Fluoride-Mediated Activation of the Respiratory Burst in Human Neutrophils

A REVERSIBLE PROCESS

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ABSTRACT Fluoride ion (F^-) is an effective activator of the respiratory burst in neutrophils, as indicated by its ability to induce O_2^- production by these cells. Other halide ions did not activate the burst, Cl^-, in particular appeared to antagonize the effect of F^- on O_2^- production. F^--stimulated O_2^- production showed a requirement for Ca^{++}, but was independent of other exogenous cations. Neither phagocytosis nor degranulation were necessary for respiratory burst activation by F^-.

The effect of F^- on the respiratory burst was reversible. Washing the cells after treatment with F^-, while they were still producing large amounts of O_2^-, returned them to the resting state. They could then be stimulated again to produce O_2^- in amounts equivalent to those originally produced. Our experiments indicated that restimulation did not represent the activation of a population of cells that had not been activated during the initial exposure to F^-, nor did it represent serial activation of different subpopulations of the O_2^--forming enzyme molecules present in a given cell. Rather, our data suggest that the entire population of O_2^--forming enzyme molecules was activated in a reversible fashion by F^-.

INTRODUCTION

The exposure of neutrophils to suitable stimuli results in the activation of an oxygen-consuming metabolic pathway which is dormant in the unstimulated cell (1–3). The cellular manifestations of this pathway include increases in oxygen uptake, superoxide production, hydrogen peroxide production, and glucose consumption by way of the hexosemonophosphate shunt, a group of events which taken together are designated the "respiratory burst". It now appears that the respiratory burst is a consequence of the activation of a membrane-bound (4–7) enzyme which catalyzes the reduction of oxygen to O_2^- at the expense of a reduced pyridine nucleotide.

The respiratory burst is activated by a remarkably wide variety of agents (3). Of primary importance in terms of the physiological role of the neutrophil are particulate agents such as opsonized bacteria, fungi, and mycoplasma, as well as opsonized zymosan (yeast cell walls) and polystyrene latex beads. A number of nonparticulate agents also activate the respiratory burst. These include such substances as anionic detergents (but not cationic or neutral detergents); antineutrophil antibodies in the presence of complement; ionophores such as A23187 and X537A, which seem to activate the burst regardless of the ionic composition of the extracellular medium; the enzyme phospholipase C, which may or may not damage the cell in the course of its action; biologically active plant products, including concanavalin A and phorbol myristate acetate; certain constituents of plasma, either free or bound to a nonphagocytosable surface; and the inorganic anion F^-. The concentrations and conditions under which these agents act vary widely.

The ability of F^- to activate the respiratory burst was discovered by Sbarra and Karnovsky (8), who were using the ion as an enolase inhibitor and noted that oxygen uptake by resting neutrophils was stimulated in its presence. The effect of F^- on the burst was studied further by Selvaraj and Sbarra (9), who concluded that it acted by precipitating with other ions within the cell as an insoluble particle that induced the burst. However, the evidence that fluoride-treated neutrophils contained internalized material was inconclusive.

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We previously reported that O_2^- production by the human neutrophils is greatly stimulated by F^- (10, 11), a finding in accord with the effect of this ion on neutrophil oxygen uptake. Recently, another group of investigators has confirmed this observation (12). In this paper we examine not only O_2^- production, but also phagocytosis and degranulation. Our experiments show that the stimulation of O_2^- production by F^- is not associated with either phagocytosis or degranulation. Moreover, fluoride activation of the O_2^--forming system is fully reversible. The latter finding in particular is of considerable interest with regard to the physiological role of neutrophils, as will be discussed.

METHODS

Materials. Macrodex (dextran 70) 6% in 0.9% NaCl and Ficoll were obtained from Pharmacia Fine Chemicals, Piscataway, N. J. Hypaque Sodium (sodium diatrizoate) 50% was purchased from Winthrop Laboratories, Sterling Drug Co., New York. Siliclad was obtained from Clay Adams, Div. Beckton, Dickinson & Co., Parsippany, N. J. Catalase (bovine liver), ovalbumin (grade V), horse heart ferricytochrome *c* (grade VI) bovine albumin (essentially fatty acid free), zymosan A, *o*-dianisidine dihydrochloride, *p*-nitrophenyl-β-D-glucuronide, disodium *p*-nitrophenyl phosphate (Sigma 104), *Micrococcus lysodeikticus*, Triton X-100, sodium pyruvate solution (0.02M), β-NADH (grade III, disodium salt), Hepes, and nitroblue tetrazolium were purchased from Sigma Chemical Co., St. Louis, Mo. Superoxide dismutase was obtained from Truett Laboratories, Dallas, Tex. Phorbol myristate acetate (PMA)[1] was purchased from Consolidated Midland Corp., Brewster, N. Y., and was stored at −70°C dissolved in dimethyl sulfoxide (2 mg/ml). Chelex 100 in the sodium form (100–200 mesh) was obtained from BioRad Laboratories, Richmond, Calif. Trypan blue stain (0.4% in 0.9% NaCl) was purchased from Grand Island Biological Co., Grand Island, N. Y. Sodium fluoride and calcium fluoride were purchased from ICN K & K Laboratories Inc., Plainview, N. Y. Other reagents were the best grade commercially available and were used without further purification.

Neutrophils and zymosan. Unless otherwise indicated, neutrophils were purified by the previously described modification (13) of the method of Skoog and Beck (14), except that hypotonic lysis was performed twice. These preparations contained >90% neutrophils. For a few experiments, cells were prepared by the Hypaque-Ficoll technique (15), which yielded preparations containing >99% neutrophils. The cells were suspended in phosphate-buffered saline (PBS) without Ca^{++} and Mg^{++} at a concentration of $3–9 \times 10^7$ cells/ml unless otherwise indicated.

Zymosan was opsonized just before use by the method of Hohn and Lehrer (16).

Measurement of O_2^- production by neutrophils. Superoxide production by neutrophils was determined by a modification of the cytochrome *c* method previously described (3). Because aqueous solutions of NaF react with glass, all incubations were performed in siliconized flasks. NaF, 0.6 M, was prepared before use and stored in polyethylene. The buffer employed was a modified Dulbecco's (17) PBS solution (PBS′) in which the concentration of NaCl was reduced so that the final salt concentrations would be physiological after the addition of NaF, and $CaCl_2$ was decreased to 0.31 mM to avoid the precipitation of CaF_2. Incubations were performed as follows: A mixture that contained PBS′, cytochrome *c*, and (when indicated) superoxide dismutase was warmed to 37°C in a shaking water bath for 10 min. The reaction was started by adding neutrophil suspension plus the desired amount of NaF. Where NaF was omitted, a corresponding amount of 0.6 M NaCl was added to restore tonicity. Immediately after starting the reaction, a 1.0-ml portion of the mixture was withdrawn with a plastic syringe and reserved in melting ice to serve as reference. The remaining portion was incubated at 37°C for 30 min (unless otherwise noted), after which another 1.0-ml aliquot was placed on ice. Neutrophils were removed by centrifugation at 1,500 *g* for 5 min at 4°C, and O_2^--dependent cytochrome *c* reduction determined spectrophotometrically, as previously described (18). Most results are expressed as nmol $O_2^-/10^6$ cells per h. The rate of O_2^- production was in fact proportional to cell concentration from 0.5 to 5×10^6 cells/ml; it did not, however, follow a linear course with time. The data are expressed per hour to facilitate comparisons, but we do not imply linearity of the reaction for that length of time.

In experiments examining O_2^- production by neutrophils in sucrose, PBS′ was replaced by a solution of the following composition: 240 mM sucrose, 10 mM Hepes, 0.5 mM $MgCl_2$, and 0.33 mM $CaCl_2$. The pH of the solution was adjusted to 7.4 with 0.5 N NaOH (or 0.5 N KOH when a Na^+-free medium was desired). In assays in which Ca^{++} was omitted, PBS′ without Ca^{++} was prepared in polypropylene containers with water that was first distilled and then deionized in an ultrapure (Mixed Bed) filter (Barnstead Co., Boston, Mass.), and disposable polystyrene pipettes were used for transferring solutions. These precautions were necessary to minimize Ca^{++} contamination from glassware and water.

Measurements of release of granule enzymes. Degranulation was determined by a modification of the method of Goldstein et al. (19). PBS′, 16.8 ml, was placed in each of three siliconized flasks and warmed to 37°C in a shaking water bath for 10 min. 0.6 ml of neutrophil suspension (2.7×10^8 cells/ml) was then added to each flask, followed by 0.6 ml of the agent to be tested (0.6 M NaF, 0.6 M NaCl or PMA [150 μg/ml PBS′]). The PMA flask also received 0.6 ml of 0.6 M NaCl, to equalize tonicity. Immediately after the addition of these agents, 4.0-ml and 2.0-ml portions of the reaction mixtures were reserved on ice. Additional 2.0-ml portions were withdrawn after incubation for 30 min at 37°C. Cells were removed from the 2.0-ml portions by centrifugation at 1,500 *g* for 10 min at 4°C, whereas the 4.0-ml portions were sonicated at 0°C for 2 min (four 30-s pulses) with a Sonifier cell disrupter (model W140, Heat Systems-Ultrasonics, Inc., Plainview, N. Y.). The following assays were performed on both the sonicated 4-ml samples and the supernates from the 2-ml samples: myeloperoxidase (20), β-glucuronidase (21), lysozyme (22), lactic dehydrogenase (23), and protein (24). Enzyme activity in 0.1 ml of supernate was taken to represent the enzyme released by degranulation, whereas the activity in 0.1 ml of sonicate was used as a measure of the total enzyme content. Results were expressed as percent of total cellular enzyme released.

Uptake of paraffin oil by neutrophils. Paraffin oil uptake by neutrophils in the presence and absence of F^- was determined by a modification of the method of Stossel (25, 26). Paraffin oil that contained Oil Red O was emulsified with an isotonic sucrose medium (see above for formula) that contained 20 mg/ml bovine albumin. The emulsion was opsonized

[1] *Abbreviations used in this paper*: NBT, nitroblue tetrazolium; PBS, phosphate-buffered saline; PBS′, phosphate-buffered saline with modified salt concentrations for use with sodium fluoride.

by incubating with an equal volume of fresh human serum for 15 min at 37°C. To measure phagocytosis, reaction mixtures were prepared that contained 8.34 ml isotonic sucrose medium, 0.33 ml neutrophil suspension (2.7×10^8 cells/ml isotonic sucrose medium), and 0.33 ml of either 0.6 M NaF or 0.6 M NaCl. The reactions were started by adding 1.0 ml of opsonized emulsion and the mixtures were incubated at 37°C in a shaking water bath. At the times indicated in Fig. 4, 0.9-ml aliquots were removed and mixed with 5 ml ice-cold saline that contained 1 mM *N*-ethylmaleimide. Cells were washed and Oil Red O extracted and measured as previously described (25).

Nitroblue tetrazolium (NBT) stain. A modification of the NBT method of Baehner and Nathan (27) was used to determine whether activation of the respiratory pathway had occurred in individual cells. Solutions of NBT were prepared by dissolving 100 mg of NBT in 100 ml PBS′ and filtering (0.44 μm Millipore filter, Millipore Corp., Bedford, Mass.) to remove undissolved residue. The NBT-staining reaction was performed in a manner similar to the O_2^- assay described above, except that PBS′-NBT was substituted for the PBS′, and cytochrome *c* was omitted. The samples were removed at the times indicated in Table V, placed on ice, and then examined in a hemacytometer. Those neutrophils showing blue formazan deposits were recorded as positive.

RESULTS

The effect of F^- on O_2^- production by neutrophils. The results in Table I show that 20 mM F^- is a potent stimulus for O_2^- production by neutrophils. The fact that cytochrome *c* reduction by this system was greatly inhibited by active superoxide dismutase but was almost unaffected by autoclaved dismutase, catalase, and albumin indicates that O_2^- was indeed the electron donor responsible for most of the cytochrome *c* reduction that took place in the reaction mixture. (The slight effect of autoclaved dismutase is probably caused by the copper released from the denatured enzyme [28]). Negligible cytochrome *c* reduction occurred when F^- was omitted. Similarly, little absorbance change was seen at 550 nm when the neutrophils were omitted, or inactivated by boiling before incubation, or when cytochrome *c* was omitted from the reaction mixture. Cell viability as determined by trypan blue exclusion was not affected by 20 mM F^- during a 50 min incubation at 37°C.

TABLE I
Stimulation by Fluoride of O_2^--Dependent Cytochrome c Reduction by Neutrophils

Reaction mixture	Cytochrome *c* reduction
	nmol/10⁶ cells/h
Complete	69.1±0.9 SE
+ Superoxide dismutase	6.6±0.5
+ Autoclaved dismutase*	59.5±1.7
+ Catalase	68.7±1.3
+ Albumin	68.3±1.7
Omit F^-	1.1±0.1
Omit cells	0.1±0.1
Boiled cells‡	1.4±0.3

The complete reaction mixture contained, in a volume of 3.0 ml, neutrophils (3×10^6 cells/ml), cytochrome *c* (80 μM), and NaF (20 mM). Where indicated, superoxide dismutase, autoclaved superoxide dismutase, catalase, or ovalbumin was present at a concentration of 20 μg/ml. Reaction mixtures were incubated 30 min at 37°C. Cytochrome *c* reduction was determined as described in Methods. The data represent the results from four cell preparations.

* Inactivated by autoclaving for 20 min at 121°C.

‡ Cells were heated for 1 min in boiling water before use.

TABLE II
Effect of Halides and a Particulate Fluoride on O_2^- Production by Neutrophils

Agent	O_2^- produced	
	Experiment 1	Experiment 2
	nmol/10⁶ cells/h	
F^-	84.4	78.0
Br^-	0.2	0.0
I^-	0.5	0.2
CaF_2	1.3	0.3
None	0.6	0.3

For each assay, duplicate reaction mixtures were prepared, each containing, in a volume of 10.5 ml, neutrophils (1.5×10^6/ml), cytochrome *c* (80 μM), and one of the agents under investigation. In one of each pair of reaction mixtures, superoxide dismutase (10 μg/ml) was included, whereas in the other no dismutase was present. NaF, NaBr, and NaI were each present at 20 mM. In the case of CaF_2, which is insoluble, each reaction mixture contained 0.78 mg/ml of the CaF_2 crystals. Superoxide-dependent cytochrome *c* reduction was determined as described in Methods. The amount of O_2^- produced in each of the two experiments is shown in the table, and is expressed on the basis of 1 h, although the incubation was for 30 min only (see Methods).

The neutrophil preparations used for the experiments in Table I were generally contaminated to the extent of ≅10% by mononuclear cells, mostly lymphocytes. To establish unequivocally that F^- is able to stimulate O_2^- production by neutrophils themselves, a highly purified preparation of these cells was obtained by the Hypaque-Ficoll technique of Boyum (15). The production of O_2^- by these cells in the presence of F^- was determined. The Hypaque-Ficoll preparation, which contained >99% polymorphonuclear leukocytes, produced 55.3 nmol $O_2^-/10^6$ cells per h under the conditions described in Table I. Neutrophils prepared simultaneously from the same donor, but by the standard procedure rather than the Hypaque-Ficoll technique, contained 10% contaminating mononuclear cells, and produced 50.7 nmol $O_2^-/10^6$ cells per h. These findings demonstrate that the neutrophil itself produces O_2^- in response to F^-.

Other halides were tested for their ability to stimulate neutrophil O_2^- production (Table II). Neither Br^- nor I^- had any effect on this process at a concentration of 20 mM. Although Cl^- was not specifically tested, it, too, must be without effect, because all the experiments so far reported were carried out with the cells suspended in 0.14 M Cl^-, and activation was not observed unless F^- was present as well. F^- is thus unique among the halide ions in its ability to stimulate human neutrophils. The poorly soluble salt CaF_2, which might be present in incubation mixtures as the result of a reaction between separately added ions, also failed to activate the cells. In control experiments not shown, neutrophils remained viable (as judged by trypan blue exclusion) after 40 min of incubation with each of the agents listed in Table II.

The cation requirement for F^--mediated neutrophil activation was examined by the omission of various cations from the incubation medium. The results of these experiments (Table III) indicate that activation can take place in the absence of exogenous K^+ or Mg^{++}. Calcium, however, is required for activation by F^-; omission of this cation reduced O_2^- production to <7% of control. The requirement for this ion as a function of concentration is indicated in Fig. 1. The fact that this curve does not reach a plateau suggests that the maximum effect of Ca^{++} was not expressed even at the highest concentration employed in these experiments. Higher concentrations could not be achieved because of precipitation of CaF_2 in the incubation mixtures.

TABLE III
Cation Requirement for Fluoride Stimulation of O_2^- Production by Neutrophils

Reaction mixture	O_2^- produced	
	Experiment 1	Experiment 2
	nmol/10⁶ cells/h	
Complete	40.7	30.5
Omit K^+	40.3	32.8
Omit Mg^{++}	36.9	32.5
Omit Ca^{++}	2.5	0.8

Superoxide-dependent cytochrome *c* reduction was determined as described in Methods using duplicate reaction mixtures that contained, in a volume of 9.0 ml, neutrophils (3×10^6 cells/ml) cytochrome *c* (80 μM), and NaF (20 mM). Superoxide dismutase (10 μg/ml) was included in only one of each duplicate. The complete reaction mixture was made with PBS'. In the assays where K^+ was omitted, a special PBS' was used instead in which equivalent molar amounts of NaCl and NaH_2PO_4 substituted for KCl and KH_2PO_4, respectively. In the cases where either Mg^{++} or Ca^{++} was omitted, $MgCl_2$ or $CaCl_2$ was not included in the PBS' (see Methods for special technique used to obtain Ca^{++}-free PBS'). Incubations were conducted at 37°C for 60 min, and O_2^- production determined as described in Methods.

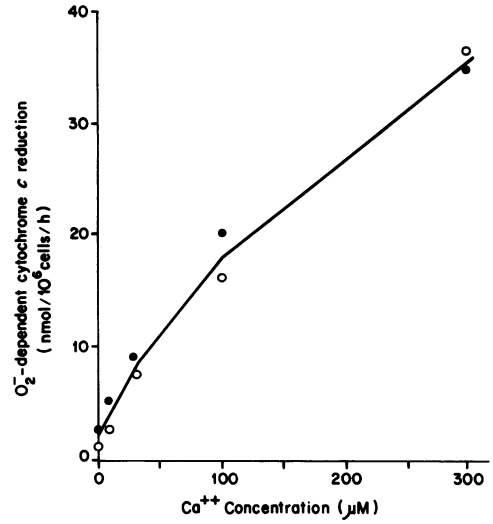

FIGURE 1 The effect of calcium concentration on O_2^- production by neutrophils stimulated with NaF. Experiments were carried out exactly as described in Table III using PBS' without Ca^{++} to which indicated concentrations of $CaCl_2$ were added. Closed symbols, experiment 1; open symbols, experiment 2. Different cell preparations were used for the two experiments.

To characterize the effect of F^- on neutrophils further, O_2^- production was measured as a function of F^- concentration and time. These measurements, presented in Fig. 2, disclosed an unexpected feature. There was a pronounced delay between the addition of F^- and the onset of O_2^- production, a delay which could not be overcome at any F^- concentration employed in these experiments. Inhibition by F^-, evident as a decline in the total O_2^- production at greater than 18 mM F^- (Fig. 2, lower panel), prevented the use of higher concentrations.[2] Furthermore, the length of the delay was sharply dependent on F^- concentration. For example, when the F^- concentration is halved from 20 mM to 10 mM, the delay in O_2^- production increased from ≅10 to ≅40 min.

Although it was not possible to eliminate the delay when the cells were incubated in PBS', it could be shortened by performing the incubation at greatly diminished salt concentrations. This suggests that the delay might be caused, at least in part, by competition between F^- and the inert anion Cl^- for the burst-activating system. Fig. 3 shows the course of O_2^- production by cells activated in isotonic sucrose free of salts except for 10 mM Na^+-Hepes buffer, 0.33 mM $CaCl_2$, 0.5 mM $MgCl_2$, and NaF at the concentration

[2] This decrease in total O_2^- production may be caused, at least in part, by clumping of cells at the higher F^- concentrations. (Craddock, P. Personal communication.)

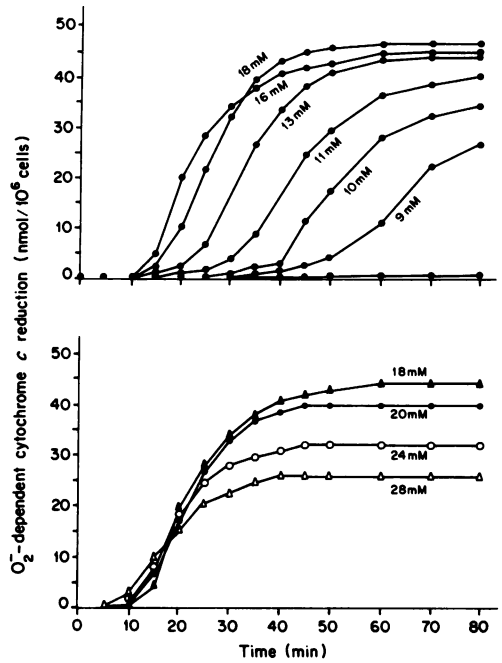

FIGURE 2 The effect of fluoride concentration on O_2^- production by neutrophils. Superoxide-dependent cytochrome *c* reduction was determined as described in Methods. Reaction mixtures contained, in a volume of 16.0 ml, neutrophils (2 × 10^6 cells/ml), cytochrome *c* (80 μM), and NaF at the concentrations indicated. Superoxide dismutase was present in a duplicate of each reaction mixture at a concentration of 10 μg/ml. For those incubation mixtures in which NaF concentration was <28 mM, an appropriate amount of NaCl was added to replace the omitted NaF. Upper panel, NaF concentrations between 0 and 18 mM. Lower panel, same experiment, NaF concentrations between 18 and 28 mM. The experiment was performed three times with three different preparations of cells. The figure shows a representative experiment.

shown (Mg^{++} was subsequently found not to be required). The potency of F^- as both activator and inhibitor is exaggerated under these conditions. For example, cells incubated in sucrose with 2 mM F^- produced 13 nmol O_2^- by 30 min (curve 4), whereas cells in PBS' with 4.5 times the concentration of F^- required 60 min to produce the same quantity of O_2^- (curve 2, Fig. 2). Inhibition by F^- became apparent in sucrose at 10 mM F^- and was pronounced at 20 mM F^-. In contrast, inhibition in PBS' began to appear only at 18–20 mM F^-. In terms of the delay, the use of 20 mM F^- in sucrose medium shortened it to only 1 min (see Fig. 3 inset). This value may be close to the intrinsic time period necessary for activation to take place, because other groups have reported a reproducible delay of 0.5–1 min between stimulation of neutrophils and the onset of the respiratory burst (29, 30).

The finding that neutrophils can be activated in sucrose confirms the idea that (apart from low concentrations of Ca^{++}) specific exogenous cations are not required for the stimulation of the burst. Experiments with K^+-Hepes and KF in sucrose showed that Na^+, too, was dispensible (not shown). It also reopens the question as to whether halide ions besides F^- are able to stimulate the burst. It could be argued, for example, that because at least part of the delay seen with 20 mM F^- in PBS' may be a result of competition between F^- and Cl^-, then the failure to activate with Br^- and I^- could be caused by a similar but more efficient competitive effect of Cl^-. We found, however, that cells in sucrose were not activated by 20 mM Br^- or I^-, indicating that the ions themselves were in fact not capable of stimulating the cells.

Phagocytosis and degranulation in the presence of F^-. Data acquired over the past few years has suggested that the activation of the respiratory burst occurs independently of both phagocytosis and degranulation. Results below provide support for this view.

The effect of F^- on phagocytosis was examined with opsonized Oil Red O-labeled paraffin oil droplets as the ingestible particle (25) (Fig. 4). When neutrophils in sucrose were incubated with F^- at a concentration that stimulated vigorous O_2^- production by the cells, phagocytosis was virtually abolished. Conversely, Cl^-, which had no effect on O_2^- production by resting cells, permitted active phagocytosis to take place.

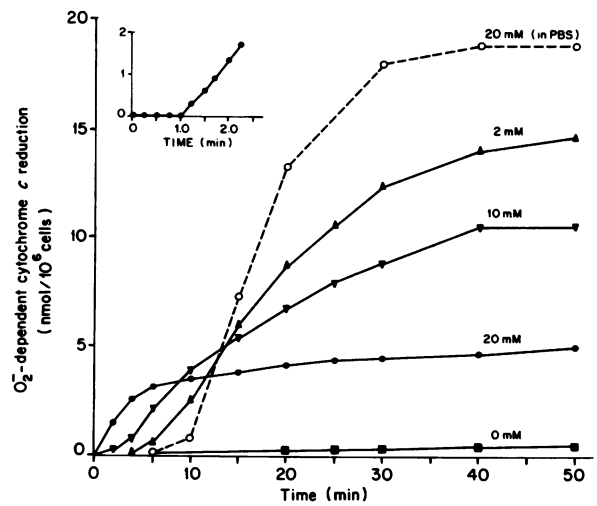

FIGURE 3 The effect of fluoride concentration on O_2^- production by neutrophils incubated in isotonic sucrose. Superoxide-dependent cytochrome *c* reduction was determined as described in Methods using the modification in which an isotonic sucrose solution is substituted for PBS'. For comparison, one reaction was performed using PBS'. Each reaction mixture contained, in a volume of 9.0 ml, neutrophils (3 × 10^6/ml), cytochrome *c* (80 μM), and NaF at the concentrations indicated below. Superoxide dismutase, when included, was present at a concentration of 10 μg/ml. Inset: higher resolution time-course of O_2^- production in isotonic sucrose with 20 mM F^-. The figure shows a representative experiment. The experiment was performed three times with three different preparations of cells.

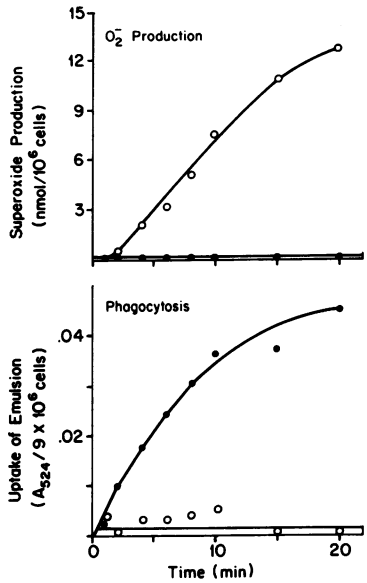

FIGURE 4 The effect of fluoride and chloride on both phagocytosis and O_2^- production by neutrophils. Phagocytosis was measured as described in Methods. Superoxide production was determined simultaneously in reaction mixtures that were identical to those used for phagocytosis except that cytochrome *c* (80 μm) was present, the concentration of neutrophils was reduced to 3 × 10^6 cells/ml, and the paraffin oil emulsion was replaced by an equal volume of a solution made by mixing 1 part serum with 1 part bovine albumin solution (20 mg/ml isotonic sucrose). Top panel, O_2^- production. Bottom panel, phagocytosis. Open symbols, 20 mM NaF present. Closed symbols, NaCl replaced NaF. The experiment was performed in duplicate with two different preparations of cells. The figure shows a representative experiment.

These findings show that the activation of O_2^- production by F^- occurs independently of phagocytosis. Although phagocytosis was not necessary for activation of the respiratory burst, it is conceivable that degranulation is required for this process to occur. Degranulation normally takes place in conjunction with phagocytosis, the granules fusing with the internalized plasma membrane to deliver their contents into the phagosome. However, degranulation of nonphagocytizing cells, with extracellular release of granule contents, is a well-recognized phenomenon (19, 31–34). It is possible that even though it blocks phagocytosis, F^- may induce the fusion of the granules with the plasma membrane, and that it is this event that is responsible for the initiation of O_2^- production. To examine this possibility, measurements were made of the liberation of granule contents into the medium upon exposure of neutrophils to F^-; this method has been used in the past to assess degranulation in response to various stimuli (19, 34). The results (Table IV) show that neither azurophilic granules (myeloperoxidase and β-glucuronidase as indicators) nor specific granules (lysozyme as indicator) release their contents when neutrophils are exposed to F^-. In contrast, the exposure of these cells to PMA causes them to release lysozyme, confirming the report of Estensen et al. (34) that this agent induces the fusion of specific but not azurophilic granules with the plasma membrane. The release of lactic dehydrogenase, a cytoplasmic enzyme, was used to evaluate nonspecific cytolysis. The data confirm the trypan blue results indicating that 20 mM F^- did not disturb cellular integrity under the conditions of these experiments. Thus, activation of O_2^- production by F^- requires neither phagocytosis nor degranulation.

Reversibility of F^--mediated activation of O_2^- production by neutrophils. One question that has not been answered to date is whether the respiratory burst requires the continuous presence of the stimulus, or whether, once initiated, it can proceed in the absence of the stimulus. We investigated this question by exposing cells to F^- for various intervals of time, washing them to remove the F^-, and then assaying the washed cells for O_2^- production. For this experiment, an aliquot of cells from a single cell preparation was assayed for O_2^- production in the presence of F^- using cytochrome *c* as the trapping agent under the usual conditions. The remaining cells were stimulated with F^- in the absence of cytochrome *c* for various intervals of time in separate reaction flasks. At the appropriate times, the flasks were placed on ice to stop the reactions and the cells were washed, resuspended in fluoride-free medium, and assayed for O_2^- production by the usual method. In this manner, O_2^- production by the same population of fluoride-stimulated cells could be compared under two sets of conditions: one, in which exposure to F^- was continued even after the cells had been stimulated, and the other, in which the F^- was removed at some point after stimulation.

TABLE IV
Effect of Fluoride on Release of Granule Enzymes into Extracellular Medium

Enzyme	Incubation conditions		
	Control	+F^-	+PMA
		% Release	
Myeloperoxidase	0.6±0.2 SE	0.7±0.2	1.1±0.2
β-Glucuronidase	2.3±0.6	3.0±0.5	5.4±1.1
Lysozyme	1.8±1.0	2.5±1.8	69.9±14.3
Lactic dehydrogenase	6.7±0.8	6.1±1.0	3.5±0.7

Enzyme release was measured as described in Methods. Total enzyme contents per 10^6 cells were: myeloperoxidase 83.4±2.9 SE; β-glucuronidase 0.190±0.012; lysozyme 9.07 ±1.47; lactic dehydrogenase 17.0±3.3. All activities except lysozyme are expressed in milliunits (the amount of enzyme that converts 1 nmol substrate in 1 min under the conditions given in Methods). Lysozyme activity is expressed in units defined in reference 24. Data are from three experiments, each performed with a different preparation of cells.

Fig. 5 shows the results of this experiment. The upper panel shows O_2^- production in the continuous presence of F^-, whereas the lower panel shows O_2^- production by cells exposed to F^- for the times noted, then assayed in the absence of that agent. It is evident that cells that were exposed to F^- long enough to activate the burst were able to make O_2^- for a considerable period even after exogenous F^- was removed. For example, cells in which O_2^- production had been stimulated by a 15-min exposure to F^- (arrow 6, upper panel of Fig. 5) were still capable of generating O_2^- after being washed and transferred to fluoride-free medium (curve 6, lower panel). This example also demonstrates, however, that the total quantity of O_2^- produced by cells after the removal of F^- (in this case, 7 nmol O_2^-) was substantially less than the amount made by the same cells when they were continuously exposed to F^- (again in this case, 20 nmol O_2^-). This result indicates that for the respiratory burst to be maintained and to reach completion, at least with F^- as the stimulus, the continuous presence of the activating agent is required.

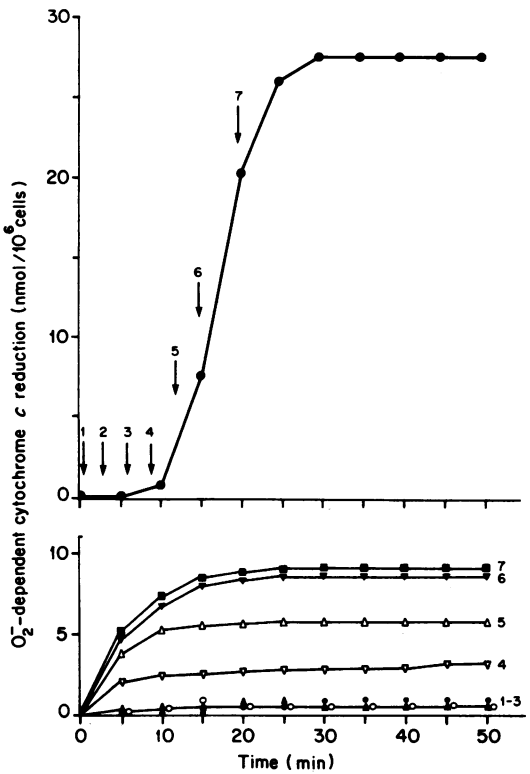

FIGURE 5 Superoxide production by fluoride-stimulated neutrophils after removal from fluoride. Nine identical reaction mixtures were prepared in siliconized flasks as described in the O_2^--assay section of Methods (with PBS') except that cytochrome *c* was omitted from the first seven and superoxide dismutase (10 μg/ml) was included in the ninth. Flasks 1–7 were used to expose cells to F^- for various intervals of time after which the F^- was washed away. Flasks 8 and 9 were used to monitor O_2^- production. Each flask contained, in a volume of 12.0 ml, neutrophils (3×10^6 cells/ml) and NaF (20 mM). Flasks 8 and 9 also contained cytochrome *c* (80 μM). The first seven flasks were incubated for various lengths of time as indicated by the seven arrows in the upper panel. At each of these times one of the seven reaction flasks was placed in melting ice. At each of these times, also, 1.0-ml aliquots were removed from flasks 8 and 9 for O_2^- measurements. The upper panel shows the amount of O_2^- generated at each of the times one of the seven reactions was terminated (arrows 1–7). The cells from each of the first seven flasks were washed twice with 10 ml PBS without Ca^{++} and Mg^{++} and resuspended in 12.0 ml of prewarmed medium that contained cytochrome *c* but not NaF. Superoxide production by each of the seven samples of cells was then measured and the results are shown in the lower panel. Each curve is numbered according to the sample of cells it represents. The experiment was performed twice with two different cell preparations. A representative experiment is shown in the figure.

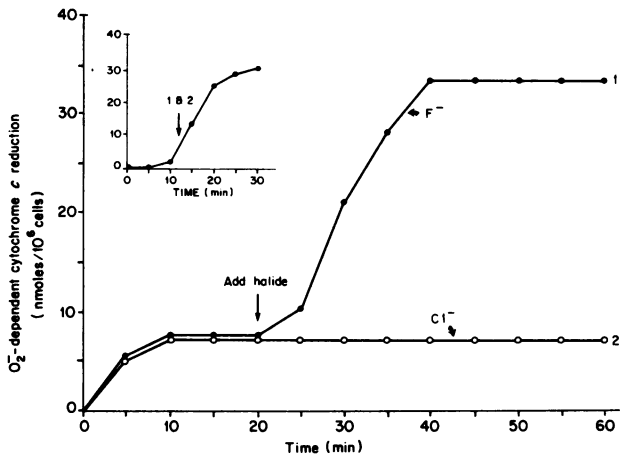

FIGURE 6 The effect of re-exposure to fluoride on previously stimulated neutrophils after they have returned to resting metabolism. The experiment was carried out in a manner similar to that described in Fig. 5. Two reaction mixtures were used to monitor O_2^- production by cells exposed to F^- for the first time and the results are shown in the inset. Simultaneously, cells in two other reaction flasks (1 and 2; vol 15.0 ml each) were exposed to F^- long enough to activate the respiratory burst. The arrow in the inset indicates the point at which flasks 1 and 2 were placed on ice to terminate F^- stimulation. Cells from these flasks were washed and then resuspended in 15.0 ml of fresh fluoride-free media that contained 80 μM cytochrome *c*. Superoxide production by each of the two samples of cells was followed for 20 min and is shown in the figure. At the point indicated by the arrow, a small aliquot of NaF was added to the cells from flask 1 (final [F^-] was 20 mM). An equal dose of NaCl was added to the cells from flask 2. Superoxide production by these cells is shown by curves 1 and 2, respectively. The experiment was performed twice with two different cell preparations. A representative experiment is shown in the figure.

The difference between the total amount of O_2^- produced by stimulated cells incubated in the presence vs. the absence of F^- suggested that the mechanism for termination of O_2^- production might differ in the two situations. In particular, it raised the possibility that the termination of O_2^- production in the absence of F^- could result from a process whereby the O_2^--forming system

TABLE V

Determination of NBT-Positive Neutrophils during Two Successive Activations with Fluoride

Reaction conditions	Experiment 1		Experiment 2	
	O_2^- produced	NBT positive	O_2^- produced	NBT positive
	nmol/10⁶ cells/h	%	*nmol/10⁶ cells/h*	%
First treatment				
With F^-	90.1	100	32.0	94
Omit F^-	0.5	0	0.3	0
Second treatment				
With F^-	85.2	92	36.0	89
Omit F^-	0.1	0	0.1	1

For the first treatment, cells (3×10^6/ml) were incubated either in the presence or absence of NaF (20 mM) for 15 min. The amount of O_2^- produced and the percentage of cells stained with NBT during the 15 min incubation were determined as described in Methods. Simultaneously, a flask with the same preparation of cells (24 ml; 3×10^6 cells/ml) was similarly exposed to 20 mM NaF for 15 min except that cytochrome *c* and NBT were omitted. In this case, the reaction was terminated and the cells washed as described in Fig. 5. The cells were then resuspended in 24 ml warm PBS′ (containing no F^- or cytochrome *c*) and allowed to return to the resting state for 20 min at 37°C. They were again centrifuged, resuspended in 0.8 ml PBS′ without Ca^{++} and Mg^{++}, and then assayed for O_2^- production and NBT staining in the presence and absence of F^- just as was done for the first treatment. In experiment 1, cells were prepared by the standard technique and contained 85% neutrophils. In experiment 2, cells were purified by the Hypaque-Ficoll technique and contained 99% neutrophils.

in the neutrophil was restored to its resting state. If this were true, it might be expected that re-exposure of the cells to F^- after the cessation of O_2^- production would again stimulate the O_2^--forming system.

This possibility was tested in the experiment presented in Fig. 6. In this experiment, two parallel incubations were carried out in which O_2^- production by neutrophils was measured in the absence of F^- with cells that had been stimulated by F^- for 12 min and then washed. After O_2^- formation by the washed cells had stopped, one incubation flask was supplemented with NaF (final concentration 20 mM), while a similar quantity of NaCl was added to the other. The figure shows that O_2^- production resumed in the flask to which F^- was added, but did not resume in the other. In experiments not shown here, O_2^- production also resumed when opsonized zymosan was added to the cells instead of F^-. The maximum rate of O_2^- production by cells stimulated by F^- for the second time was the same as the maximum rate achieved during the original activation step, as shown in the inset. These findings suggest that the activation of the O_2^- forming system in neutrophils is reversible, because under suitable conditions, O_2^- production by these cells can be sequentially started, stopped, and then started again.

Whereas reversibility of activation is one interpretation of the data in Figs. 5 and 6, it is not the only one. It is possible, for example, that during the first exposure to F^- not all the cells were stimulated, and that the second burst in O_2^- production resulted from the effect of F^- on cells that were not stimulated the first time. To test this possibility, a modification of the NBT-reduction method of Baehner and Nathan (27) was used to determine the fraction of cells stimulated during each incubation with F^-. Neutrophils stimulated in the presence of NBT reduce the dye to an insoluble blue formazan which remains associated with cells that have undergone the respiratory burst. This precipitate, which is easily seen under the microscope, can serve as a marker permitting the state of stimulation of individual cells to be evaluated. The results of Table V show that during the first exposure, nearly all the F^- treated neutrophils were affected, although none of the Cl^- treated cells were. When the same measurements were made on cells that had been preactivated with F^- and then incubated in an F^--free medium long enough to restore resting oxygen metabolism, comparable results were obtained; that is, almost all such cells were affected by a second exposure to F^-, whereas none showed formazan deposits when incubated with Cl^-. The fact that over 90% of the neutrophils were stimulated during each of two successive exposures to F^- indicates that the same cells that were stimulated during the first F^- exposure must have also been stimulated during the second.

Although the foregoing experiments show that the same population of cells can be stimulated twice, they do not firmly establish that the activation of the O_2^--forming oxidase is reversible, i.e., that the oxidase itself can be turned on, off, and then on again. It could be argued that during the first exposure to F^-, only a fraction of the oxidase was activated by the time the F^- was removed. The O_2^- production during the second F^- incubation, then, could have been the result of F^- activating the remaining unused enzyme. To test this possibility, an experiment was done to determine whether the length of the first F^- exposure has any effect on the size of the second burst of O_2^-. The rationale for this experiment, if the above argument is correct, is that an increase in the length of the first F^- incubation should activate a larger fraction of the oxidase. This, in turn, should result in a diminished amount of O_2^- produced during the second F^- exposure because fewer unused enzyme molecules would be available for activation.

For this experiment, a series of aliquots of neutrophils was obtained which had been exposed to F^- for various lengths of time; three aliquots (A, B, and C) for each length of exposure. Aliquot A was used to determine the amount of O_2^- that had been produced during the incubation with F^-. Cells from B and C were washed to remove the F^-. Those from B were then resuspended in fresh medium that contained

TABLE VI

Reversibility of Fluoride-Induced Respiratory Burst

Duration of first exposure to F^-, *min*	O_2^- Produced									
	5	10	15	20	25	30	35	40	50	60
	nmol O_2^-/10^6 cells									
First exposure to F^- (set A)	0.0	1.3	13.2	29.0	36.7	41.6	46.2	48.8	53.3	55.2
Second exposure to F^- (set B)	47.6	51.5	43.5	28.6	20.2	15.9	11.5	*	*	*
Sum (A + B)‡	47.6	52.8	56.7	57.6	56.9	57.5	57.7			
Re-exposure to F^- for 40 min after returning to resting state (set C)	39.6	51.6	55.4	52.2	50.8	44.7	40.0	*	*	*

Neutrophils were stimulated with F^- for the first time at 37°C in a siliconized 500-ml flask that contained, in a volume of 186 ml, neutrophils (10^6 cells/ml), cytochrome *c* (100 μM), and NaF (20 mM). Superoxide production was determined on aliquots of this mixture (set A) at 5-min intervals up to 60 min. In addition, pairs of aliquots (for sets B and C) were removed (B, 9 ml; C, 15 ml) at 5-min intervals (between 5 and 35 min, inclusive) and placed on ice to stop the reaction. To promote more rapid cooling, 1.5 vol of ice-cold PBS without Ca^{++} and Mg^{++} were added to each aliquot. The chilled cells were then centrifuged and washed twice with 10 ml PBS without Ca^{++} and Mg^{++}. Cells from B were resuspended in 9 ml warm PBS′ that contained cytochrome *c* (100 μM) and NaF (20 mM), and O_2^- production was then measured. The second line of the table shows the amount of O_2^- made by these cells. Cells from set C were resuspended in 15 ml of fluoride-free PBS′ that contained cytochrome *c* (100 μM) only and allowed to return to the resting state for 20 min. At that point, the reaction mixture was divided into two equal parts and NaF was added to one half (final concentration 20 mM) and NaCl to the other. Superoxide production was then measured during the next 40 min and the results are shown in the last line of the table. In all incubations shown in this table, cytochrome *c* was in at least twofold excess as confirmed by dithionite reduction of reaction samples. The results shown in this table are from a representative experiment. The experiment was performed four times with four different preparations of cells.

* Aliquots (for B and C) of cells exposed to F^- for these times were not analyzed.

‡ This sum represents the total O_2^- made in 60 min at 37°C. In every case, O_2^- production by set B had ceased by the end of the incubation.

20 mM F^-, and O_2^- production measured. Cells from C, on the other hand, were resuspended in medium that did not contain F^- and were incubated for 20 min to allow the cells to return to the resting state. At that point, F^- was added and O_2^- production determined.

The results of this experiment are shown in Table VI. The first line of the table shows the amount of O_2^- produced by each of the aliquots of neutrophils incubated with F^- for the times indicated. These results are very similar to those presented in Figs. 2 and 5. The next line shows the amount of O_2^- generated by the cells from the B aliquots, i.e., the cells immediately re-exposed to F^- after washing. This number represents the amount of O_2^- produced in a time period equal to 60 min minus the length of the first exposure. It is apparent that the more O_2^- produced during the first exposure to F^-, the less was produced during the second. The sum of the two values was constant, and was with one exception equal to the maximum amount of O_2^- produced in 60 min by cells in which exposure to F^- was not interrupted. These results show that, if added immediately after the washing of stimulated cells, F^- permits O_2^- production to continue as if the exposure to the agent had never been interrupted.

The results also demonstrate that when cells are washed in the cold to remove F^-, and then resuspended, their subsequent respiratory behavior is dictated by the presence or absence of F^- in the new medium, rather than by changes in the properties of the oxidase caused by the washing procedure. This conclusion is strengthened by the fact that the curves describing the time course for O_2^- production during the pre- and postwash incubations of cells from aliquot B could be superimposed on the corresponding portions of the curve obtained with aliquot A. It therefore appears that cooling the cells merely arrests whatever respiratory burst-related metabolic events are taking place, without affecting the enzymes responsible for these processes.

In sharp contrast to these results are the findings shown in the last line of Table VI with cells from the C aliquots. The data show that if the second F^- exposure was delayed for 20 min after washing, i.e., if the cells were incubated at 37°C without F^- to allow them to return to the resting state, then they made as much O_2^- when the F^- was again added as expected if they had never been activated before. The situation was not influenced by the extent of prior activation. An example may help to clarify this: cells exposed to F^- for 10 min during the first activation step produced only 1.3 nmol O_2^-/10^6 cells, whereas those incubated for 25 min produced 36.7 nmol O_2^-/10^6 cells (the latter value represents 65% completion of the O_2^- burst) (set A). Despite this large difference in the extent of the first activation, both sets of cells, after returning to rest, produced within experimental error the same amount of O_2^- when restimulated with F^- (51.6 and 50.8 nmol O_2^-/10^6 cells during 40 min) (set C). An identical aliquot of cells, but one that had never been activated before, pro-

duced 48.8 nmol O_2^-/10^6 cells during the same period of time on exposure to 20 mM F^- (set A, 40 min point). These results make it highly unlikely that the O_2^- seen during the second F^- activation is produced by enzyme molecules not activated during the first F^- incubation, but suggest instead that it is produced by O_2^--forming enzyme molecules that had been turned on, then off, and then on again.

DISCUSSION

Several features regarding the stimulation of neutrophil O_2^- production by F^- are noteworthy. The first is the very broad range of conditions under which it takes place. O_2^- production is stimulated when the ionic composition of the suspending medium is similar to that of extracellular fluid (PBS'), when its ionic composition is qualitatively different, but the ionic strength is about the same (omission of Mg^{++} of K^+ from PBS'), and when the concentration of exogenously added electrolytes is negligible (isotonic sucrose medium). The only ion that appears to be essential for stimulation is Ca^{++}, and that only at very low concentrations. This insensitivity to the extracellular environment is not surprising because the neutrophil is capable of functioning in regions of inflammation, where the environment is likely to differ from that of blood and other healthy tissues.

Another point of interest is the question of whether phagocytosis or degranulation is required for activation of the respiratory burst. Many previously published reports have indicated that burst activation can be experimentally dissociated from both phagocytosis (4, 6, 19, 33, 35–44) and degranulation (19, 34, 45, 46). We have made similar observations: F^- stimulates O_2^- production without inducing degranulation and at the same time abolishes phagocytosis. We conclude on the basis of both our experiments and the studies mentioned above that activation of the respiratory burst is not dependent on either phagocytosis or degranulation.

The results presented in Tables V and VI and in Fig. 6 provide very strong evidence for the reversibility of the activation of respiratory burst. The results of the NBT experiments indicate that a given neutrophil can be activated, returned to rest, and then activated again. Furthermore, these results apparently do represent reversible activation per se, rather than activation of different portions of the O_2^--forming system during each of the two consecutive incubations with F^-. This is indicated by the fact that under suitable conditions the quantity of O_2^- generated during a second incubation with F^- was equal to the maximum that could be produced during the first stimulation. Others have reported reversible activation of granulocyte respiration in response to various stimuli, but the distinction between reversible stimulation and sequential partial stimulation occurring during each of consecutive incubations was not established (35, 47).

In physiological terms, the ability of a neutrophil to return to the resting state after activation in vivo would be of value. It is likely, for instance, that from time to time a neutrophil is activated accidentally by transient exposure to an activating agent at a location far distant from a site of inflammation. If this cell continued to express the respiratory burst after the activating agent had been removed from its vicinity, it would probably be destroyed by its own metabolic products, and in addition could inflict damage on nearby healthy tissues by the O_2^- and H_2O_2 liberated during the course of the burst. The ability of the cell to shut off the burst both restricts this damage and preserves it as a functional unit capable of participating in an inflammatory process at some later time.

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