BIOCHEMICAL MEDICINE 25, 106–113 (1981)

Effects of Fluoride on the Oxidative Metabolism of Human Neutrophils

LAWRENCE R. DECHATELET,[1] THIMYSTA L. CAMPBELL, MARY ALICE WESTRICK, AND PAMELA S. SHIRLEY

Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, North Carolina 27103

Received August 8, 1980

Phagocytosis by human neutrophils is accompanied by marked alterations in oxidative metabolism collectively referred to as the "respiratory burst." These alterations include increased oxygen consumption, hydrogen peroxide production, hexose monophosphate shunt activity, nitroblue tetrazolium dye reduction, superoxide production, and the generation of chemiluminescence (1). A number of soluble stimuli will likewise trigger this burst. These include, but are not limited to, detergents (2), phospholipases (3), phorbol myristate acetate (PMA) (4), and concanavalin A (5). Recently, evidence has been presented that high concentrations of sodium fluoride (20 mM) will likewise stimulate the respiratory burst; this observation has been based primarily on the ability of F^- to stimulate superoxide production (6,7), and fluoride has been suggested as a useful probe for investigating the mechanism of the inititation of the respiratory burst. The present communication compares the effect of fluoride on superoxide production by human neutrophils to that on a number of other parameters classically associated with the respiratory burst.

MATERIALS AND METHODS

Isolation of Cells

Neutrophils were isolated from heparinized peripheral blood of normal volunteer donors as previously described (8). Briefly, erythrocytes were sedimented with plasma gel (HTI Corp., Buffalo, N.Y.) and leukocytes obtained from the supernate by centrifugation at 160*g* for 8 min at 4°C. They were washed once with a modified Dulbecco's phosphate-buffered

[1] To whom inquiries should be addressed.

0006-2944/81/010106-08$02.00/0

saline (PBS′) in which the $CaCl_2$ was decreased to 0.31 mM as described by Curnutte *et al.* (7). All experimental manipulations performed in this study employed this buffer which was required to avoid the precipitation of CaF_2. Contaminating erythrocytes were removed by a brief exposure to distilled water, isotonicity was restored by the addition of hypertonic saline, and the cells were collected by centrifugation. Total and differential counts were performed and the cells suspended in PBS′ to the desired final concentration (usually 5×10^6 neutrophils/ml). This procedure routinely yielded a preparation containing 80% neutrophils; viability was greater than 95% as judged by the exclusion of trypan blue dye.

Metabolic Assays

The oxidation of [1-^{14}C]glucose to $^{14}CO_2$ was determined as previously described (4). Glucose oxidation was measured in resting cells or in cells incubated with opsonized zymosan (2.8 mg/ml) phorbol myristate acetate (1 μg/ml) or sodium fluoride (20 mM). The reaction was initiated by the addition of 5×10^6 PMNL in PBS′ and stopped after varying periods of time by the addition of 5% trichloroacetic acid. $^{14}CO_2$ liberated during the course of the incubation was trapped in a center well containing hyamine hydroxide and radioactivity determined in a liquid scintillation spectrometer. The generation of superoxide anion under various conditions was determined by means of the superoxide dismutase (SOD)-inhibitable reduction of cytochrome *c* as described by Babior *et al.* (9). The incubation volume was increased to 3.0 ml to make the conditions comparable to those in the assay for glucose oxidation. Results are expressed as the change in absorbance at 550 nm/5×10^6 PMNL. The ability of cells to incorporate ^{125}I into trichloroacetic acid-insoluble material was measured with the various stimuli as previously described (10). Nitroblue tetrazolium reduction by the cells was determined by the quantitative procedure of Baehner and Nathan (11). Generation of chemiluminescence was determined by means of a liquid scintillation spectrometer operated in the out-of-coincidence mode. This reaction was performed in plastic minivials in a total volume of 2.0 ml employing 1×10^5 PMNL/vial and 1×10^{-6} M luminol (12) at ambient temperature. Luminol was necessary in order to measure the chemiluminescent response induced by soluble stimuli.

RESULTS

Figure 1 illustrates the time course of superoxide production by human neutrophils activated by a number of stimuli. The soluble stimulus, phorbol myristate acetate, and the phagocytic challenge, opsonized zymosan, both resulted in a marked and rapid production of O_2^- which was maximal in 10 to 20 min. In contrast, O_2^- production induced by 20 mM F^- showed a lag period of 10 min followed by a much more gradual generation of superoxide. All three stimuli were examined in parallel in this experiment

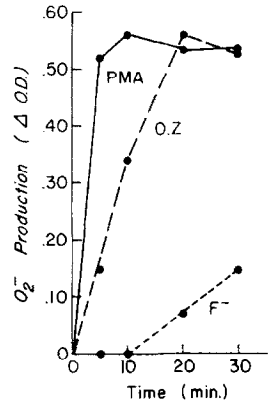

FIG. 1. Superoxide production as a function of time of incubation by neutrophils exposed to opsonized zymosan, phorbol myristate acetate, or fluoride. Each point represents the mean of triplicate determinations; this experiment was performed at one time with the same source of cells for each stimulus.

using the same source of cells. Similar observations were made in from 6 to 12 experiments where PMA or opsonized zymosan challenge always showed a very rapid and linear rate of O_2^- production, while fluoride invariably showed a slower rate of O_2^- production which was always preceded by a lag time of approximately 10 min. This apparent difference in kinetics led us to examine several other parameters associated with the respiratory burst. Table 1 illustrates glucose oxidation and superoxide production run in parallel from 5 to 30 min. Very little superoxide was produced by resting cells while a rapid rate of production which was maximal within 10 min was induced by phagocytosis of opsonized zymosan. In accord with the data in Fig. 1, the addition of 20 mM fluoride initiated a slower rate of O_2^- production after a lag time of

TABLE 1
GLUCOSE OXIDATION AND SUPEROXIDE PRODUCTION BY HUMAN NEUTROPHILS INDUCED BY A VARIETY OF STIMULI

	Oxidation of [1-^{14}C]glucose (cpm)			Superoxide production (ΔOD 550 nm)		
Time	Resting cells	Opsonized zymosan	NaF	Resting cells	Opsonized zymosan	NaF
5	37	1441	35	0.005	0.224	0.007
10	155	2442	61	0.000	0.387	0.036
15	487	3119	109	0.004	0.389	0.142
30	990	5369	243	0.014	0.378	0.220

Note. All values represent the mean of triplicate determinations and are corrected for zero-time values (glucose oxidation—98 cpm; O_2^- production (ΔOD − 0.012). All assays were performed in parallel in this experiment using a single source of cells but represent a large number of single experiments (from 6 to 12) depending upon the specific conditions.

approximately 10 min. The situation was quite different with respect to [1-^{14}C] glucose oxidation. The addition of opsonized zymosan resulted in an immediate burst of hexose monophosphate shunt activity which continued to increase over the period of 30 min. In contrast, the addition of 20 mM NaF did not result in increased glucose oxidation; in fact the activity of resting cells was significantly inhibited by this concentration. Thus, there is a discordance between the effect of fluoride on superoxide production and that of glucose oxidation.

This discrepancy would be trivial if fluoride were directly inhibiting the enzymes of the hexose monophosphate shunt. To test this hypothesis, we examined the oxidation of [1-^{14}C] glucose in a neutrophil sonicate containing ATP and NADP. Results are illustrated in Table 2. The presence of fluoride was only very slightly inhibitory when tested in a cell-free system. Only 10% inhibition was noted at a concentration of 20 mM F^- after a 1-hr incubation; this contrasts with the situation in intact resting cells where the same concentration resulted in a 75% inhibition within 30 min (Table 1).

Chemiluminescence by intact neutrophils is illustrated in Fig. 2. In the luminol-enhanced system, 1 μg/ml PMA induced a marked and rapid increase in chemiluminescence. The addition of 20 mM F^- alone had no effect on chemiluminescence; when the two were added together, fluoride resulted in a small, but significant, inhibition of the PMA-induced response.

The ability of neutrophils to covalently attach iodine to protein under various conditions is illustrated in Table 3. Resting cells incorporated very little ^{125}I into protein, while the addition of opsonized zymosan resulted in a very large degree of incorporation. The soluble stimulus, PMA, was not nearly as effective as opsonized zymosan in promoting iodination but still gave substantially higher activity than the resting cells. In contrast, 20 mM F^- did not cause significant incorporation of ^{125}I under the experimental conditions employed.

TABLE 2

OXIDATION OF [1-^{14}C]GLUCOSE IN SONIC EXTRACT OF HUMAN NEUTROPHILS

Fluoride concentration (mM)	Glucose oxidation (cpm)
0.0	220,623
5.0	215,495
10.0	207,059
15.0	198,528
20.0	198,596

Note. Each value represents the mean of closely agreeing triplicate determinations. Each flask contained 1 mM ATP, 0.2 mM NADP, and sonicate representing 2×10^6 neutrophils. The incubation was performed for 1 hr at 37°C.

TABLE 3
IODINATION BY HUMAN NEUTROPHILS

Condition	^{125}I-Incorporated (cpm)	
	Experiment 1	Experiment 2
Resting cells	520	1,281
+ Opsonized zymosan	39,283	51,289
+ PMA	10,975	10,798
+ F^-	1,382	1,411

Note. The two experiments were performed on different days with different cell donors. Each value represents the mean of triplicate determinations for each experiment.

DISCUSSION

Sbarra and Karnovsky (13) first observed that fluoride could stimulate oxygen uptake by resting cells. This observation was extended by Curnutte and Babior (6) who demonstrated a marked enhancement of superoxide production by neutrophils exposed to 20 mM fluoride. In this initial report, O_2^- production was very rapid and linear with time for 10 min, at which point maximal production was observed. In a more recent paper, Curnutte *et al.* (7) published kinetic curves similar to that depicted in Fig. 1 with a lag period of approximately 10 min; no reasons for the discrepancy between these and the earlier results were discussed. The authors ascribed the lag period to a competition between Cl^- in the buffer and the F^- and demonstrated that the lag was very much reduced if the reaction were run in isotonic sucrose comtaining very low concentrations

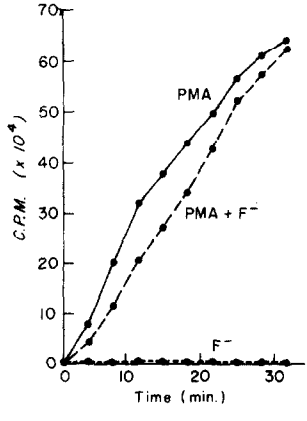

FIG. 2. Generation of chemiluminescence by neutrophils in the presence of PMA (1 μg/ml), fluoride (20 mM), or PMA plus fluoride. The luminol-enhanced system was used to measure chemiluminescence. Each value represents the mean of triplicate determinations. Background values (cells in PBS alone) were not different from those seen in the presence of fluoride.

of Cl^-. Under these conditions, however, the maximal amount of superoxide generated was also reduced (7).

Since the respiratory burst is characterized by a number of other metabolic alterations in the cell, we examined the effect of fluoride on some of these. The results in Table 1 demonstrate that under conditions where 20 mM F^- stimulated superoxide production by resting cells, the same concentration actually inhibited the oxidation of [1-^{14}C] glucose, a measure of hexose monophosphate shunt activity. This inhibition of glucose oxidation could not be ascribed to a direct effect of fluoride on the enzymes involved in glucose oxidation (hexokinase, glucose-6-phosphate dehydrogenase, or 6-phosphogluconate dehydrogenase) since only a marginal inhibition was observed in a cell-free system supplied with ATP and NADP (Table 2). These data are consistent with those of Gabler and Leong (14) who observed that fluoride inhibited the increased oxidation of [1-^{14}C] glucose induced by phagocytosis of latex particles.

Similarly, 20 mM fluoride did not augment neutrophil chemiluminescence in the luminol-enhanced system under conditions where another soluble stimulus, PMA, caused marked enhancement of this phenomenon (Fig. 2). This is in contrast to the results of Harvath *et al.* (15) who reported that F^- did stimulate chemiluminescence by both canine and human leukocytes. This generation of chemiluminescence by F^- was quite unusual in that the maximal response was only about 14,000 cpm with fluoride as opposed to 122,000 cpm with zymosan. Further, the chemiluminescent response to fluoride was very temperature dependent with no activity observed at ambient (25°C) temperature. These results are quite unlike those observed with other materials which stimulate the respiratory burst. Our observations are in essential agreement with those of Cheson *et al.* (16) who likewise failed to observe chemiluminescence in cells challenged with F^-. They ascribed this to the fact that F^- was a soluble stimulus and particulate material was needed for the required secondary emissions. The use of luminol in the present experiments demonstrates that this is not an adequate explanation. PMA resulted in a very pronounced stimulation of chemiluminescence (Fig. 2); similar results were obtained with the plant lectin concanavalin A (data not shown). Under these same conditions, F^- was completely without effect.

Other oxidative measurements were anomalous in cells challenged with fluoride. Iodination is markedly stimulated in cells challenged with opsonized zymosan and significantly enhanced in cells challenged with PMA (Table 3). The difference in these two challenges is likely due to the availability of a substrate for iodination. The zymosan particle can provide an excellent substrate while PMA must induce either iodination of the cell itself, or proteins in the medium which are external to the cell. Although significant iodination was observed with PMA, there was little or no effect observed in the presence of 20 mM F^-.

In experiments not shown, we examined the quantitative NBT dye reduction in resting cells and cells stimulated with either fluoride or opsonized zymosan. Results were similar to those reported here for iodination; little reduction occurred with resting cells and this was not significantly enhanced by the addition of F^-, but was greatly increased in the presence of opsonized zymosan. This is consistent with the reported data of Gabler and Leong (14) who demonstrated that F^- inhibited the NBT reduction of neutrophils incubated with latex particles.

Thus, fluoride causes human neutrophils to generate superoxide anion, although the kinetics are dissimilar to those induced by other stimuli. The relationship of this superoxide generation to the respiratory burst is questionable, however, since similar concentrations of fluoride inhibited glucose oxidation, failed to generate chemiluminescence, and failed to significantly iodinate protein or reduce NBT dye under conditions where another soluble stimulus, PMA, accomplished all these phenomena. This raises the disquieting possibility that the neutrophil may possess several means of generating O_2^- and that fluoride may stimulate a mechanism which is not truly a reflection of the respiratory burst. If this were true, the use of fluoride as a stimulus would not be helpful in dissecting events concerned with initiation of the respiratory burst.

SUMMARY

Although 20 mM fluoride stimulates neutrophils to produce superoxide anion, the kinetics of production are dissimilar to those observed with other stimuli. Further, fluoride was shown to inhibit oxidation of [1-^{14}C] glucose, and incubation of neutrophils with fluoride did not result in significant generation of chemiluminescence or iodination of cellular material, as did other soluble stimuli. These results indicate a discordance between the effect of fluoride on superoxide generation and other metabolic parameters associated with the respiratory burst. The data suggest that fluoride might not be a useful probe for investigating the molecular basis of the respiratory burst in human neutrophils.

ACKNOWLEDGMENTS

The excellent editorial assistance of Ms. Gwen Charles is gratefully acknowledged. This research was supported by Grant AI-10732 from the National Institute of Allergy and Infectious Disease.

REFERENCES

1. DeChatelet, L. R., *J. Reticuloendothel. Soc.* **24,** 73 (1978).
2. Zatti, M., and Rossi, F., *Biochim. Biophys. Acta* **148,** 553 (1967).
3. Patriarca, P., Zatti, M., Cramer, R., and Rossi, F., *Life Sci.* **9,** 841 (1970).
4. DeChatelet, L. R., Shirley, P. S., and Johnston, R. B., Jr., *Blood* **47,** 545 (1976).
5. Romeo, D., Zabucchi, G., and Rossi, F., *Nature (London)* **243,** 111 (1973).
6. Curnutte, J. T., and Babior, B. M., *Blood* **45,** 851 (1975).
7. Curnutte, J. T., Babior, B. M., and Karnovsky, M. L., *J. Clin. Invest.* **63,** 637 (1979).

8. DeChatelet, L. R., and Cooper, M. R., *Biochem. Med.* **4,** 61 (1970).
9. Babior, B. M., Kipnes, R. S., and Curnutte, J. T., *J. Clin. Invest.* **52,** 741 (1973).
10. Qualliotine, D., DeChatelet, L. R., McCall, C. E., and Cooper, M. R., *Infect. Immunity* **6,** 211 (1972).
11. Baehner, R. L., and Nathan, D. G., *N. Engl. J. Med.* **278,** 971 (1968).
12. Stevens, P., Winston, D. J., and Van Dyke, K., *Infect. Immunity* **22,** 41 (1978).
13. Sbarra, A. J., and Karnovsky, M. L., *J. Biol. Chem.* **234,** 1355 (1959).
14. Gabler, W. L., and Leong, P. A., *J. Dent. Res.* **58,** 1933 (1979).
15. Harvath, L., Amirault, H. J., and Anderson, B. R., *J. Clin. Invest.* **61,** 1145 (1978).
16. Cheson, B. D., Christensen, R. L., Sperling, R., Kohler, B. E., and Babior, B. M., *J. Clin. Invest.* **58,** 789 (1976).