Detection, Pathogenesis, and Prevention of Damage to Human Granulocytes Caused by Interaction with Nylon Wool Fiber

IMPLICATIONS FOR FILTRATION LEUKAPHERESIS

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ABSTRACT Granulocytes collected by reversible adhesion to nylon wool fiber (NWF) function relatively well in standard in vitro tests; however, they have an abnormally shortened survival time in the circulation. Assuming that this rapid disappearance represents clearance and that recognition by phagocytes is important for such clearance, we used an autologous in vitro cell:cell recognition assay to determine whether phagocytes can detect cellular changes induced by exposure of normal granulocytes to NWF. Human granulocytes incubated with NWF 1 h at 37°C, eluted with 20% acid citrate dextrose plasma, and washed stimulated the hexose monophosphate shunt activity of normal granulocytes an average of twofold (193±40% of controls), indicating a recognition response. NWF-induced granulocyte recognition was not dependent on plasma factors or activated complement components but was dependent on the time that the granulocyte was on the NWF and was maximal by 60 min of exposure. After elution from NWF, granulocytes demonstrated resting glucose oxidation rates only slightly higher than normal; however, during the first 20 min of exposure to NWF, granulocytes increased their rate of $^{14}CO_2$ production from [1-^{14}C]glucose three- to fivefold. Therefore, experiments were performed to determine whether toxic oxygen metabolites produced by NWF-adherent cells might contribute to recognition. The results showed that (*a*) normal granulocytes exposed to NWF in the presence of scavengers of superoxide anion (superoxide dismutase) or free radicals (ascorbate, mannitol, or benzoate) and washed before assay did not stimulate glucose oxidation of indicator granulocytes; and (*b*) NWF granulocytes prepared from cells unable to generate high levels of toxic oxygen metabolites, i.e. cells prepared anaerobically or from a patient with chronic granulomatous disease, also failed to stimulate indicator granulocytes. Human granulocytes placed in contact with NWF show an oxidative burst and become recognizable to other phagocytes. Free radical scavengers are effective in minimizing this recognition conferred on NWF-procured granulocytes.

INTRODUCTION

Nylon fiber filtration has become a popular method for procurement of human granulocytes for transfusion (1–5). The separation of granulocytes from blood by this method is effective because granulocytes preferentially adhere to the nylon fiber material; this permits the collection of large numbers of cells. In contrast to granulocytes collected by centrifugation procedures, granulocytes collected by the nylon wool fiber (NWF)[1] method exhibit variable abnormalities in bactericidal function and morphology (2–8). Both centrifuge- and NWF-procured granulocytes can be found at sites of infection after transfusion, but NWF-procured granulocytes can only be found in small numbers in the recipient's circulation after infusion (4, 5, 8); and, relative to centrifuge-acquired cells, only a small proportion of the infused cells reach sites of inflammation (9). The disappearance of NWF-procured granulocytes suggests that they are rapidly cleared from the circulation. Because important aspects of clearance of circulating

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[1] *Abbreviation used in this paper:* NWF, nylon wool fiber.

matter are recognition, sequestration, and endocytosis by phagocytes (10), we examined the possibility that changes caused by NWF contact renders granulocytes recognizable to other phagocytic cells. The results of this examination form the basis of this report.

METHODS

Procurement of normal granulocytes. Heparinized blood from normal persons was mixed with 1 vol of 6% dextran (formula weight 400,000) in 0.15 M NaCl solution and allowed to sediment for 1 h at room temperature. Leukocytes in the supernatant fluid were washed once with a 0.15-M NaCl solution, centrifuged at 1,000 *g* min, and suspended in 10 vol of 0.84% NH_4Cl solution for 10 min to lyse red cells. The leukocytes were washed twice more with 0.15 M NaCl solution, centrifuged at 1,000 g/min to remove platelets, and kept at ice-bath temperature. The morphology of leukocytes was examined with Wright-stained smears. On the average, 80% of the cells were granulocytes, predominantly neutrophils. This mixed leukocyte preparation is hereafter referred to as granulocytes.

Procurement of NWF granulocytes. NWF granulocytes were obtained either from whole blood by the filtration leukapheresis technique of Djerassi (1) as previously modified (7), or by allowing a normal mixed leukocyte preparation (see above) to adhere to sterile NWF removed from a filtration leukapheresis filter (Fenwall Laboratories, Deerfield, Ill.). 100,000,000 leukocytes (10^7 cells/ml) were incubated with 200 mg NWF in a 20-ml sterile plastic syringe in Hanks' balanced salt solution, pH 7.4, for 1 h at 37°C followed by washing of the NWF with 5 vol of 0.15 M NaCl solution and elution of the cells by washing the NWF with 10 vol of 20% acid citrate dextrose (National Institutes of Health formula A) in autologous plasma (pH 6.5) and gentle tapping of the syringe. Eluted NWF granulocytes were washed twice with 0.15 M NaCl solution before being tested. In some experiments, the NWF incubation medium contained one of the following: superoxide dismutase, sodium ascorbate, mannitol, catalase, or sodium benzoate (Sigma Chemical Co., St. Louis, Mo.). All solutions containing added drugs were buffered to pH 7.0 except ascorbate which was kept at pH 6.5. NWF granulocytes were then washed free of these materials with 0.15 M NaCl solution and used in the assay. For anaerobic studies, normal granulocytes were incubated with NWF at 2°C for 1 h in bubbling N_2 gas. The system was closed to air, and incubation was performed at 37°C as described for other experiments. Granulocytes from a patient shown to have chronic granulomatous disease (11) were separated and incubated under the same conditions as described for normal and NWF granulocyte preparations.

Cell:cell recognition assay. The assay is a modification of one described previously (12). 5,000,000 normal ("indicator") granulocytes, 10^6 granulocytes to be tested ("test cells"), and 1 μCi of [1-^{14}C]glucose (30.7 mCi/mmol; New England Nuclear, Boston, Mass.) were added to polypropylene scintillation vials (Packard Instrument Co., Inc., Downers Grove, Ill.). The volume was brought to 1 ml with 0.15 M NaCl solution, Hanks' buffer solution pH 7.4, or autologous plasma, and the vials were stoppered with rubber stoppers containing a suspended plastic cup (Kontes Co., Vineland, N. J.). The cup contained a 1 × 3-cm strip of filter paper moistened with 200 μl of 2 N NaOH. Vials were kept at ice-bath temperature until the reaction was started by placing them in a 37°C shaking water bath. The incubation was terminated after 15 min by injecting 0.2 ml N H_2SO_4 into the vial through the rubber stopper. After 15 min of equilibration, the stoppers were removed, and the plastic cups were placed into 5 ml of scintillation fluid (Instagel, Packard Instrument Co., Inc.). Vials containing scintillant and cups were counted for radioactivity in a refrigerated scintillation spectrometer (Packard Tricarb, Packard Instrument Co., Inc.). All assays were performed in triplicate. Optimal conditions for the assay were determined. The amount of $^{14}CO_2$ produced in the reaction was constant with time for the first 15 min of incubation whether or not carrier glucose (5 mM) or plasma was present, although absolute rates of oxidation of glucose were different; therefore all assays were performed with cells suspended in 0.15 M NaCl solution. Because base-line rates of production of $^{14}CO_2$ varied somewhat between experiments, the results are expressed as the percent increase in radioactivity liberated from the system as $^{14}CO_2$; radioactive CO_2 produced by indicator granulocytes in the system containing 5×10^6 indicator and 10^6 test granulocytes was compared to radioactive CO_2 produced by indicator granulocytes in the system containing 5×10^6 indicator and 10^6 untreated control granulocytes. The amount of $^{14}CO_2$ produced by the 5×10^6 indicator granulocytes was arbitrarily assigned 100% activity. The small SD around this value is derived from averaging the three numbers in the triplicate assay. In control and test incubations, all cells were obtained from the same donor; however, the test results were similar if normal untransfused males were used as the source of indicator granulocytes. Additionally, brief exposure of granulocytes to erythrocyte lysing solutions did not affect their performance in the test system or the test results.

RESULTS

Detection of cell recognition by the assay. The indicator granulocytes increased their glucose oxidation rates when incubated with cells treated in a variety of ways. Normal granulocytes heated to 43°C for 3 min and normal granulocytes stored for 24 h at 2°C stimulated glucose oxidation of indicator granulocytes 100–300% of controls (Table I). Fig. 1 shows that NWF granulocytes procured either by in vitro NWF incubation or by clinical filtration leukapheresis caused a twofold (193±40%) increase in $^{14}CO_2$ production by indicator granulocytes. The increase in [1-^{14}C]glucose

TABLE I

Cell: Cell Recognition Assays Using Normal, Heat-Damaged, and Stored Granulocytes

Test conditions	$^{14}CO_2$ production of indicator cells	P*
	% of control±SD	
5×10^6 Normal granulocytes + 10^6 untreated granulocytes	100±11 (n = 11)	
5×10^6 Normal granulocytes + 10^6 heat-damaged granulocytes	287±31 (n = 3)	<0.01
5×10^6 Normal granulocytes + 10^6 granulocytes stored for 24 h at 4°C	147±20 (n = 3)	<0.025

* Significance of difference between experimental and control means (Student's t test).

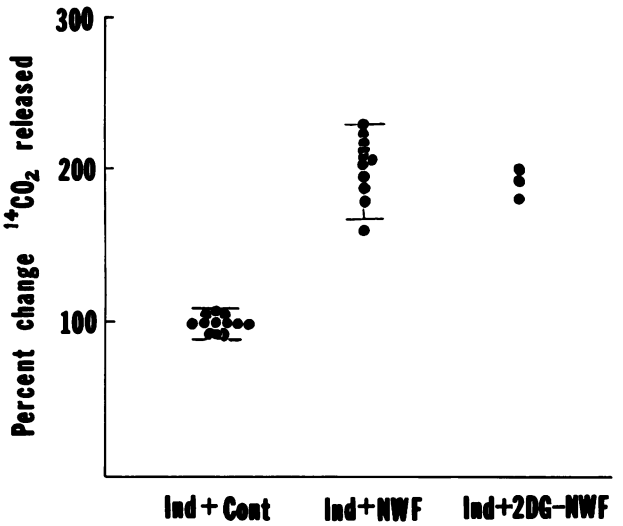

FIGURE 1 Results of studies of oxidation of [1-^{14}C]glucose to $^{14}CO_2$ by 5×10^6 indicator granulocytes plus 10^6 untreated control granulocytes (Ind + cont), 5×10^6 indicator granulocytes plus 10^6 NWF granulocytes (Ind + NWF), and 5×10^6 indicator granulocytes plus 10^6 NWF granulocytes trated with 2-deoxyglucose (Ind + 2DG-NWF). Each point is the result of an experiment with a single cell preparation done in triplicate. Bars represent 1 SD from the mean. Three of the points in the Ind + NWF column represent tests with clinical filtration leukapheresis cells.

oxidation by the indicator:NWF granulocyte mixture was not simply the result of increased metabolism of NWF granulocytes. As shown in Table II, NWF granulocytes did show an 18% higher base-line $^{14}CO_2$ production per cell than did normal unexposed granulocytes. We attributed this increase in base-line glucose oxidation to the attempts of some NWF granulocytes to respond as indicator cells; however, this slight increase in glucose utilization by NWF granulocytes cannot explain the increase in $^{14}CO_2$ in the test system for several reasons: (*a*) in each set of experiments, the base-line counts per minute of 10^6 NWF granulocytes alone was subtracted from the counts per minute of the whole indicator:NWF mixture to calculate the percent increase in radioactivity due to indicator cell metabolism; (*b*) the 18% increase in counts per minute per NWF granulocyte in the indicator:NWF granulocyte incubation mixture amounts to only 3% of the total counts in the indicator:NWF mixture; and (*c*) as shown in Fig. 1, eluted NWF granulocytes treated with 5 mM 2-deoxyglucose for 1 h at 37°C and washed free of 2-deoxyglucose before assay stimulated $^{14}CO_2$ production in the system no less than untreated NWF granulocytes. Light microscope observation of incubations containing NWF granulocytes and indicator granulocytes showed clumping. Although test and indicator granulocytes could not be identified with certainty, many areas showed neutrophils with small, dense nuclei and little cytoplasm surrounded by one or more normal-appearing neutrophils (Fig. 2). As shown in Fig. 3, the number of NWF granulocytes in the assay correlated with the amount of [1-^{14}C]glucose oxidized by the indicator cells.

Mechanism and prevention of recognition of NWF granulocytes. We looked at three possible contributing factors to NWF granulocyte recognition: (*a*) the role of plasma factors, (*b*) the length of time on NWF, and (*c*) the production of toxic oxygen metabolites by granulocytes on NWF. As shown in Table III, the indicator granulocyte assay could not distinguish granulocytes allowed to adhere to NWF in 0.15 M NaCl solution from NWF granulocytes made in fresh plasma or in plasma heated to 56°C for 30 min. Adding plasma that had been exposed to NWF for 1 h at 37°C to the assay system or preincubation of normal control granulocytes with NWF-exposed plasma did not cause an increase in $^{14}CO_2$ production in indicator granulocytes.

As shown in Table IV, the length of time granulocytes were on NWF was found to be an important factor in causing cell recognition. Granulocytes incubated with NWF for 5 min were indistinguishable in the assay system from untreated controls. Only NWF granulocytes incubated for more than 15 min were able to stimulate indicator granulocytes; however, incubations longer than 1 h did not increase this stimulation more.

Although eluted NWF granulocytes had nearly normal base-line rates of glucose oxidation, NWF granulocytes attached to NWF had substantially increased [1-^{14}C]glucose oxidation rates (Fig. 4). Therefore, we examined the possibility that oxygen metabolites produced by granulocytes while on NWF might be damaging such cells. NWF granulocytes were prepared in the presence of materials that reduce the levels of certain of these metabolites by scavenging them or by enzymatically degrading them. Fig. 5 shows results of cell:cell recognition assays done on NWF granulocytes prepared in various concentrations of sodium ascor-

TABLE II

Results of Base-Line Unstimulated [1-^{14}C]Glucose Oxidation by Normal and NWF-Exposed Granulocytes

Test conditions	$^{14}CO_2$ production	P*
	%	
5×10^6 Indicator granulocytes alone	100±6 (n = 11)	
alone	118±4 (n = 6)	<0.005
5×10^6 NWF granulocytes alone	118±4 (n = 6)	<0.005

* Significance of difference between experimental and control (Student's t test).

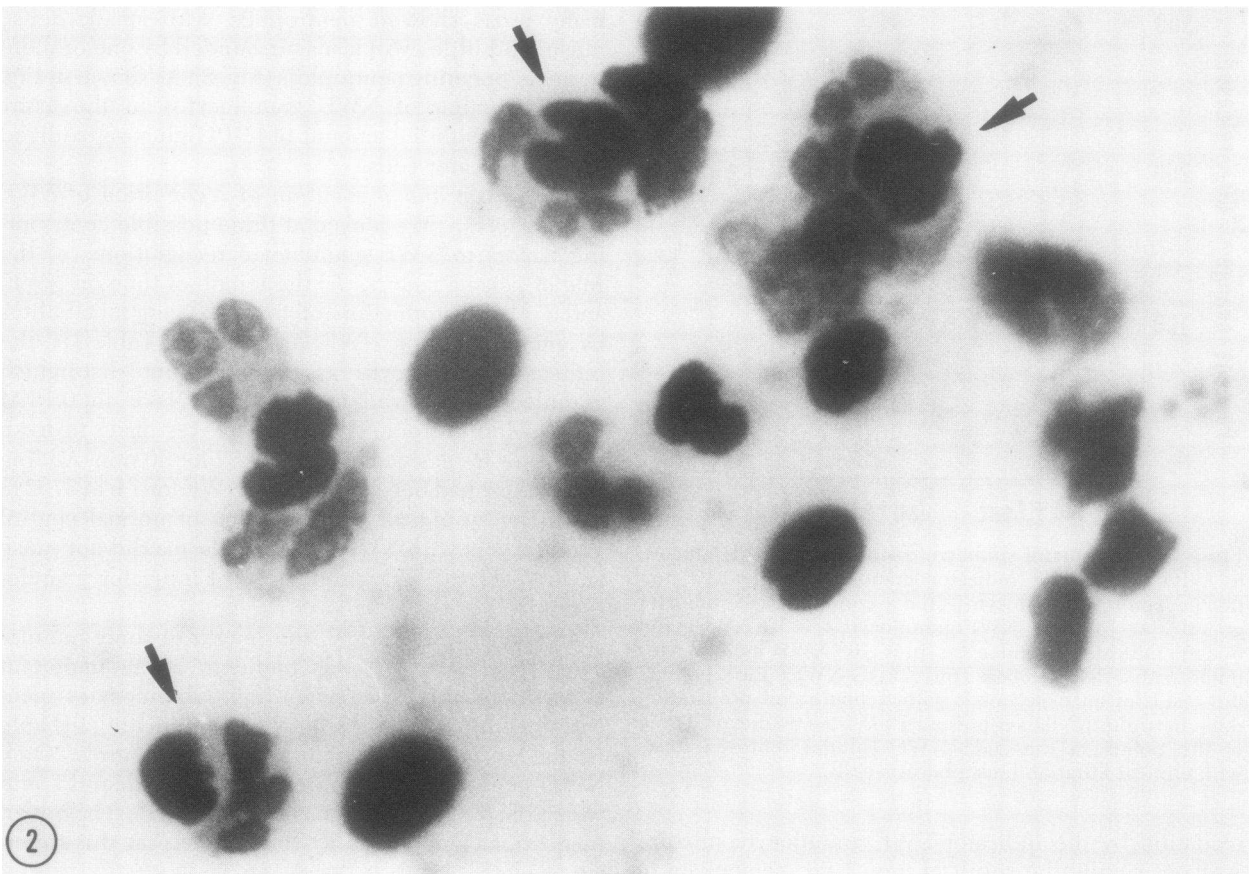

FIGURE 2 Wright-stained smear of granulocytes taken from an indicator:NWF granulocyte assay mixture after 15 min of incubation; the picture shows clumping of cells and apparent attempts at ingestion of autologous granulocytes (arrows).

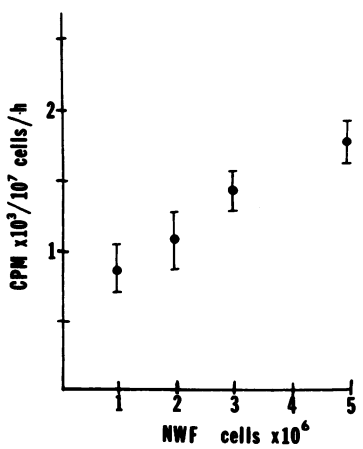

FIGURE 3 Results of oxidation of [1-^{14}C]glucose to $^{14}CO_2$ by 5×10^6 indicator granulocytes incubated with 1, 2, 3, and 5×10^6 NWF granulocytes. NWF granulocytes in this experiment were pretreated with 2-deoxyglucose as described in the text. Points represent the averages of three determinations, and bars represent 1 SD.

TABLE III

Cell: Cell Recognition Assays Using Normal Granulocytes and NWF Granulocytes Made in Different Solutions

Test cells	$^{14}CO_2$ production by indicator cells	P*
	% of control±SD	
Normal granulocytes‡	100±11 (n = 11)	
NWF granulocytes (made in 0.15 M NaCl)	193±40 (n = 11)	<0.001
NWF granulocytes (made in autologous plasma)	161±12 (n = 2)	<0.025
NWF granulocytes (made in heated plasma)	174±27 (n = 3)	<0.01
Normal granulocytes (incubated with NWF-exposed plasma)	89±19 (n = 2)	NS

* Significance of difference between experimental and control; NS = not significant (Student's t test).

‡ Test conditions are the same as described in Table I and Methods.

TABLE IV

Results of Cell: Cell Recognition Assays Performed with Normal Granulocytes Incubated with NWF for 0, 5, 15, 30, and 60 Min

Number of cells			
Normal granulocytes	NWF granulocytes	Length of incubation on NWF	$^{14}CO_2$ Production of indicator cells
		min	*% of control±SD*
6×10^6	0	—	100±11 (n = 11)
5×10^6	10^6	5	95±20 (n = 5)
5×10^6	10^6	15	155 (n = 2)
5×10^6	10^6	30	181 (n = 2)
5×10^6	10^6	60	193±40 (n = 13)

bate, superoxide dismutase, mannitol, and sodium benzoate. Sodium ascorbate had a slightly protective effect at 1 mM levels but was not more effective at higher levels. Superoxide dismutase was optimally effective at 200 μg/ml but was less effective at higher levels. Mannitol and sodium benzoate, when used in concentrations exceeding 1–2 mM, were effective in protecting NWF granulocytes. Equimolar concentrations of glucose and sucrose were not protective for NWF granulocytes. Catalase, shown to be active in degrading H_2O_2, had no protective effect on NWF granulocytes at 100, 250, and 500 μg/ml levels (Table V). Benzoate, mannitol, and ascorbate did not diminish glucose oxidation by normal granulocytes ingesting latex, nor did benzoate or mannitol affect the oxidation of [1-^{14}C]glucose by indicator granulocytes when included in the assay medium (data not shown). Table V also shows results of cell:cell recognition assays with NWF granulocytes prepared anaerobically and prepared from a patient with chronic granulomatous disease. Both of these granulocyte types have an impaired ability to generate oxygen metabolites, and NWF granulocytes prepared from these cells did not stimulate indicator granulocytes; therefore, the inability to generate high levels of oxygen metabolites was protective for NWF granulocytes and prevented their subsequent recognition by indicator granulocytes.

DISCUSSION

NWF-procured granulocytes do not function normally in vitro or remain in the circulation in vivo. We have attributed the decreased killing ability of NWF granulocytes to a deficiency of lysosomal enzymes, this deficiency arising from degranulation during contact with NWF (7). However, the reasons for the rapid disappearance of transfused NWF granulocytes are not clear from previous studies. The experiments described in this paper show that NWF granulocytes are recognized by autologous phagocytes in an in vitro cell:cell recognition system, a system originally used for the detection of human polymorphonuclear leukocytes coated with antineutrophil antibodies (12). The oxidative metabolic response of one phagocyte recognizing another is the basis of the assay, and it permits biochemical quantitation of this recognition. We have demonstrated that this assay also reproducibly detects various forms of nonimmunologic damage to granulocytes including that caused by contact of normal granulocytes with NWF. The stimulation of the hexose monophosphate shunt in this test only indicates that recognition is occurring. Recognition of NWF granulocytes by normal unexposed granulocytes occurs, and, if we are correctly interpreting the slight increase in NWF granulocytes alone, some self-recognition of NWF granulocytes also occurs. This recognition does not determine whether indicator granulocytes simply bind, or partially or completely ingest NWF granulocytes. However, extrapolating from studies of antibody-coated erythrocytes, any of these mechanisms is potentially lethal to NWF granulocytes (13–16).

Several possible mechanisms could inflict the changes on test cells that elicit recognition by indicator cells: (*a*) hydrolytic lysosomal enzymes released during NWF contact could attack the cell membrane; (*b*) activated complement components proposed to arise during the interaction of plasma and NWF could damage the granulocyte surface, or (*c*) cell alterations could result from the effects of toxic oxygen metabolites generated during NWF contact.

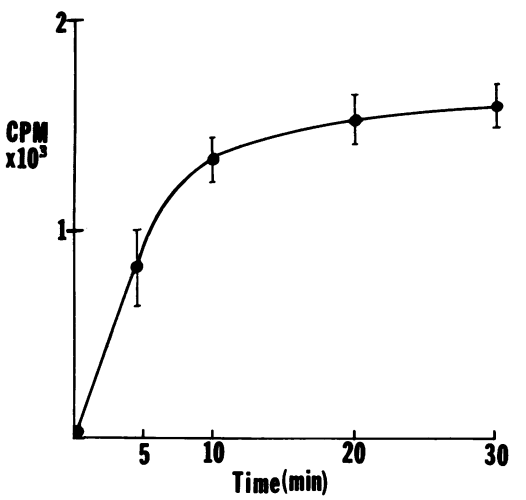

FIGURE 4 Results of oxidation of [1-^{14}C]glucose to $^{14}CO_2$ by 5×10^6 normal granulocytes incubated in 0.15 M NaCl solution with 100 mg of NWF and 1 μCi of [1-^{14}C]glucose. Figure shows cpm trapped as CO_2 vs. time. Each point is the mean of three determinations. Bars represent 1 SD. Normal granulocytes show a constant increase in $^{14}CO_2$ production for the first 20 min of incubation after which the rate of glucose oxidation slows markedly.

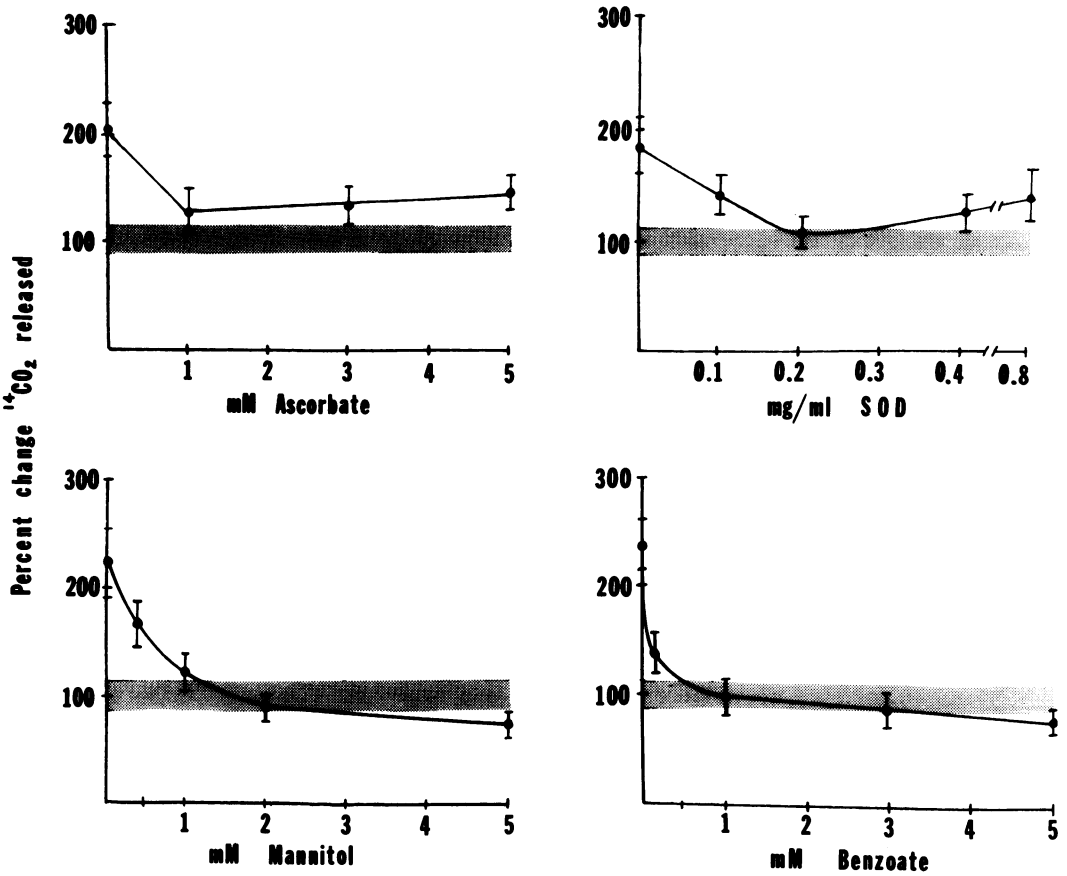

FIGURE 5 Results of cell:cell recognition assays done on NWF granulocytes prepared in various concentrations of sodium ascorbate, superoxide dismutase, mannitol, and sodium benzoate. Ordinate shows percent increase in glucose oxidation of indicator granulocytes when incubated with NWF granulocytes. Shaded area represents the range of glucose oxidation seen when untreated normal granulocytes are used as the test cells. Points within or below the shaded area indicate protection against recognition conferred by that concentration of scavenger. Points are the average of three experiments done in triplicate, and bars represent 1 SD.

Recognition of NWF granulocytes by autologous phagocytes is not dependent upon opsonins or activated complement components because the metabolic activation and subsequent recognition by autologous phagocytes can occur in the absence of autologous plasma, and exposure of normal granulocytes to NWF-exposed plasma (17) does not render them recognizable by other phagocytes in this system. It is still possible that activated complement components produced by exposure to NWF might play a role in vivo because activated complement components can cause metabolic changes in human granulocytes (18, 19); however, we were not able in these experiments to demonstrate that activated complement components were contributing to the phenomena that we observed.

These studies implicate oxygen metabolites, particularly O_2^- and hydroxyl radical as agents able to effect changes in NWF granulocytes, making them recognizable by other phagocytes. Human granulocytes can make several different oxygen metabolites including H_2O_2 (20–22) and O_2^- (23), which in turn can interact to produce hydroxyl radical, singlet oxygen and probably other radical species (20, 22). These oxygen metabolites are produced to exert microbicidal activity against ingested microorganisms (24), but a fraction of these species appear in the extracellular medium (21, 22) where they can damage host cells, including granulocytes from which they originate (20). Salin and McCord (20) demonstrated that scavengers of oxygen radicals could protect human granulocytes during phagocytosis. Our results with NWF, a noningestible material, were similar to those of Salin and McCord. Mannitol and benzoate are relatively specific hydroxyl radical scavengers (25), and they were most effective as protectants above 1 mM. The protective effect was not a result of osmotic activity because equimolar solutions

TABLE V
Cell: Cell Recognition Assays Using Normal and NWF Granulocytes Prepared under Various Conditions

Test cells	$^{14}CO_2$ Produced by indicator cells	P*
	% of control±SD	
Normal granulocytes‡	100±11 (n = 11)	—
NWF granulocytes	193±40 (n = 11)	<0.001
NWF granulocytes (N_2)	96±9 (n = 3)	NS
NWF granulocytes (CGD)	90	—
NWF granulocytes (catalase 100 μg/ml)	221±19 (n = 5)	<0.001
NWF granulocytes (catalase 250 μg/ml)	185±37 (n = 5)	<0.01
NWF granulocytes (catalase 500 μg/ml)	203±22 (n = 5)	<0.001

Results of cell:cell recognition assays performed with 10^6 normal untreated granulocytes, 10^6 NWF granulocytes, 10^6 NWF granulocytes prepared under anaerobic conditions (N_2), 10^6 NWF granulocytes prepared from the blood of a patient with chronic granulomatous disease (CGD), and 10^6 NWF granulocytes prepared in various concentrations of catalase.

* Significance of difference between experimental and control; NS = not significant (Student's t test).

‡ Test conditions are the same as described in Table I and Methods.

of glucose and sucrose did not protect NWF granulocytes from recognition. Sodium ascorbate was poorly effective as a protectant because it is a less effective free radical scavenger than mannitol and benzoate, and because at higher concentrations it can by itself be an oxidant. Superoxide dismutase was optimally protective for NWF granulocytes at 200 μg/ml; however, it was less effective at higher concentrations. Catalase did not protect NWF granulocytes from recognition. Possible explanations for the less dramatic effect of the enzyme scavengers are that (*a*) being enzymes, these compounds might be expected to be active only above certain substrate concentrations; this might leave small amounts of H_2O_2 and O_2^- available for free radical generation; and (*b*) steric factors may limit the effectiveness of the relatively large enzyme molecules at the cell surface.

Because the recognition assay involves the detection of an oxidative response by the recognizing cell, the apparent protective effects could have been the result of an influence of residual traces of the agents not removed during the washing process on the oxidative response of indicator granulocytes. However, benzoate, mannitol, and ascorbate did not diminish glucose oxidation by normal granulocytes which were ingesting latex particles; these agents also did not affect the metabolism of indicator granulocytes when they were included in the assay medium. Therefore, the drugs primarily exert their effects by reducing extracellular free radical levels generated at the time of NWF contact. This interpretation was also supported by nonpharmacologic experiments with granulocytes unable to generate high levels of oxygen metabolites. Granulocytes incubated with NWF under anaerobic conditions (26, 27) and NWF cells prepared from the blood of a patient with chronic granulomatous disease (28, 29) did not develop characteristics that made them recognizable to indicator granulocytes; thus the inability to generate high levels of toxic oxygen metabolites was protective for the granulocyte exposed to NWF.

A primary role for the release of lysosomal enzymes in the production of cell:cell recognition seems unlikely because the release of granule enzymes from granulocytes occurs normally under anaerobic conditions (30); it is not markedly impaired in the granulocytes of our patient with chronic granulomatous disease (31), and oxygen radical scavengers do not inhibit the activity of lysosomal hydrolases (32). Furthermore, NWF can affect the granulocyte in the presence of serum that contains potent inhibitors of lysosomal proteases (32, 33); however, the possibility that oxygen metabolites act indirectly by releasing lysosomal enzymes has not been excluded.

We have shown that cell alterations undetectable by conventional granulocyte function tests occur when normal granulocytes are exposed to NWF. If such subtle changes recognized by autologous phagocytes in vitro are occurring in vivo, the paradox that NWF granulocytes function relatively well in vitro but fail to survive normally in vivo might be explained. This hypothesis is testable; if it is confirmed in experiments involving transfusion of granulocytes into humans, it suggests a strategy for pharmacologically improving the intravascular survival of NWF-procured granulocytes.

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