Chemiluminescence of Human and Canine Polymorphonuclear Leukocytes in the Absence of Phagocytosis

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ABSTRACT Polymorphonuclear leukocytes (PMNs) have increased oxidative metabolism during phagocytosis and emit light (chemiluminescence, CL) as a result of metabolic activation. The present study examined PMN CL in the absence of phagocytosis using sodium fluoride (NaF), a nonparticulate agent and known stimulator of cellular oxidative metabolism. Normal human and canine PMNs were assayed in a CL spectrometer which permitted continuous sample mixing and constant temperature regulation during CL measurement. PMNs treated with 20 mM NaF demonstrated maximum CL responses of 10,000–20,000 cpm above background, 13–17 min after addition of NaF at 37°C. Temperature regulation of reaction mixtures was found to be a critical factor in assaying PMN CL responses to NaF, because a small decrease in temperature (i.e. 1.5°C) substantially depressed and delayed the CL response. Superoxide anion production correlated closely with CL responses in NaF-treated human PMNs. CL responses were completely suppressed in the presence of the oxidative metabolic inhibitors, iodoacetamide, and *N*-ethylmalemide; and were partially suppressed in the presence of either superoxide dismutase or sodium azide.

CL responses of NaF-treated PMNs were significantly lower than responses generated by PMNs phagocytizing opsonized yeast. When NaF was evaluated for its effect on light generation from a singlet oxygen dependent CL reaction, it was found that NaF did not quench singlet oxygen light. This study demonstrates that PMN CL can occur in the absence of phagocytosis, and it proposes that a nonphagocytic PMN CL assay may be useful in evaluating leukocyte metabolic defects.

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INTRODUCTION

Polymorphonuclear leukocytes (PMNs) activated during phagocytosis produce light emitting chemical responses or chemiluminescence (CL).[1] Allen et al. (1) first described CL in PMNs phagocytizing opsonized bacteria, and later reports have confirmed PMN CL using opsonized zymosan (2). PMNs engaged in phagocytosis have substantial increases in oxygen consumption and glucose oxidation via the hexose monophosphate shunt (3–5). It has been demonstrated that PMNs can generate superoxide anion, O_2^- (6–8), hydrogen peroxide, H_2O_2 (9), hydroxyl radical, $\cdot OH$ (10), and singlet molecular oxygen, $^1O_2$ (11, 12) as a result of increased oxidative metabolism. PMN light emission is, in part, dependent upon the relaxation of $^1O_2$ or excited-state compounds that are formed by their interaction with $^1O_2$ (1, 11–15). Additional light generating reactions, however, may be produced by the other oxidative intermediates listed above. Thus far, reports of PMN CL have correlated light emission with cells metabolically activated by phagocytosis.

Sodium fluoride (NaF) has been shown to be a potent stimulator of hexose monophosphate shunt activity in PMNs. Sbarra and Karnovsky (3) demonstrated that guinea pig peritoneal cells treated with 20 mM NaF had significantly enhanced oxygen consumption and hexose monophosphate shunt activity; and, in a later

[1] *Abbreviations used in this paper*: CL, chemiluminescence; HBSS, Hanks' balanced salt solution; PMN, polymorphonuclear leukocytes; SOD, superoxide dismutase.

study Selvaraj and Sbarra (16) reported that NaF-treated PMNs had significantly depressed phagocytosis with simultaneous increase in HMS activity. More recently, Curnutte et al. (17) and Cheson et al. (18) have demonstrated that human PMNs treated with 20 mM NaF have substantial increases in O_2^- generation.

This study investigated CL responses of canine and human PMNs treated with NaF using a chemiluminescence spectrometer specifically designed to permit CL measurement with continuous sample mixing and constant temperature regulation at preset levels (19). PMNs were evaluated for their CL responses to NaF in the presence of the oxidative metabolic inhibitors, *N*-ethylmalemide and iodoacetamide, and in the presence of the enzyme, superoxide dismutase, and the $^1O_2$ quencher, sodium azide. PMNs were examined morphologically, and functionally for changes in viability, phagocytosis, and chemotaxis during NaF treatment. An experiment was also performed to evaluate the effect of fluoride ion (F^-) in a cell-free $^1O_2$-dependent CL reaction of hypochlorite (OCl^-) plus H_2O_2 (20, 21).

METHODS

Isolation of leukocytes. Leukocytes were procurred from heparinized human or canine blood (10 U heparin/ml of blood) by adding 2 ml of plasmagel (Roger Bellon Laboratories, Neuilly, France) to each 10 ml of blood. Samples were incubated at 37°C for 30–60 min and the leukocyte-rich supernatant fluid was removed. Cells were centrifuged at 200 *g* for 10 min and washed with 30 ml of Hanks' balanced salt solution (HBSS). Contaminating erythrocytes were removed by suspending the cell pellet in 3–10 ml of ice-cold isotonic ammonium chloride (NH_4Cl) solution (0.155 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) for 10 min (22). The cells were washed twice with 30 ml of HBSS, and resuspended to a cell concentration of 10^6 PMNs/ml HBSS.

Isolation of platelets. Platelets, free of contaminating leukocytes, were obtained from a normal canine donor using a continuous flow centrifuge (Haemonetics 30; Haemonetics Corp., Boston, Mass.). Platelets were washed twice in HBSS and diluted to a concentration of 4×10^7/ml of HBSS, which corresponded to the concentration present in plasmagel-separated leukocyte preparations.

Cyclophosphamide-treated leukocyte donors. Dogs were treated with a single intravenous injection of cyclophosphamide (Cytoxan; Mead Johnson & Co., Evansville, Ind.) at a dose of 40 mg/kg body wt (23). PMNs were tested for CL immediately before drug administration and 3 days after cyclophosphamide injection.

Chemicals and reagents used in CL experiments. All inorganic chemicals (reagent grade, Fisher Scientific Co., Pittsburgh, Pa.), were prepared in HBSS immediately before PMN CL experiments. NaF was dissolved in HBSS in plastic tubes and used in CL experiments in a final concentration of 20 mM. Sodium azide (NaN_3) was used at a concentration of 0.1 mM in PMN experiments. Superoxide anion dismutase (SOD; Truett Labs, Dallas, Tex.) was prepared in a stock solution of 1,000 μg/ml HBSS. The final concentration of SOD in CL experiments was 100 μg/reaction vial. Two protein solutions were used as control protein solutions in experiments evaluating the effect of SOD on CL responses. One solution contained 1,000 μg of bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) per milliliter HBSS, and a second solution contained 1,000 μg of denatured SOD (denatured by boiling for 120 min) per milliliter HBSS. Two inhibitors of oxidative metabolism, 0.1 mM *N*-ethylmalemide and 2.0 mM iodoacetamide were tested. In some experiments PMNs were treated with cytochalasin B (Sigma Chemical Co.) in a final concentration of 5 μg/ml HBSS containing 0.1% dimethyl sulfoxide (Matheson, Coleman, and Bell, East Rutherford, N. J.). Cells were incubated with cytochalasin B for 10 min at 37°C before use in CL experiments.

$^1O_2$ was produced by mixing 0.5% sodium hypochlorite (NaOCl; J. T. Baker Chemical Co., Phillipsburg, N. J.), and 0.3% hydrogen peroxide (H_2O_2) (Fisher Scientific Co.) in H_2O.

Phagocytic particles. Commercial baker's yeast was suspended in saline and boiled for 10 min. The suspension was filtered four times through gauze and the concentration was adjusted to 4×10^7 yeast/ml of HBSS for phagocytosis experiments, and 5×10^8 yeast/ml for CL experiments. Yeast were opsonized by mixing at 37°C for 10 min in 15% autologous serum and HBSS.

Chemiluminescence measurement. PMN CL was measured in a chemiluminescence spectrometer (19). The spectrometer contains the light sensing and electronic components of a Packard Tri Carb scintillation spectrometer (model 3380, Packard Instrument Co., Inc., Downers Grove, Ill.) and incorporates the photomultiplier tube (RCA 4501/V3, RCA Solid State, Somerville, N. J.) in a lighttight chamber. Counting vials are placed in a carousel machined from an aluminum block which has an embedded heating element so that the reaction vials can be maintained at a preset temperature. In addition, magnetic mixers are located under each of the reaction vial positions to keep PMNs suspended and well-oxygenated during CL measurement. Rubber septa injection ports are located over reaction vials to permit both dark adaption of reaction vials and measurement of CL responses immediately after injection of reactants.

In this study PMN CL measurements were made using polyethylene scintillation vials (Minivials, Rochester Scientific Co., Rochester, N. Y.) with continuous mixing of PMNs throughout the experiment. Unless otherwise indicated, in the text, reactions were monitored at 37°C; and background counts obtained from unstimulated granulocytes were subtracted from the values obtained during the CL experiment. All CL experiments were carried out in a dark room.

The final reaction volume was 6.0 ml/vial. Cell concentration for PMN CL measurements was 5×10^6 PMNs/vial. Emitted light is expressed as counts per unit time.

CL measurement of OCl^- plus H_2O_2. A light generating reaction of NaOCl plus H_2O_2 described by Seliger (20) and Khan and Kasha (21) was used to evaluate the effect of F^- on $^1O_2$ light-dependent CL. Reactions were conducted by injecting 2.5 ml of 0.3% H_2O_2 into vials containing 3.0 ml of 0.5% NaOCl in H_2O. H_2O_2 was injected into reaction vials through rubber septa injection ports of the CL spectrometer by using 3.0 ml syringes. The reaction was monitored for 10 s beginning immediately after injection of H_2O_2. NaF was added to the 0.5% NaOCl-H_2O solutions in concentrations of 1, 5, 20, 100, and 500 mM before injection of H_2O_2. Control reaction mixtures containing 3.0 ml of 0.5% NaOCl in H_2O and 2.5 ml of H_2O_2 were assayed in the presence and absence of 0.1 M NaN_3. The results are reported as counts per initial 10 s interval of the reaction.

Assay of superoxide (O_2^-). PMN generation of O_2^- was approximated by using a modification of the method of Babior et al. (6). The assay measures ferricytochrome reduction by PMNs and is inhibited by SOD. Only superoxide which

reacts with cytochrome *c* under the assay conditions is measured.

Reaction mixtures contained 1×10^6 PMNs and 0.1 mM ferricytochrome *c* (horse heart, type VI, Sigma Chemical Co.). Yeast in a concentration of 5×10^7 yeast/reaction vial (opsonized with 15% autologous donor serum) and 20 mM NaF were tested in the assay for their effect on O_2^- production by PMNs. Controls consisted of PMNs with 0.1 mM ferricytochrome *c* in HBSS. Reaction vials were run in pairs with and without SOD (15 μg/ml). The final volume of the reaction mixtures was 2.0 ml; and 5 pairs of tubes were set up for each stimulant. All reactants (except yeast or NaF) were added to plastic tubes and preincubated at 37°C in a shaker water bath for 10 min. Yeast or NaF were added at zero time, and one pair of tubes was transferred immediately to an ice bath (zero time), and at designated time intervals. After completing the experiment, all tubes were centrifuged at 2,500 *g* for 15 min at 4°C. Supernates were measured spectrophotometrically between 540 and 560 nm using 0.1 mM ferricytochrome *c* as the blank. Nanomoles of cytochrome *c* reduced were calculated from the increases in absorbance using $E\Delta = 21.0$ cm^{-1} mM^{-1} (24). Superoxide anion formation was determined from the difference in the rate of cytochrome *c* reduction in paired samples (with and without SOD). Results are expressed as nmol/10^6 PMNs.

Chemotaxis. Normal PMNs and PMNs treated with 20 mM NaF were assessed for their chemotactic responses to autologous serum. Cells were preincubated at 37°C for 20 min in HBSS (controls) or in HBSS with 20 mM NaF. After incubation, cells were centrifuged at 200 *g* for 10 min to remove extracellular NaF and adjusted to a final concentration of 5×10^6 PMNs/ml HBSS. Control cells were centrifuged and resuspended under identical conditions.

Chemotaxis was assessed in blind well lucite chemotaxis chambers (Neuro Probe, Inc., Bethesda, Md.). The lower compartment contained 0.10 ml of either HBSS (negative control), or 20% autologous serum (positive control). The compartments were separated by a 5-μm membrane filter (Millipore Corp., Bedford, Mass.), and 0.1 ml of the PMN suspension was added to the upper chamber compartment. Chambers were incubated at 37°C for 2 h. Filters were removed, stained using the procedure of Boyden (25), and the bottom surface of the filters were scanned. Triplicate filters were used in these experiments and five randomly selected fields were counted at ×400. Results are expressed as the mean±1 SE of the mean for triplicate filters.

Phagocytosis. Normal and 20 mM NaF-treated PMNs were compared for their ability to phagocytize yeast using a modified method of Maaløe (26) previously described by Andersen et al. (27). Cells were adjusted to a concentration of 5×10^6 cells/ml of HBSS containing 200 mg glucose/100 ml and 40% autologous serum. Equal volumes of the yeast suspension (0.4 ml) and cell suspension (0.4 ml) were mixed in plastic tubes in a shaker water bath for 10 or 30 min at 37°C. After incubation, 0.1 ml of 0.9% EDTA (in 0.9% NaCl) was added to each tube and tubes were centrifuged at 200 *g* for 5 min. The pellets were resuspended in HBSS, and smears of the suspension were stained with Wright's-Giemsa stain. Results are reported as the percentage of cells containing phagocytized yeast from 200 cells counted.

Cell viability. Cell viability was assessed by trypan blue exclusion (0.1% trypan blue dye, Grand Island Biological Co., Grand Island, N. Y.).

Electron microscopy. Ultrastructural morphology of normal and 20 mM NaF-treated PMNs was compared by incubating PMNs at 37°C for 20 min in either HBSS or 20 mM NaF in HBSS. Cells were centrifuged at 200 *g* for 10 min and the pellets were then placed in a solution of 3% glutaraldehyde and 0.1 M PO_4 buffer for 2 h at 4°C. Pellets were rinsed with 0.2 M sucrose in 0.1 M PO_4 buffer, immersed in 2% OsO_4 in 0.1 M PO_4 buffer, dehydrated with acetone, and embedded in epon. Specimens were stained with uranyl-lead acetate and examined with an RCA-EMU 4A microscope (RCA Solid State).

Data analysis. Integral areas of CL graphs were measured by using a compensating polar planimeter, LASICO L 30AB (Los Angeles Scientific Instrument Co., Inc., Los Angeles, Calif.). In experiments that evaluated suppressive effects of reagents on CL reactions, the total area under CL curves was measured and expressed as the percent of suppression of the control CL response.

Data were analyzed using the Student's two tailed *t* test for independent means (NS, $P > 0.05$; [28]). Data are expressed as the mean±1 SE Mean unless otherwise stated in figure or table legends.

RESULTS

Chemiluminescence of NaF-treated PMNs. Normal human and canine PMNs treated with 20 mM NaF consistently produced a CL response when measured at 37°C. A preliminary experiment testing various concentrations of NaF (1, 10, 20, 50, and 100 mM NaF) indicated that an optimal CL response occurred with a concentration of 20 mM. Maximum CL occurred 13–17 min after the addition of 20 mM NaF, and was 10,000–20,000 cpm above background CL. Human cells had a maximum CL response of 12,600±1,200 cpm ($n = 5$), and canine cells had a CL response of 15,100 ±1,600 cpm ($n = 5$). CL responses of NaF-treated cells were ≅1/10 of responses generated by the same cells exposed to yeast (opsonized with 15% autologous serum). The maximal CL responses of PMNs in the presence of yeast were 126,700±4,600 cpm ($n = 4$) for humans and 121,700±5,000 cpm ($n = 4$) for canines;

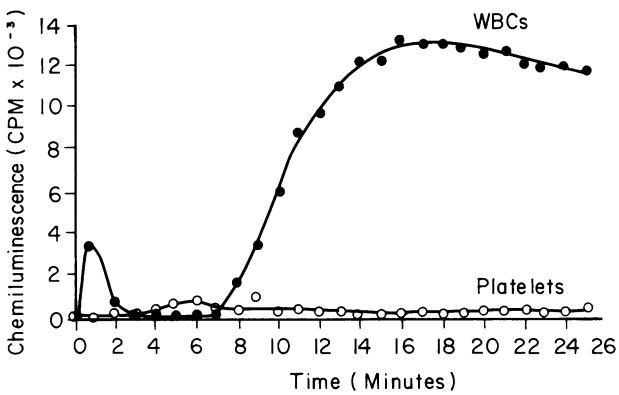

FIGURE 1 CL response of 20 mM NaF-treated leukocytes and platelets procurred by a continuous flow blood cell separator. The platelet preparation (○) contained 2×10^8 platelets/vial and was free of leukocytes; the leukocytes preparation (●) contained 85% PMNs and 15% mononuclear cells in a concentration of 5×10^6 PMNs/vial. PMNs had a base-line CL value of 4,600 cpm and platelet CL base-line value was 2,800 cpm. WBCs, leukocytes.

and the maximum CL occurred 2–4 min after the addition of opsonized yeast. Background CL values which were subtracted from the data in this study had a mean of 5,600 cpm with a range of 3,000–7,100 cpm.

Previous studies have demonstrated generation of CL by mononuclear leukocytes and platelets (29, 30). Because PMN preparations from plasmagel-sedimented blood contained both mononuclear leukocytes and platelets, experiments were undertaken to verify that PMNs were principally responsible for the increased CL observed after NaF treatment. One study (Fig. 1) separated platelets from leukocytes of a canine donor using a continuous blood cell separator (Haemonetics Corp.). Platelets free of leukocytes and treated with 20 mM NaF failed to show a CL response, whereas a leukocyte preparation containing 85% PMNs and 15% mononuclear leukocytes had a maximum CL response of 13,000 cpm 16 min after NaF treatment (Fig. 1).

A second experiment tested a cell preparation of 99% PMNs from a canine donor. The donor was treated with a single intravenous dose of cyclophosphamide (40 mg/kg) and 3 days later the peripheral blood cells were used in a CL experiment with NaF. We have previously used cyclophosphamide in dogs, as a method of producing transient bone marrow suppression (23), and have found that 3 days after cyclophosphamide treatment, mononuclear leukocyte concentration is markedly depressed whereas PMNs, although depressed in total numbers, remain viable. A donor was evaluated in this study that had 99% PMNs and 1% mononuclear cells. When treated with 20 mM NaF the CL maximum was 19,000 cpm, 13 min after addition of NaF.

The results of these experiments demonstrate that the CL response generated by leukocytes treated with 20 mM NaF was due principally to PMNs and not mononuclear leukocytes or platelets.

Temperature dependence of NaF-stimulated CL response. PMNs treated with 20 mM NaF generated a CL response when the reaction was performed at 37°C (Fig. 2). When cells were maintained at 35.5°C the CL response was decreased, and the maximum light emission occurred 30 min or more after the addition of NaF. PMNs tested at ambient temperature (25°C) failed to generate a CL response over a 30-min period. These observations indicate that NaF-induced PMN CL is a temperature-dependent reaction; and a small decrease in temperature (i.e. 1.5°C) substantially delays the response. As a result of these findings, all PMNs in this study were maintained at 37°C during the CL reaction.

Effect of oxidative metabolic inhibitors on NaF-induced CL. The sulfhydryl reagents, *N*-ethylmalemide and iodoacetamide have been shown to be potent inhibitors of PMN oxidative metabolism and O_2^- production (31). Both reagents were tested for their inhibitory effect on NaF-induced PMN CL. Both, 2.0 mM iodoacetamide and 0.1 mM *N*-ethylmalemide completely suppressed the CL response of PMNs treated with 20 mM NaF. These data indicate that PMNs with chemically impaired oxidative metabolism fail to be activated by 20 mM NaF.

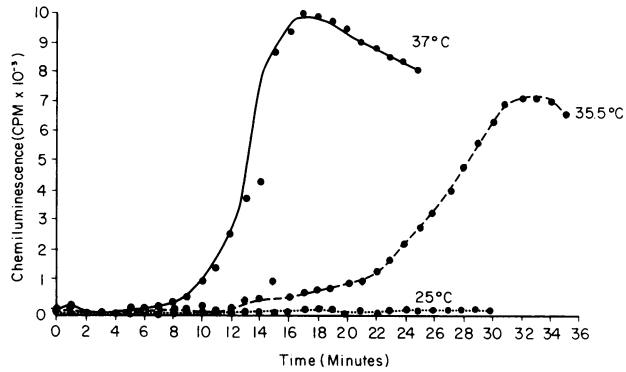

FIGURE 2 Effect of temperature on CL response of PMNs to 20 mM NaF. 5×10^6 PMNs from the same donor were assayed at 37°C (——), 35.5°C (– – –), and 25°C (. . .). Base-line CL value of PMNs was 4,300 cpm.

Effect of superoxide dismutase and sodium azide on NaF-induced CL. SOD has been shown to depress CL responses of phagocytizing PMNs (2). PMNs treated with NaF were tested for CL in the presence and absence of SOD (100 μg). The CL response of canine PMNs ($n = 3$) was suppressed by 37±11% of control NaF-treated values (Fig. 3). Human PMN CL responses ($n = 3$) were also suppressed by 35±10% of control values with the addition of 100 μg SOD to the reaction mixture. The suppressive effect of SOD could not be attributed to a nonspecific suppressive effect by protein because protein solutions of bovine serum albumin (100 μg) and heat-denatured SOD (100 μg) both failed to suppress the CL response of NaF-treated PMNs. In this experiment, the percentage of suppression was calculated by measuring the integral areas of the CL curves.

Sodium azide (NaN_3) is both an inhibitor of myeloperoxidase (32) and a scavenger of $^1O_2$ (33). Canine and human PMNs were examined in this study for the effect of 0.1 mM NaN_3 on NaF-induced PMN CL. NaN_3 partially suppressed the CL response of NaF-treated human and canine PMNs by 34±11% of control values (Fig. 3). When both 0.1 mM NaN_3 and 100 μg SOD were added to the reaction mixture the CL response of PMNs to NaF treatment was completely suppressed.

Quantitation of O_2^- from NaF-treated PMNs. NaF has been reported to significantly increase O_2^- generation by PMNs (17, 18). The present study compared O_2^- production of NaF-treated PMNs with PMNs

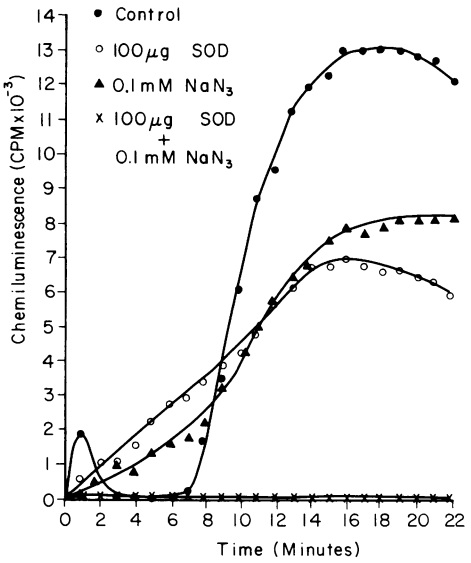

FIGURE 3 Effect of SOD and sodium azide (NaN_3) on CL response of PMNs treated with NaF at 37°C. Control CL response of 5×10^6 PMNs in 20 mM NaF (●) was compared with autologous PMNs treated with 20 mM NaF and 100 μg SOD (○); 0.1 mM NaN_3 (▲); a combination of 100 μg SOD and 0.1 mM NaN_3 (×). Protein solutions containing 100 μg of bovine serum albumin were similar to control CL responses.

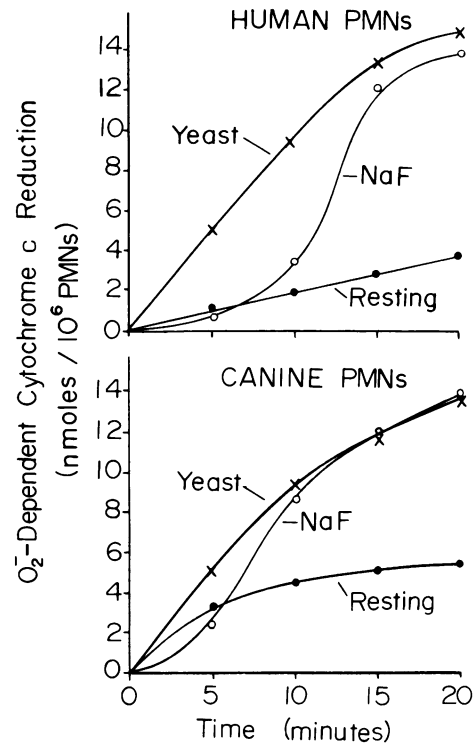

FIGURE 4 O_2^- production of resting, phagocytic, and 20 mM NaF-treated human and canine PMNs at 37°C. SOD inhibitable cytochrome *c* reduction was used to quantitate O_2^- produced by 1×10^6 PMNs in the presence of 0.1 mM cytochrome *c* in HBSS. Unstimulated or resting PMNs (●) were compared with cells treated with 20 mM NaF (○) and with PMNs mixed with 5×10^7 opsonized yeast (×). Data are the means of three different human and canine donors.

phagocytizing opsonized yeast and with unstimulated (resting) PMNs of the same donor. O_2^- was quantitated using a modified method of Babior et al. (6). PMNs were evaluated from three human and three canine donors; and the mean values of O_2^- production over a 20-min period are graphed in Fig. 4.

Human PMNs treated with NaF demonstrated a substantial increase in O_2^- production 10–15 min after NaF treatment (Fig. 4, top). O_2^- production was four-fold higher in NaF-treated cells compared to resting cells at 15 and 20 min (significant, $P < 0.05$). In contrast, human PMNs incubated with opsonized yeast had a linear increase in O_2^- production during the first 15 min of incubation, which was significantly greater than O_2^- production of resting cells throughout the 20 min incubation period ($P < 0.05$). In this experiment, O_2^- production from human PMNs either treated with NaF or phagocytizing opsonized yeast was not significantly different 15 and 20 min after incubation at 37°C.

Canine PMNs either treated with NaF or phagocytizing yeast had increased O_2^- production 10–20 min after incubation which was 2- to 2.5-fold greater than O_2^- production by resting cells (Fig. 4, bottom). The largest increase in O_2^- production by NaF-treated canine cells occurred 5–10 min after incubation. Under the assay conditions of this experiment it was found that canine PMNs either treated with NaF or phagocytizing yeast had almost identical O_2^- production 15–20 min after incubation.

The results of these experiments indicate that O_2^- production by canine and human PMNs is similar in cells treated with NaF and phagocytizing yeast when measured 15–20 min after incubation. The primary difference between phagocytic and NaF-treated PMN O_2^- production occurred during the first 5–10 min of incubation. NaF-treated human PMNs had a lag period of ≅10 min, that is similar to the results reported by Cheson et al. (18); and NaF-treated canine PMNs had a lag period of ≅5 min before O_2^- production increased.

Effect of fluoride ion (F^-) on $^1O_2$-dependent CL. Because NaF-induced PMN CL responses in these studies were substantially lower than CL responses from the same PMNs in the presence of yeast, a cell-free light generating reaction was examined in the presence and absence of NaF to determine whether F^- had a direct inhibition on $^1O_2$-dependent light emission.

A $^1O_2$-producing reaction of hypochlorite (OCl^-) and hydrogen peroxide (H_2O_2) has been described as

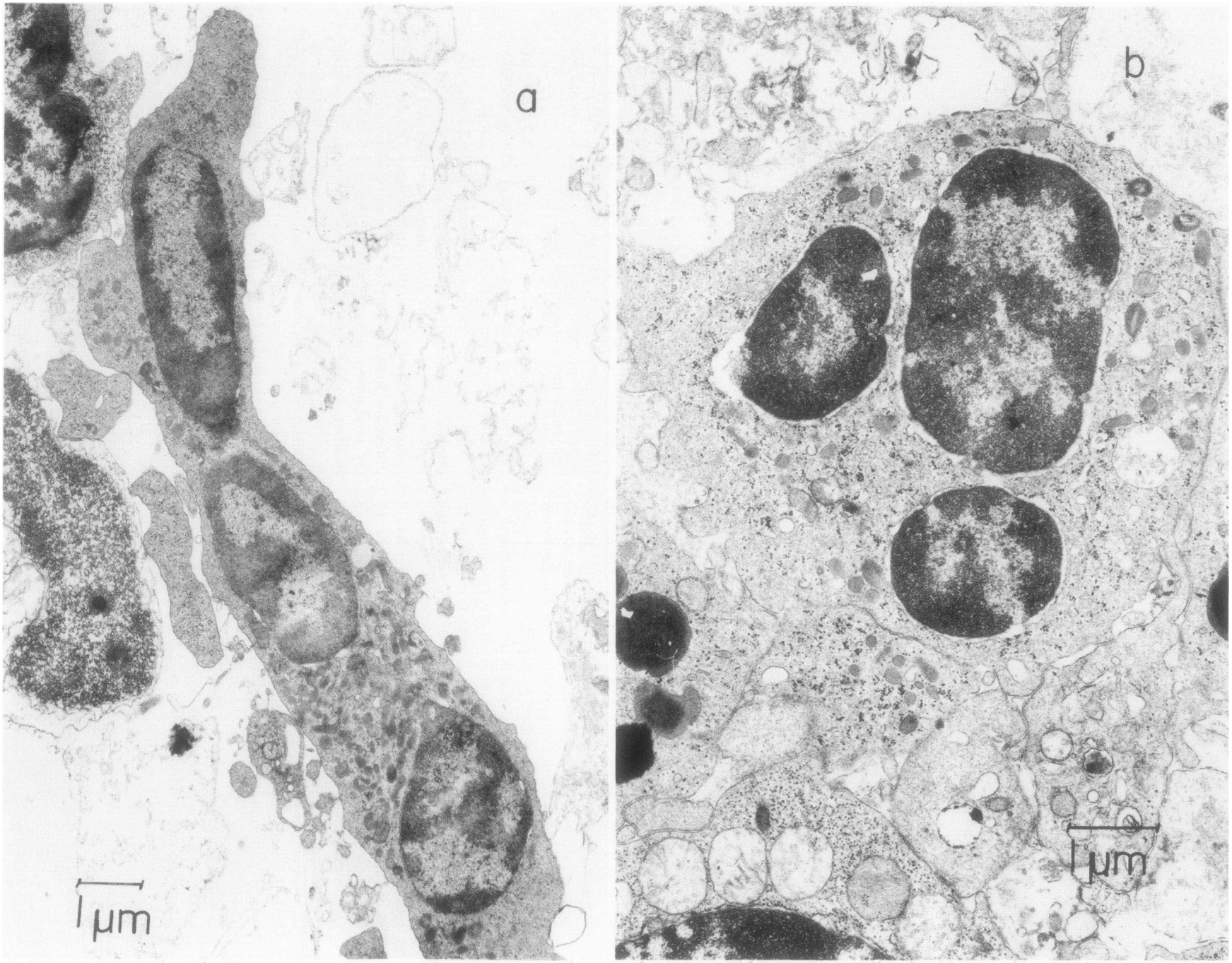
a
1 µm
b
1 µm

TABLE I
Effect of F^- on $^1O_2$ Chemiluminescence

Reaction*	Chemiluminescence
	Counts/initial 10 s of reaction±1 SEM
Control	358,355±6,165
1 mM NaF	368,245±9,000
5 mM NaF	395,350±3,150
20 mM NaF	371,230±7,180
100 mM NaF	368,580±6,800
500 mM NaF	385,120±14,350

* Reactions consisted of injecting 2.5 ml of 0.3% H_2O_2 into vials containing 3.0 ml of 0.5% NaOCl in H_2O. Results are reported as the mean±1 SEM for three experiments. Data are expressed as counts recorded during the first 10 s after H_2O_2 injection.

TABLE II
*Effect of 20 mM NaF on Human PMN Chemotaxis**

Cell suspension	Chemoattractant	Cells per high power field
Normal	Hanks' BSS	1±1
20 mM NaF-treated		1±1
Normal	20% Autologous serum	171±10
20 mM NaF-treated		27±8

* Cells were treated with NaF, centrifuged after a 20-min incubation period, and resuspended in NaF-free HBSS. Results are expressed as means±1 SEM cells per high power field of triplicate filters from four different PMN donors.

a potent CL reaction in which $^1O_2$ serves as the primary light generating species (20, 21). This reaction was measured in the absence of NaF and in the presence of a range of F^- concentrations. The data in Table I summarize the results of three experiments and are expressed as the mean±1 SEM in counts per initial 10 s interval of the reaction. Control reaction mixtures containing 2.5 ml of 0.5% NaOCl and 2.5 ml of 0.3% H_2O_2 in 0.5 ml H_2O produced a CL response of 358,355 ±6,165 counts/10 s. The CL response of control reactions was suppressed by 96% when 0.1 M of the $^1O_2$ quencher, NaN_3, was added. It was found that F^- concentrations of 1 mM, 5 mM, 20 mM, 100 mM, and 500 mM failed to suppress the CL response of this $^1O_2$ light generating reaction. It appears from this reaction that F^- does not directly quench $^1O_2$ light emission.

Effect of NaF on function and morphology of PMNs. Viability as assessed by trypan blue dye exclusion, was not significantly altered when human or canine PMNs were incubated with 20 mM NaF for 20 min at 37°C. Untreated and NaF-treated cells were ≥90% viable after the incubation period.

NaF has been reported to depress PMN phagocytosis (3). PMNs incubated for 20 min with 20 mM NaF were compared with untreated PMNs for phagocytosis of opsonized yeast. PMNs were incubated with opsonized yeast and examined 10 and 30 min after addition of yeast particles. Phagocytosis was stopped at 10- and 30-min periods by the addition of 0.1 ml of 0.9% EDTA to the reaction which effectively reduced phagocytosis in this test system by 90%. Treatment of human and canine PMNs with NaF depressed phagocytosis of opsonized yeast at both time periods tested. After a 10-min incubation period with yeast, phagocytosis was decreased by 15% in human PMNs (controls, 90±2%; NaF treated, 75±2%) and by 13% in canine PMNs (controls: 88±1%; NaF treated, 75±1%). The results obtained after a 30-min incubation period were similar to results at 10 min. After a 30-min incubation period phagocytosis was decreased by 18% with human PMNs and by 15% with canine PMNs. NaF treatment significantly depressed phagocytosis of yeast at both time intervals, $P < 0.05$. It was also observed that control cells had three or more yeast per PMN whereas most NaF-treated cells had two or less yeast per PMN. NaF treatment, therefore, depressed both the number of phagocytic PMNs and the number of phagocytized yeast per PMN.

PMN chemotaxis was significantly depressed when cells were incubated with NaF at 37°C ($P < 0.05$). Results of experiments using human PMNs are summarized in Table II. When NaF-treated PMNs were centrifuged after the 20 min incubation period and resuspended in HBSS without NaF for the chemotaxis assay, the chemotactic response to autologous serum was depressed by 88%. These studies indicate that NaF-treated PMNs have depressed phagocytosis and substantially impaired chemotaxis.

Morphologic comparisons were made between control PMNs and NaF-treated PMNs with light microscopy and electron microscopy. The most significant difference between the two groups was in the cytoplasmic structure. NaF-treated PMNs had

FIGURE 5 Electronmicrographs of human peripheral blood cells incubated for 20 min at 37°C (*a*) with 20 mM NaF in HBSS illustrating the elongated and distorted cytoplasmic morphology and (*b*) with HBSS alone illustrating normal round to oval cytoplasmic morphology. The bar represents 1 μm.

elongated or distended cytoplasmic cell morphology (Fig. 5*a*), while control PMNs had consistently round or oval cytoplasmic cell morphology (Fig. 5*b*). NaF treatment did not promote PMN degranulation under the conditions used in this study.

Although reaction mixtures of PMNs and NaF did not contain a particulate agent to stimulate CL, the cell preparations appeared to contain fragments of cytoplasm and cellular debris. To eliminate the possibility that CL responses of NaF-treated PMNs were due to phagocytosis of cellular debris, an experiment was designed to use an agent known to paralyze phagocytosis. PMNs were incubated for 10 min at 37°C with cytochalasin B (34) immediately before NaF stimulation. Cytochalasin B treatment did not depress the CL responses of PMNs to NaF and did not alter the base-line CL values of unstimulated PMNs.

DISCUSSION

CL assays have significant potential as both a means for evaluating mechanisms of leukocyte phagocytosis and microbial-opsonin interaction, and as a method for investigating oxidative metabolism of leukocytes. A number of studies have used CL as a measure of PMN phagocytosis (1, 2, 35), and other reports have evaluated CL of phagocytizing monocytes (29) and eosinophils (36). In CL assays where the leukocyte to particle ratio has been kept constant, opsonic capacities of various sera for some microorganisms have been evaluated (37–39). PMN CL reactions and corresponding O_2^- production from myeloperoxidase-deficient leukocytes have also been examined to further understand the microbicidal mechanisms of PMNs (40). Studies using CL reactions as a method for evaluating leukocyte function or microbial-opsonin interaction have thus far utilized leukocytes activated during the phagocytic process. The present study proposes that the use of nonphagocytizing PMNs activated with a nonparticulate agent, such as NaF, has potential as a CL method for studying PMN oxidative metabolism independent of phagocytosis. By comparing CL responses from phagocytizing PMNs with responses from metabolically activated nonphagocytic PMNs it may be possible to further understand metabolic and phagocytic defects in leukocytes.

Viable leukocytes treated with 20 mM NaF have substantially increased HMS activity (3). As a result, marked increases in O_2^- generation accompany leukocyte stimulation with NaF (17, 18). Because reports have indicated that O_2^- is associated with CL responses of phagocytizing PMNs (2) and mononuclear cells (29), it would seem likely that leukocytes stimulated by NaF have potential for light emitting reactions. The present study has shown that NaF-treated PMNs from human and canine peripheral blood generate a consistent CL response of 10,000–20,000 cpm above background. The temperature at which CL is measured is a critical factor in assaying NaF-treated PMNs. When measured at 37°C the maximum response occurred 13–17 min after the addition of NaF; however, a small decrease in temperature (i.e. 1.5°C) depressed the CL response and delayed the response by ≅15 min. No CL response was observed when PMNs were assayed at room temperature (25°C). The temperature dependency of this reaction could possibly explain the negative CL findings of a previous report that examined NaF-treated PMNs (18). Temperature effects have also been described in studies of PMN CL during phagocytosis (19, 29). Those studies have shown that PMN CL responses are depressed by 50% or more when tested at 25°C as compared to 37°C. It is apparent from these studies that CL responses of cell preparations are optimal at physiologic temperatures.

Early experiments with peritoneal phagocytes used 20 mM NaF as an inhibitor of glycolysis to study the phagocytic process (3, 16). It was reported that F^- enhanced hexose monophosphate shunt activity and simultaneously suppressed phagocytosis. Our present study indicates that although the dye exclusion ability of F^--treated PMNs remained unchanged, two cell membrane associated functions of PMNs were significantly depressed. Phagocytosis of yeast was depressed by 13–18% and chemotaxis was depressed by 88% in NaF-treated cells. PMNs treated with F^- had distorted and elongated cytoplasm when examined morphologically. Our studies also indicate that NaF-treated cells had enhanced oxidative metabolism which appears to have been responsible for the observed CL response. When two different sulfhydryl metabolic inhibitors, *N*-ethylmalemide and iodoacetamide, were used to suppress oxidative metabolism in PMNs, the CL response to NaF was completely inhibited. It appears that F^- has diverse effects on PMNs; although dye exclusion ability remains unchanged after NaF treatment, phagocytosis and chemotaxis are significantly depressed whereas oxidative metabolism is substantially increased.

In the present study it was found that normal PMNs phagocytizing yeast generated significantly more light than NaF-stimulated PMNs from the same donor, and that both NaF treatment and phagocytosis stimulated substantial increases in O_2^- production. O_2^- generation (presumably, extracellular O_2^- measured by the cytochrome *c* assay) was not significantly different in phagocytic or F^--activated PMNs when assayed 15–20 min after the addition of yeast or NaF. The maximum CL response of NaF-treated cells correlated closely with increased O_2^- production of human PMNs. Despite the similarity in extracellular O_2^- concentration of phagocytic and F^--activated PMNs, CL of PMNs phagocytizing yeast was 10-fold higher than CL of

PMNs treated with NaF. At present, the reasons for the difference in CL of phagocytic and F^--activated cells are not completely clear. We examined the possibility of NaF quenching $^1O_2$ light by using a cell-free CL reaction of NaOCl and H_2O_2 (20, 21), and found that NaF did not appear to directly quench $^1O_2$-dependent CL. It is possible that the light emitted by PMNs phagocytizing yeast was greater than light produced by NaF-stimulated PMNs due to the reactions of oxidative intermediates with yeast cell walls (40) which could produce secondary light generating reactions (41).

Although the CL response of NaF-treated PMNs was lower than the response generated by phagocytizing PMNs, F^--activated PMNs consistently generated light in the absence of phagocytosis, and the CL response was accompanied by an increase in O_2^- production at 37°C. Light emission by F^--activated cells was depressed with SOD and the $^1O_2$ quencher, NaN_3, suggesting that both O_2^- and $^1O_2$ are, at least indirectly, contributing to the response. It is likely that other nonparticulate agents can stimulate leukocyte oxidative metabolism and CL responses in PMNs. An agent such as phorbol myristate acetate, recently reported to promote alveolar macrophage CL (42), could have possible usefulness in PMN CL assays. It is also conceivable that a combination of stimulating agents with a nonparticulate substrate could be used to activate nonphagocytizing PMNs and achieve a greater CL response than cells activated by F^- alone. These are necessary considerations in developing this CL reaction as a useful assay, and they are currently being evaluated in our laboratory.

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