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Failure to Detect Superoxide in Human Neutrophils Stimulated With Latex Particles[21]

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Summary

Human neutrophils stimulated with either latex particles or opsonized zymosan exhibited equivalent rates of net oxygen consumption as well as hydrogen peroxide release. The quantity of superoxide (O_2^-) detected in latex-stimulated neutrophils was less than 2% of that seen with opsonized zymosan stimulation, and only several-fold greater than that of resting cells. The failure to detect O_2^- in the latex-stimulated neutrophils was due neither to latex acting as a O_2^- scavenger nor to its interference with the O_2^--forming system of the neutrophil. An intracellular site of O_2^- generation could not be demonstrated. NADPH oxidase activity in cells exposed to latex particles was only 10% of that seen in cells comparably activated with opsonized zymosan. Latex particles have the unusual property of stimulating the respiratory burst of the human neutrophil without the extracellular release of O_2^-. The potential physiologic importance of this finding is discussed.

Abbreviations

O_2^-, superoxide
O_2, oxygen
H_2O_2, hydrogen peroxide
SOD, superoxide dismutase
PBS, phosphate buffered saline
PBS$^-$, phosphate-buffered saline without Ca^{++} and Mg^{++}
HEPES, *N*-2-hydroxyethyl piperazine-N′-2-′ethanesulfonic acid

Superoxide production is an integral component of the respiratory burst that occurs in human neutrophils when exposed to a variety of phagocytosable particles or other soluble stimuli (4, 7). In the first report that neutrophils generate O_2^-, Babior, *et al.* (1) found that neutrophils phagocytizing latex particles exhibited a modest, but statistically significant, increase in O_2^- production. Subsequent reports, however, have indicated that other phagocytosable particles stimulate O_2^- production in quantities much larger than that observed with latex (7). This finding suggested that either latex particles were a relatively weak stimulus of the respiratory burst as a whole, or that O_2^- production was selectively dissociated from other components of the respiratory burst. The results presented below indicate that latex particles are, in fact, a potent stimulus for the respiratory burst, but that only small amounts of O_2^- are detected.

MATERIALS AND METHODS

Materials. Ferricytochrome *c* (Type VI), zymosan, superoxide dismutase (2900 U/mg), xanthine oxidase, and purine were obtained from Sigma Chemical Co., St. Louis, MO. Polystyrene/toluene particles (2.77 μm diameter) were purchased from Dow Chemical Corp., Midland, MI. Macrodex (dextran 70) (6% in 0.9% NaCl) and Hypaque-Ficoll (Ficoll-Paque) were purchased from Pharmacia, Piscataway, NJ. All other reagents were the highest quality commercially available and were used without further purification.

Preparation of neutrophils and phagocytosable particles. Neutrophils were harvested from fresh human blood obtained from normal adult donors by the dextran-sedimentation method previously described (8). After hypotonic lysis of contaminating erythrocytes, the neutrophils were further purified by centrifugation over Hypaque-Ficoll (17), which yielded a greater than 99% pure preparation. Zymosan particles were opsonized with fresh autologous human serum just before use by the method of Hohn and Lehrer (11) and then suspended in PBS at a concentration of 60 mg/ml (4.56×10^9 particles/ml). Latex particles were dialyzed three times against 1 liter 0.9% NaCl at 8°C for a total of 48 h, and then stored at 4°C in suspension in 0.9% NaCl at a concentration of 3.0×10^9 particles/ml.

Determination of neutrophil oxygen consumption, O_2^- production, and H_2O_2 generation. Oxygen consumption was measured using a Model 53 Biological Oxygen Monitor (Yellow Springs Instrument Co., Yellow Springs, OH) calibrated using the phenylhydrazine oxidation method (13). Before the start of each determination, 0.1 ml cell suspension was added to 2.9 ml PBS (containing 1 mM sodium azide and 7.5 mM glucose, prewarmed to 37°C) in a siliconized sample chamber equipped with a magnetic stirrer and allowed to equilibrate for 2 min. Oxygen consumption during phagocytosis was determined by introducing 0.1 ml of prewarmed particle suspension into the sample chamber. Preliminary experiments showed that maximal oxygen consumption occurred with final particle concentrations of 10^8 particles/ml for the latex beads and 1.5×10^8 particles/ml for opsonized zymosan. Using optimal concentrations of particles, the maximal rate of O_2 consumption was linear over at least a 10-fold range of neutrophil concentrations ($1.6–16 \times 10^6$ cells/ml). Based on these results, all oxygen consumption assays were performed with 6.6×10^6 cells/ml. Azide did not affect the rate of O_2 consumption.

Superoxide production was determined concurrently with O_2 consumption by the method of Curnutte and Babior (8) using two additional sample chambers with magnetic stirrers. Each of the two reaction mixtures contained cytochrome *c* (120 μM), neutrophils (0.5×10^6 cells/ml for opsonized zymosan studies and 5×10^6 cells/ml for latex particles), sodium azide (0.7 mM), and phagocytosable particles at the concentrations listed above for O_2 consumption assays. One reaction mixture contained SOD (20 μg/ml). The rate of O_2^- production was linear over a cell concentration range of $0.5–21 \times 10^6$ cells/ml. Azide did not affect the rate of O_2^- generation.

H_2O_2 production was determined by modification of the thiocyanate method of Thurman *et al.* (18). Reaction mixtures were identical to those used in O_2 consumption studies except that the neutrophil concentration was 1.5×10^7 cells/ml (the assay was linear with cell concentration between $0.5–1.5 \times 10^7$ cells/ml). The reaction was terminated after 4-min incubation by placing a 1.0-cc aliquot on ice and adding 0.2 ml 80% (w/v) trichloroacetic acid. Samples were spun at $2000 \times g$ for 5 min and 300 μl of

supernatant was assayed and compared to a standard curve of H_2O_2.

Determination of O_2^- production by neutrophil subcellular 27,000 × g particles. Neutrophils (5×10^8) were preincubated in 2.9 ml PBS at 37°C for 3 min in vials containing magnetic stirrers. Each reaction was started by adding 0.1 ml of PBS, opsonized zymosan suspension as described above for oxygen consumption, or latex particles and terminated after 3 min by adding an equal volume of ice cold PBS to the reaction mixture and placing it on ice. The cells were then sonicated and fractionated by previously published methods (17) to obtain the 27,000 × *g* particulate fraction, which was then assayed for O_2^- production by the cytochrome *c* assay (12) in a Perkin-Elmer Model 552 double beam spectrophotometer. In experiments where the 27,000 × *g* particles were solubilized by detergent, Triton X-100 (0.4%) and FAD (0.2 mM) were present in the O_2^- assay curvettes as previously described (9, 12).

RESULTS

Figure 1 (*top panel*) shows a comparison of the time courses of O_2 consumption and O_2^- production by neutrophils at rest and stimulated with opsonized zymosan. Both measurements were performed in parallel under similar conditions on cells from a single donor in order to permit direct comparisons. Resting cells in this experiment consumed little oxygen (0.3 nmole/min/10^7 cells) and produced O_2^- at a rate less than 1 nmole/min/10^7 cells. In cells exposed to opsonized zymosan, dramatic increases in both O_2 consumption and O_2^- production were observed with rates that were several hundred-fold greater than those observed for resting cells. Superoxide production began 10 sec after the addition of zymosan, reached its maximum rate for 40 sec, and then remained linear for approximately 4 min, after which time the rate slowly decreased. The diminished rate of O_2^- production was not due to the exhaustion of oxidized cytochrome *c* in the reactioin mixture because doubling the concentration of cytochrome *c* did not influence the detection of O_2^-. The time course for O_2 consumption was parallel to that observed for O_2^- generation (22).

In marked contrast to the results with opsonized zymosan are those results obtained using comparably sized latex particles (Fig. 1, *lower panel*). Although the measured O_2 consumption was increased nearly as much as with zymosan, only 1% as much O_2^- was detected (Table 1) (23). Attempts to recover more O_2^- by doubling the cytochrome *c* concentration or extending the duration of incubation were unsuccessful. Even though little O_2^- was detected in latex-stimulated neutrophils, one of the other products of the respiratory burst, H_2O_2, was readily measurable. As shown in Table 1, 30% (±4% S.D.) of the oxygen consumed was recovered as H_2O_2 in the case of latex, whereas 52% (±5% S.D.) was recovered when zymosan was the stimulus. These values are in excellent agreement with previously reported recoveries of H_2O_2 (*e.g.*, Table VIII in reference 15).

The data in Figure 1 and Table 1 show that in the presence of opsonized zymosan, neutrophils generate nearly two moles of O_2^- for each mole of O_2 consumed. This stoichiometry is found

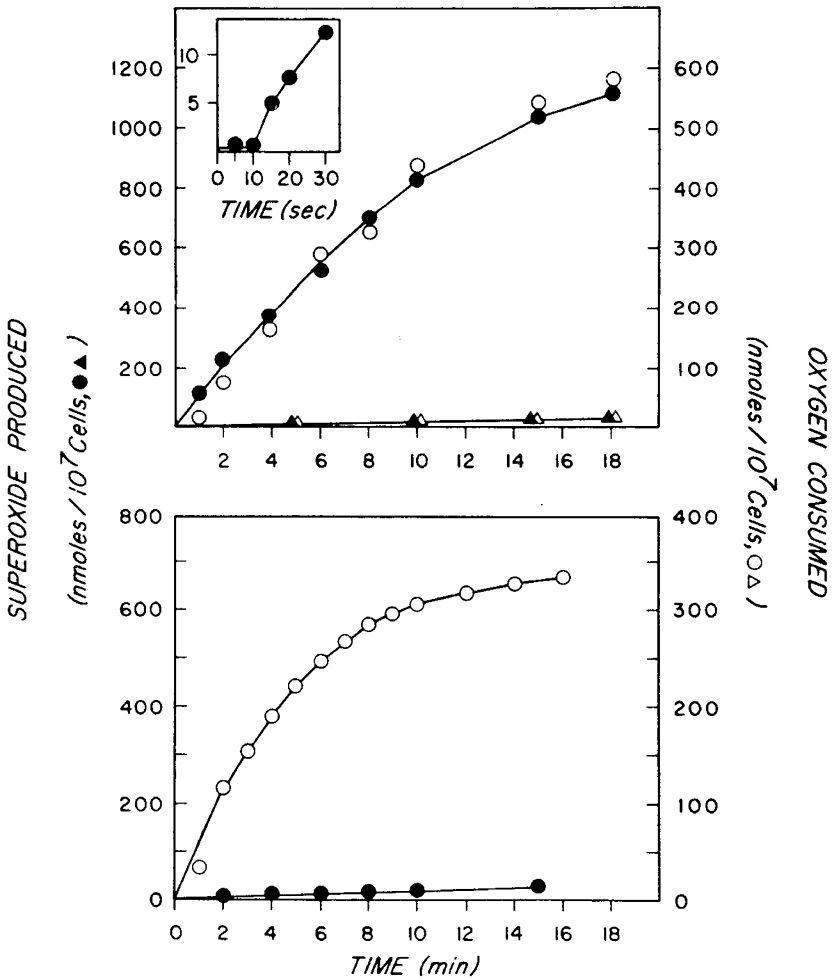

Fig. 1. Oxygen consumption and O_2^- production by resting and stimulated cells. Oxygen consumption and O_2^- production were determined as described in "Materials and Methods." The experiment was performed with three different preparations of cells: (○, △), oxygen consumption and (●, ▲), O_2^- production. The figure shows a representative experiment. *Top panel:* triangles, resting cells and circles, opsonized zymosan-stimulated cells. *Inset:* higher resolution time course of O_2^- production with opsonized zymosan as stimulus (cell concentration 7×10^6 cells/ml). *Bottom panel:* latex particles as stimulus.

Table 1. *Comparison of the respiratory bursts of human neutrophils stimulated with either opsonized zymosan or latex particles (nmole/4 min/10^7 cells)*[1]

Stimulus	Oxygen consumed	O_2^- produced	H_2O_2 produced
None	1.0 ± 0.4	0.6 ± 0.2	0
Zymosan	229.6 ± 18.2	420.0 ± 29.7	119.0 ± 11.6
Latex	169.0 ± 16.0	2.8 ± 2.8	50.3 ± 6.5

[1] Oxygen consumption, O_2^- production, and H_2O_2 production were determined as described in "Materials and Methods." The data represent the mean ± S.E. from three different preparations of cells. The values in the table reflect the amounts of oxygen consumed, O_2^- produced, or H_2O_2 produced during the first 4 min of the reaction.

because the oxygen electrode used in these experiments measures only *net* oxygen consumption and is unable to detect that portion of oxygen which is first consumed and then later recycled back to oxygen (2). At least several reactions known to occur in the neutrophil can recycle oxygen, most notably the dismutation of O_2^- and the breakdown of H_2O_2 by catalase:

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad \text{(a)}$$

$$2H_2O_2 \rightarrow 2H_2O + O_2 \quad \text{(b)}$$

At present it is not possible to correct for all the recycling reactions and arrive at an accurate *gross* oxygen consumption; however, the observation here that 183% of the net oxygen consumed is recovered as O_2^- suggests that O_2^- is the major product of the respiratory burst in zymosan-stimulated cells and that a substantial portion of the O_2^- can be detected under our assay conditions. Because the latex-stimulated neutrophils are assayed under the same conditions, it is unlikely that the low rates of O_2^- production in these cells are a reflection of a failure to detect O_2^-. This matter is explored further in experiments described below.

The phenomenon of oxygen recycling raises an important issue regarding the latex data in Table 1. If the latex-stimulated cells in fact do *not* produce O_2^- (and the catalatic breakdown of H_2O_2 is blocked by azide in the medium), then little oxygen recycling should occur and the measured oxygen uptake of 169 nmoles/4 min/10^7 cells should approximate the true oxygen consumption. With zymosan, however, there should be appreciable recycling of oxygen. If all the O_2^- produced by the zymosan cell undergoes spontaneous dismutation by reaction (a), then 210 nmoles of oxygen will be recycled and not reflected in the measured net oxygen consumption. The gross oxygen uptake would then be estimated at 440 nmoles O_2/min/10^7 cells (229.6 nmoles O_2/min/10^7 cells measured, plus 210 nmoles O_2/min/10^7 cells presumed recycled from O_2^-); thus, oxygen uptake in the latex-treated cells is only 38% of that seen with zymosan-stimulated neutrophils. This calculation helps explain why the measured rate of H_2O_2 production with latex in Table 1 is only 42% of that observed with zymosan. When corrected for the difference in the magnitudes of the respiratory bursts (assuming that little O_2^- is made by latex cells), the rates of H_2O_2 production with both stimuli are equivalent. As commented above, however, there are other reactions by which O_2^- could be metabolized and thus affect these calculations. For example, an oxidized molecule in the cell (X^{ox}) could be reduced by O_2^- in the following reaction:

$$O_2^- + X^{ox} \rightarrow 0_2 + X^{red}$$

The contribution of this type of reaction must be minor, given the close agreement of the H_2O_2 recoveries.

A control experiment was performed to assure that the observed difference in O_2^- production between latex- and zymosan-stimulated cells in Table 1 was not due to the greater than 2-fold difference in the magnitudes of the respiratory bursts. The concentration of opsonized zymosan was decreased to 0.4 mg/ml, resulting in a drop in the rate of oxygen consumption (22.4 ± 2.3 nmoles O_2/min/10^7 cells) to a level which, after correcting for recycling of oxygen, was equal to that observed with latex (40.6 ± 3.6 nmoles O_2/min/10^7 cells). Under these conditions, the rate of O_2^- production with latex was still less than 5% of that seen with zymosan as the stimulus (36.1 ± 2.3 nmoles O_2^-/min/10^7 cells).

One possible explanation for the low rate of O_2^- production by latex-stimulated cells is that latex may function as a O_2^- scavenger, thus preventing the detection of O_2^- by the cytochrome *c* assay. To test this possibility, the effect of latex particles on the detection of O_2^- produced by xanthine oxidase and purine, a known O_2^--generating system, was examined. At rates of O_2^- generation equivalent to those seen in stimulated neutrophils, the ability of cytochrome *c* (80 μM) to measure O_2^- was totally unaffected by the presence of latex particles (data not shown). Another possible explanation is that superoxide dismutase might be released from latex-stimulated cells and thereby interfere with O_2^- detection. To test this possibility, opsonized zymosan was added to latex-stimulated cells either 10 sec or 4 min after the latex was added. In neither case was there any change in the recovery of O_2^- produced as a result of the zymosan stimulation (data not shown) (24). If there was an appreciable amount of SOD activity present in the incubation medium to interfere with O_2^- detection in the latex case, then it should have affected the zymosan experiment in a like manner. Yet another possibility to explain the data is that latex damages the O_2^--generating system in the neutrophil. This, however, is also unlikely because, in the experiments in which zymosan was added to latex-stimulated cells, O_2^- generation commenced after the addition of zymosan without a lag and at a rate which was the same as if the latex had never been present. If latex particles were inhibiting the O_2^--forming system, then some diminution of O_2^- production should have been observed when zymosan was added 4 min after the addition of latex.

The 27,000 × *g* particulate fraction from activated human neutrophils is known to contain an NADPH-dependent O_2^--generating system (12). We examined this fraction prepared from cells stimulated with opsonized zymosan or latex particles to determine whether the differences observed on the whole cell level also applied to this subcellular fraction. The particulate fraction from zymosan-stimulated cells produced 14.1 ± 7.9 S.E. nmoles O_2^-/min/mg protein (n = 3). In marked contrast, the same fraction obtained from cells stimulated with latex particles generated only 1.4 ± 0.5 S.E. nmoles O_2^-/min/mg protein.

The addition of detergent to the 27,000 × *g* particulate preparation augments the O_2^--generating capacity of this cell-free system (12). We confirmed that the addition of a Triton X-100 (0.4%) and FAD (0.2 mM) increased O_2^- production by particles from zymosan-activated neutrophils 230 ± 25% (mean ± S.D., n = 3) as compared to the same untreated 27,000 × g particles. Particulate preparation from latex-stimulated cells showed a similar proportionate increase in O_2^--generating activity, 290 ± 45.2%. This augmented activity in the latex-stimulated preparation still did not account for the widely discrepant O_2^- rates seen in the corresponding intact cells.

DISCUSSION

Under conditions in which opsonized zymosan and latex comparably stimulate O_2 uptake as well as H_2O_2 release in human neutrophils, O_2^- generation by latex-stimulated cells is only 2% of that observed with zymosan stimulation. This difference in O_2^- detected is not due to latex particles either scavenging O_2^- or interfering with the O_2^--generating system of this cell.

In quantitative terms, the levels of O_2^- produced by latex-stimulated cells in this study are in accord with previously published reports (1, 19). Our results differ from those of Weening *et al.* (20) and Segal and Meshulam (16) who found substantial O_2^- production in neutrophils activated with serum-treated or IgG-coated latex particles, in contrast to the unmodified latex particles employed in this study. It is likely that the coated latex particles had surface properties which enabled them to stimulate O_2^- production.

An intracellular site of O_2^- production is an unlikely explanation for the observed low levels of O_2^- production in latex-stimulated cells for several reasons. First, the O_2^- assay employed in these experiments is highly efficient. In the case of zymosan-stimulated cells, 183% of the net oxygen consumed was recovered as O_2^- (Table 1). This stoichiometry was consistent throughout the entire time course of the incubation (Fig. 1). In contrast, less than 2% of the O_2 consumed by latex-stimulated cells was detected as O_2^- under identical experimental conditions. The high recovery of O_2^- in the zymosan-stimulated cells suggests that under the assay conditions employed, a substantial portion of the O_2^- generated can be detected. Second, cytochemical studies by Briggs *et al.* (5, 6), using cerium precipitation as a method for localizing H_2O_2 demonstrated that both zymosan- and latex-stimulated human neutrophils have H_2O_2 present both around phagocytic vacuoles and on the plasma membrane. Control experiments in these studies showed that the surface-bound reaction product did not result from the diffusion of H_2O_2 from within the cell. These results suggest that an oxidase activity is present on the plasma membrane of latex-stimulated human neutrophils and that if this oxidase generates O_2^-, this anion should be detectable with the cytochrome *c* assay employed in our experiments. Finally, the NADPH-dependent superoxide generating system in the 27,000 $\times$ *g* particulate fraction of zymosan-treated cells exhibited little activity when prepared from neutrophils stimulated with latex particles.

Taken together, the above arguments suggest that little or no O_2^- is produced at intracellular sites within latex-stimulated neutrophils; however, this possibility cannot be definitely excluded and remains speculative. Intracellular O_2^- generation has not been measured, because no such assay is sufficiently sensitive to measure the levels of O_2^- believed to be present within neutrophils.

The experiments herein demonstrate that latex particles have the unusual property of markedly stimulating the respiratory burst without causing the release of *extracellular* O_2^-. In this regard, the latex particle is a unique respiratory burst stimulus (7). Although the failure to release O_2^- into the extracellular medium may be a property peculiar to latex-stimulated neutrophils, it is also possible that it may represent a more general property of the phagocyte. The nature of the ingested particle may determine whether O_2^- is released into the extracellular medium or simply confined to the phagolysosome (or possibly, not made at all). This property would have potentially important implications in inflammation. The toxicity of a latex-like stimulus may well differ from a potent O_2^--generating agonist with resultant changes in damage to host tissue or pathogens. The search for physiologic stimuli, which activate the respiratory burst as a latex particle, is underway to further explore these questions.

We regard at least two models as possible explanations for a respiratory burst without O_2^- production. In the first, a single enzyme is responsible for both O_2^- and H_2O_2 production, but the relative quantities of each produced is determined by the physical state of the enzyme. The precedent for this model is xanthine oxidase (10, 14), an enzyme which generates O_2^- and H_2O_2. The relative amounts of O_2^- and H_2O_2 produced is determined by the reductive state of xanthine oxidase - H_2O_2 favored in the higher reductive state. Of interest in this regard are the data with NADH oxidase from guinea pig neutrophils (3). Both O_2^- and H_2O_2 are produced by this enzyme. Approximately 15% of the electrons derived from NADH are channeled into the univalent reduction of oxygen to O_2^- whereas the rest of the electrons are apparently donated to oxygen in pairs to form H_2O_2. At present it is not known whether the relative quantities of O_2^- and H_2O_2 can be experimentally controlled. In the second model, the enzyme which produces O_2^- during the respiratory burst is distinct from an enzyme which produces H_2O_2 exclusively. With this model, the latex stimulation only triggers the latter enzyme, whereas with zymosan stimulation, both enzymes are activated.

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22. For technical reasons related to the delay in re-equilibration of the oxygen electrode after the addition of opsonized zymosan, values for O_2 consumption could not be accurately obtained during the first minute.
23. In contrast to unopsonized latex particles (as used in these experiments), unopsonized zymosan neither increases oxygen consumption nor O_2^- generation over resting values.
24. The effects of zymosan and latex on oxygen consumption were not additive: the rate of oxygen consumption with both stimuli present was the same as with zymosan alone.

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