5 × 10^7 PMN). Number in parentheses indicates number of experiments.

TABLE IV

*Effect of Neuraminidase on NBT Reduction by Human PMN**

	Resting cells		Phagocytosing cells		
	Control	Neuraminidase	Control	Neuraminidase	Heat-inactivated neuraminidase
OD absorbance at 515 nm	0.046 ± 0.005 (4)	0.052 ± 0.008 (4)	0.280 ± 0.033 (4)	0.177 ± 0.046 (4)	0.273 ± 0.045 (3)
P value (vs. control)		>0.5		<0.02	>0.8

* PMN were preincubated with and without 0.11 U/ml neuraminidase for 20 min at 37°C, then phagocytosis was induced with latex particles. NBT reduction was determined after 15 min. The results are expressed as mean ± standard error of the mean (OD absorbance at 515 nm/15 min/2.5 × 10^6 PMN). Number in parentheses indicates number of experiments.

while it inhibited phagocytosis-associated stimulation of superoxide production. Therefore it was important to determine whether membrane sialic acid is also essential for the stimulation of O_2^- production by some mechanisms other than phagocytosis. Con A is a plant lectin. It has been shown that Con A stimulates HMS activity and H_2O_2 production by resting PMN (7, 24). As shown in Table V, Con A stimulated SOD-inhibitable ferricytochrome C reduction by human PMN. This stimulation was partially prevented by α-methylglucose, a competitive inhibitor of Con A binding. Pretreatment of PMN with neuraminidase completely prevented the stimulation of O_2^- production by Con A.

Effect of Desialylation on HMS Activity of Human PMN. Stimulation of HMS activity accompanies phagocytosis of particles by PMN (1). Since desialylation prevented the stimulation of O_2^- production associated with phagocytosis, its effect on HMS activity was also studied. As shown in Table VI, desialylation had no effect on [1-^{14}C]glucose and [6-^{14}C]glucose oxidation by either resting or phagocytosing PMN. Thus, desialylation had no effect on the phagocytosis-associated stimulation of the HMS activity.

Discussion

The results presented in this study indicate that surface sialic acid is essential for the stimulation of superoxide production by human PMN during phagocytosis or by Con A. Removal of sialic acid from cell membrane did not affect the capacity of PMN to ingest latex particles. This later finding is also true for the phagocytosis of bacteria (12, 19). Karnovsky and co-workers (19, 22) have demonstrated that only one-third to one-half of the total cellular sialic acid can

TABLE V
*Effect of Neuraminidase on Con A-Stimulated SOD-Inhibitable Ferricytochrome C Reduction by Human PMN**

	Control	Con A	Con A α-methylglucose	Con A (desialylated PMN)
OD absorbance at 550 mu	0.043 ± 0.016 (3)	0.107 ± 0.003 (3)	0.065 ± 0.009 (3)	0.035 ± 0.017 (3)
P value		<0.02 (vs. control)	<0.02 (vs. Con A)	<0.001 (vs. Con A)

* The results are expressed on the same basis as in Table III. Concentrations used: Con A, 100 μg/ml; α-methylglucose, 50 mM; and neuraminidase, 0.11 U/ml.

TABLE VI
*Effect of Neuraminidase on Glucose Oxidation by Human PMN**

	Resting cells	Phagocytosing cells
[1-^{14}C]glucose oxidation (5 mM)		
Control	428 ± 84 (6)	1,476 ± 285 (6)
Neuraminidase	501 ± 56 (6)	1,544 ± 266 (6)
P value (vs. control)	>0.6	>0.8
[6-^{14}C]glucose oxidation (0.5 mM)		
Control	300 ± 138 (4)	870 ± 228 (4)
Neuraminidase	374 ± 138 (4)	863 ± 258 (4)
P value (vs. control)	>0.6	>0.8

* The results are expressed as mean ± standard error of the mean (dpm) of the $[^{14}C]O_2$ released. PMN were incubated with and without neuraminidase (0.11 U/ml) for 20 min at 37°C. Phagocytosis was induced with latex particles. $[^{14}C]O_2$ release was determined after incubation for 1 h. The radioactivity added to each flask was 0.1 μCi for [1-^{14}C]glucose and 0.2 μCi for [6-^{14}C]glucose. Number in parentheses indicates the number of experiments.

be removed by neuraminidase. The total cellular sialic acid of PMN as determined by these investigators (22) is very similar to that of our reults. Thus, in this study we removed 40–60% of the neuraminidase-sensitive sialic acid. Since it is believed that neuraminidase does not penetrate the plasma membrane (22), it seems reasonable to conclude that the plasma membrane is actively involved in the production of superoxide or the activation of the superoxide-generating mechanism of PMN during phagocytosis.

In this study superoxide was measured by two different methods, namely, SOD-inhibitable ferricytochrome C reduction and NBT reduction. The addition of SOD makes the measurement specific for superoxide (3). In the experiment with NBT reduction, SOD was not used. However, Baehner et al. (23) have shown that during phagocytosis, O_2^- accounts for about 60% of NBT reduction, the rest being due to the presence of an as yet uncharacterized reductase. Therefore, if one assumes that the inhibition of NBT reduction during phagocytosis by desialylation is due to the inhibition of O_2^- production, then the degree of inhibition of O_2^- production during phagocytosis by desialylation, measured by both methods, is essentially the same (about 90%).

The sequence of oxidative metabolic changes associated with phagocytosis has been the subject of intensive investigations. Superoxide production is probably the initial step (3, 23). In the presence of hydrogen donor, these unstable intermediates (O_2^-) undergo dismutation, either spontaneously or enzymatically, producing H_2O_2 and oxygen (3, 23, 25). Whether H_2O_2 production is exclusively through O_2^- as an intermediate or can be generated directly from

oxygen is still an unsettled question (25). Stimulation of glucose oxidation via HMS and iodination of ingested particles are dependent on H_2O_2 (23). In this study we showed that desialylation markedly inhibited phagocytosis-associated O_2^- production, while the activation of HMS activity was not affected. In previous studies (14, 26) we have shown that inhibition of surface sulfhydryl groups inhibited phagocytosis-associated stimulation of HMS activity and H_2O_2 production, while phagocytosis of particles and superoxide production are not affected. Our observations are best explained by the hypothesis that superoxide production and HMS activation during phagocytosis are controlled by separate membrane mechanisms requiring intact surface sialic acid and sulfhydryl groups, respectively.

Leukocytes from patients with chronic granulomatous disease (CGD), while retaining normal phagocytic capacity, fail to stimulate HMS activity, and H_2O_2 (27) and O_2^- production (28) during phagocytosis. In addition, CGD granulocytes have very low resting oxidative metabolism (27). Baehner and co-workers (29, 30) have previously suggested that a deficiency of NADH oxidase in the soluble fraction of CGD granulocytes is responsible for the metabolic derangement in this disease. However, their results cannot be confirmed by other investigators (27), who have found normal NADH and NADPH oxidase activities in CGD granulocytes. Recent evidence (31–33) has repeatedly demonstrated that O_2^- and H_2O_2 production by CGD granulocyte lysate or particulate fraction are normal. A deficiency of O_2^- and H_2O_2 production by cell-free preparations from CGD granulocytes can be demonstrated only after granulocytes have been previously stimulated with particles (31, 32, 34). These observations strongly suggest the possibility of a defect in the activation during phagocytosis of the enzyme or enzymes of oxidative metabolism in CGD granulocytes. Whether this defect will be found to be on the plasma membrane or located intracellularly is not clear. Further study of the mechanisms by which membranes control granulocyte oxidative metabolism may help us to better understand the pathophysiology of CGD.

Summary

The effect of desialylation on phagocytosis of latex particles and oxidative metabolism of human polymorphonuclear leukocytes was studied. Removal of 20% total leukocyte sialic acid by bacterial neuraminidase had no effect on phagocytosis of latex particles and phagocytosis-associated activation of hexose monophosphate shunt in human polymorphonuclear leukocytes. In contrast, desialylation prevented the stimulation of superoxide production either by phagocytosis or by concanavalin A. It is concluded that membrane sialic acid is essential for the stimulation of superoxide production by human polymorphonuclear leukocytes.

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