Levels of DNA Strand Breaks and Superoxide in Phorbol Ester-Treated Human Granulocytes

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Abstract Phorbol ester treatment of granulocytes triggers release of superoxide ($O_2^{\cdot-}$) and a concomitant burst of DNA strand breaks. The relationship between the amount of $O_2^{\cdot-}$ and the number of DNA breaks has not previously been explored. To quantify the relatively large amount of $O_2^{\cdot-}$ generated over a 40-min period by 1×10^6 granulocytes/mL, a discontinuous "10-min pulse" method employing cytochrome c was used; 140 nmol $O_2^{\cdot-}$ per 1×10^6 cells was detected. DNA strand breaks were quantified by fluorimetric analysis of DNA unwinding (FADU). To vary the level of $O_2^{\cdot-}$ released by cells, inhibitors of the respiratory burst were used. Sodium fluoride (1–10 mM) and staurosporine (2–10 nM) both inhibited $O_2^{\cdot-}$ production. In both cases, however, inhibition of strand breakage was considerably more pronounced than inhibition of $O_2^{\cdot-}$. Zinc chloride (50–200 μM) inhibited both $O_2^{\cdot-}$ and DNA breaks, approximately equally. Dinophysistoxin-1 (okadaic acid) inhibited $O_2^{\cdot-}$ production more effectively than it inhibited DNA breaks. $O_2^{\cdot-}$ dismutes to H_2O_2, a reactive oxygen species known to cause DNA breaks. The addition of catalase to remove extracellular H_2O_2 had no effect on DNA breakage. Using pulse field gel electrophoresis, few double-stranded breaks were detected compared to the number detected by FADU, indicating that about 95% of breaks were single-stranded. The level of DNA breaks is not directly related to the amount of extracellular $O_2^{\cdot-}$ or H_2O_2 in PMA-stimulated granulocytes. We conclude that either an intracellular pool of these reactive oxygen species is involved in breakage or that the metabolic inhibitors are affecting a novel strand break pathway. J. Cell. Biochem. 66:219–228, 1997. © 1997 Wiley-Liss, Inc.

Key words: DNA strand breaks; superoxide; granulocytes; human; okadaic acid; fluoride; staurosporine; phorbol myristate acetate; hydrogen peroxide

So-called professional phagocytes (neutrophils, eosinophils, monocytes, and macrophages) possess a membrane-bound enzyme complex (the respiratory burst oxidase or NADPH oxidase), which is capable of catalysing the one-electron reduction of diatomic oxygen (O_2) to superoxide anion radical ($O_2^{\cdot-}$) [Cross, 1990; Cross and Jones, 1991; Segal and Abo, 1993; McPhail and Harvath, 1993; Umeki, 1994]. The NADPH oxidase is dormant in non-activated phagocytes but can be activated by many stimuli including the tumour promoter, phorbol myristate acetate (PMA). Large amounts of $O_2^{\cdot-}$ are released by granulocytes in response to PMA and its quantitative measurement can pose difficulties for technical reasons. Estimates of the time course of $O_2^{\cdot-}$ production by PMA-stimulated human granulocytes have varied from <20 min [Jandl et al., 1978; Klebanoff, 1968] to >3 hours [Nathan, 1987, 1989; Black et al., 1991a,b].

Stimulation of granulocytes by PMA is also known to induce strand breaks in nuclear DNA over a 40-min period [Shacter et al., 1988, 1990; Chong et al., 1989; Schraufstatter et al., 1988; Schwab et al., 1988; Dutton and Bowden, 1985; Singh et al., 1985; Birnboim, 1982, 1983]. Although the mechanism(s) of strand breakage is still not adequately understood, it is presumed to involve $O_2^{\cdot-}$ or some related oxygen radical [Birnboim and Biggar, 1982], perhaps by a direct "chemical" attack on DNA by hydroxyl radi-

Abbreviations used: BSS, balanced salt solution; DMSO, dimethyl sulfoxide; DSB, double-strand DNA breaks; DTX-1, dinophysistoxin-1 (35-methylokadaic acid); FADU, fluorimetric analysis of DNA unwinding; PFGE, pulsed field gel electrophoresis; PMA, phorbol myristate acetate; SSB, single-strand DNA breaks.

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cal (OH·) or ferryl radicals, or by reactive lipid species [Bertoncini and Meneghini, 1995; Halliwell and Gutteridge, 1990, 1992]. We and others have proposed a pathway, triggered by oxyradicals, that activates an endonuclease and leads to DNA strand breakage [Ueda and Shah, 1992; Birnboim and Kanabus-Kaminska, 1985]. In order to provide additional evidence concerning such a pathway, we have performed a quantitative study of the amount of $O_2^{\cdot-}$ produced and the number of DNA breaks formed in human granulocytes stimulated with PMA. If $O_2^{\cdot-}$ is a source of OH· or other such reactive species, then DNA breaks might be expected to be proportional to the amount of $O_2^{\cdot-}$ generated. If triggering a pathway leading to endonuclease activation, then such a relationship would not necessarily be expected. Using pharmacological agents to vary the amount of $O_2^{\cdot-}$ produced, we demonstrate that the number of DNA breaks produced per unit $O_2^{\cdot-}$ was not constant for different agents.

MATERIALS AND METHODS

Reagents

Superoxide dismutase was obtained from DDI Pharmaceuticals, Mountain View, CA. Catalase and staurosporine were from Sigma Chemical Co., St. Louis, MO. PMA (phorbol-12-myristate-13-acetate) was supplied by LC Services Corp., Woburn, MA; it was stored in aliquots at −20°C in DMSO at 1×10^{-4} M and 1×10^{-3} M. For daily use, a stock was prepared at 5×10^{-5} M in DMSO. Cytochrome c (equine heart) was from ICN Biomedicals, St. Laurent, Quebec. Sodium fluoride (stored in a plastic tube as a 1 M stock in water at −20°C) and zinc chloride were from Fisher Scientific, Ottawa, ON. Dinophysistoxin-1 (DTX-1, 35-methylokadaic acid) was a gift from Dr. Hirota Fujiki of the Cancer Prevention Division, National Cancer Center Research Institute, Tokyo, Japan.

Cell Isolation and Treatment

Human granulocytes were purified from the peripheral blood of normal volunteers as described elsewhere [Birnboim, 1990]. In brief, granulocytes and erythrocytes were first separated from mononuclear cells on a Ficoll-Hypaque density gradient. The erythrocyte/granulocyte layer was treated with ammonium chloride and hypotonic solutions to lyse erythrocytes. The final preparation consisted of >97% granulocytes. Purified cells were resuspended at 1×10^6/mL in a balanced salt solution (BSS): 137 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 10 mM HEPES, 5 mM glucose, pH 7.4. Where indicated, PMA was added at 50 nM unless otherwise specified; inhibitors were added at the same time as PMA and incubations were at 37°C.

Analysis of DNA Strand Breaks

Two types of DNA strand break analyses were carried out. The FADU method (fluorimetric analysis of DNA unwinding [Birnboim, 1990; Birnboim and Jevcak, 1981]) measures both single-strand breaks (SSB) and double-strand breaks (DSB). One Q_d unit corresponds to 120 strand breaks per cell, and the response for ionizing radiation is linear up to at least 70 Q_d units [McWilliams et al., 1983] (Birnboim, unpublished data). Pulsed field gel electrophoresis (PFGE), which detects primarily DSB, was carried out as described elsewhere [Sandhu and Birnboim, 1993]. Cells were suspended at 1×10^6 cells/mL in BSS containing 10 μg/mL catalase.

Superoxide Production

Two different methods for measurement of $O_2^{\cdot-}$ production were compared, as described in Results. Both are based upon the ability of $O_2^{\cdot-}$ to reduce oxidized cytochrome c in a superoxide dismutase-inhibitable fashion [Markert et al., 1984]. Absorbance measurements were carried out in a Perkin-Elmer (Norwalk, CT) Lambda 5 double-beam spectrophotometer. For *continuous* measurement of $O_2^{\cdot-}$ production by granulocytes, both the reference and the sample cuvettes contained the following (in 1 mL of BSS): $0.25–1.0 \times 10^6$ granulocytes, 40–160 μM cytochrome c, and 10 μg/mL catalase. The reference cuvette additionally contained 25 μg/mL superoxide dismutase. Cuvettes were maintained at 37°C and the respiratory burst was started by the addition of 1 μL of PMA.

For the *discontinuous 10-min pulse* method, 5 mL of cells (approximately 1×10^6/mL in BSS) were incubated at 37°C in a polypropylene tube. PMA and inhibitors, where indicated, were added to the cells at 0-time. At appropriate intervals, 0.25 mL of cell suspension was transferred (in triplicate) to 1.5 mL polypropylene microfuge tubes containing 25 μL of a solu-

tion of cytochrome c (1.6 mM) and catalase (10 μg/mL). The cell concentrations shown in the figure legends are those after this dilution. A 0.25 mL aliquot of cell suspension was also added to a "control" tube containing 25 μg superoxide dismutase in addition to cytochrome c and catalase. After a 10-min period of incubation at 37°C, the tubes were chilled on ice. At the end of the total incubation period, all tubes were centrifuged for 10 s to deposit the cells and the absorbance at 550 nm of a ⅓ dilution of the clarified supernatant was made. Calculation of $O_2^{-\cdot}$ is based upon $\Delta A_{550} = 0.021$ for the reduction of 1 nmol/mL of ferricytochrome c [Massey, 1959].

Potential Interference of Sodium Fluoride in the Detection of $O_2^{-\cdot}$ by Cytochrome c

Solutions of cytochrome c (80 μM) in BSS were prepared without or with sodium fluoride (10 mM) and preincubated for at least 10 min at room temperature. Then 1 mL was transferred to a cuvette and 6-hydroxydopamine (20 μL of a freshly prepared 1 mg/mL stock in cold, degassed water) was added as a source of $O_2^{-\cdot}$ [Gee and Davison, 1989]. Xanthine oxidase/xanthine cannot be used since fluoride inhibits this $O_2^{-\cdot}$ generating system [Beyer and Fridovich, 1987; Beyer et al., 1986]. Immediately following mixing, the cuvette was transferred to a spectrophotometer and ΔA_{550} was monitored for 60 s. Four sets of readings were taken, alternating between samples without or with sodium fluoride.

RESULTS

Measurement of $O_2^{-\cdot}$

The conventional *continuous* method for quantitation of extracellular $O_2^{-\cdot}$ produced by granulocytes utilizes the increase in absorbance at 550 nm associated with the reduction of extracellular ferricytochrome c, as measured in a double-beam spectrophotometer. While relatively simple, it is reliable only for measuring relatively small amounts of $O_2^{-\cdot}$ produced by relatively few cells [Markert et al., 1984]. The limitation of the *continuous* method is illustrated in Figure 1A. Granulocytes were stimulated with PMA to release $O_2^{-\cdot}$ and the increase in A_{550} was measured. After the change in absorbance had levelled off, the amount of unreduced ferricytochrome c was determined by the addition of the strong reducing agent, dithionite. As can be seen, the amount of $O_2^{-\cdot}$ produced was seriously underestimated at 1×10^6 cells/mL for 40 and 80 μM cytochrome c (curves 1 and 2, respectively). Only for 0.25×10^6 granulocytes/mL at 80 μM cytochrome c did the method appear to provide a reasonable measure of $O_2^{-\cdot}$ produced (curve 3). Cytochrome c reduction continued for at least 30 min under these conditions. The ΔA_{550} (corresponding to about 100 nmol $O_2^{-\cdot}$ per 1×10^6 cells per 30 min) is comparable to that reported by others [Markert et al., 1984; Black et al., 1991a,b]. Further indication that this method can be misleading at a higher cell concentration is illustrated by a study of the effect of 10 mM sodium fluoride on $O_2^{-\cdot}$ production by PMA-stimulated granulocytes (Fig. 1B). The cell number was 1×10^6/mL and $O_2^{-\cdot}$ production was monitored in the presence of 40, 80, or 160 μM cytochrome c (curves 1, 2, or 3, respectively). The apparent level of inhibition was strongly dependent on the amount of cytochrome c present. Inhibition by sodium fluoride was obvious only at 160 μM cytochrome c. These data indicate that the *continuous* method for assaying $O_2^{-\cdot}$ production by higher concentrations of granulocytes should be used with caution.

Because of this difficulty, we turned to a *discontinuous pulse* method for measuring $O_2^{-\cdot}$ production in PMA-stimulated granulocytes [Birnboim and Kanabus-Kaminska, 1985]. With this method, problems associated with (1) excessive light scattering due to high numbers of cells and (2) depletion of substrate (cytochrome c) are avoided since absorbance measurements are carried out using clear solutions and cells are exposed to cytochrome c for only a fraction (pulse time) of the total period of PMA stimulation. For the experiment of Figure 1C, 1×10^6 cells/mL were stimulated by PMA in the absence or presence of 10 mM sodium fluoride; the results differed significantly from those of Figure 1B: (1) $O_2^{-\cdot}$ production was seen to continue for appreciably longer, at least 40 min. (2) The total amount of $O_2^{-\cdot}$ produced was appreciably greater (150 nmol per 1×10^6 cells). (3) the degree of inhibition by sodium fluoride was more evident, 47.7% at 40 min for the pulse method compared to 33.0% at 10 min for the *continuous* method (Fig. 1B, curve 3). At shorter times (5–10 min), the two methods gave similar results.

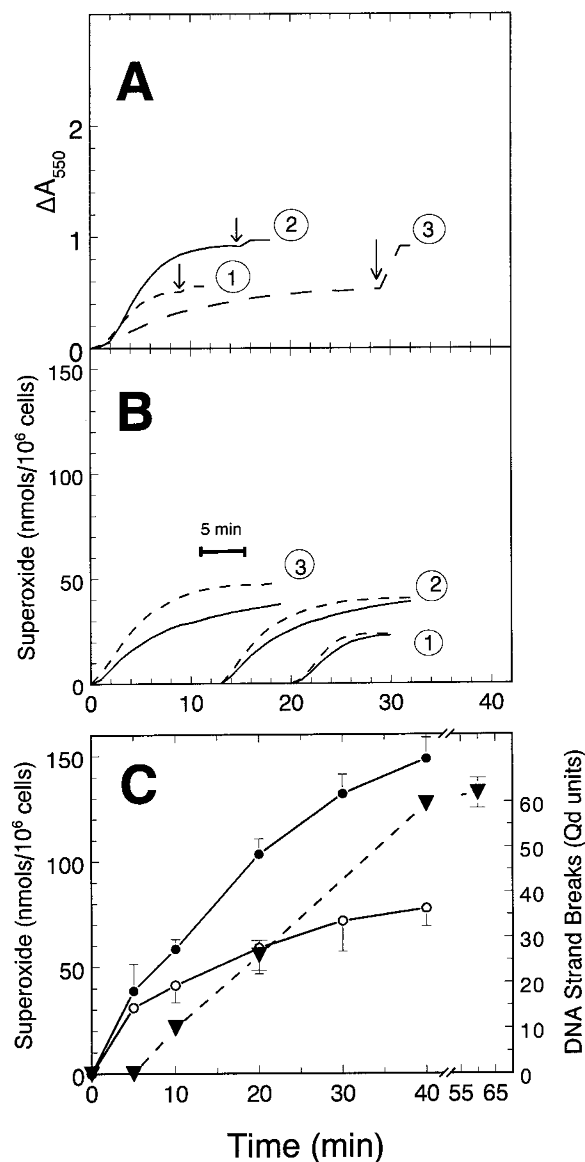

Fig. 1. Time course of $O_2^{\cdot -}$ production and DNA strand breaks in PMA-stimulated granulocytes, in the absence and presence of sodium fluoride. **A:** $O_2^{\cdot -}$ production measured by the continuous reduction of ferricytochrome c (ΔA_{550}). ①, 40 μM cytochrome c, 1 × 10^6 cells/mL; ②, 80 μM cytochrome c, 1 × 10^6 cells/mL; ③, 80 μM cytochrome c, 0.25 × 10^6 cells/mL. Arrows indicate the addition of dithionite to cause full reduction of the substrate. **B:** Effect of sodium fluoride on $O_2^{\cdot -}$ production, expressed on a per-cell basis, as measured by the continuous reduction of ferricytochrome c. Cell concentration was 1 × 10^6/mL. Cytochrome c concentration was ①, 40 μM, ②, 80 μM or ③, 160 μM. Dashed line, no sodium fluoride; solid line, 10 mM sodium fluoride. **C:** Time course of $O_2^{\cdot -}$ production, as measured by the pulse method, and DNA strand breaks in PMA-stimulated granulocytes. Cell concentration was 1 × 10^6/mL. Each symbol represents the cumulative amount of $O_2^{\cdot -}$ detected to that time point. For example, the amount of $O_2^{\cdot -}$ detected in the 10 to 20 min time interval is the difference between the 10 and 20 min points. ●, no sodium fluoride; ○, 10 mM sodium fluoride. Averages ± SEM for 4–14 experiments are shown. ▼, DNA strand breaks, as measured by the FADU method. Other details are described in Materials and Methods.

Kinetics of DNA Strand Breakage in Relation to $O_2^{\cdot -}$ Levels

Having established procedures for measurement of $O_2^{\cdot -}$ by PMA-stimulated granulocytes at 1 × 10^6/mL (conditions needed for DNA strand break determination), we explored the time course of DNA breakage in relation to $O_2^{\cdot -}$ production (Fig. 1C, broken line). The onset of DNA breaks occurred about 5 min after the onset of $O_2^{\cdot -}$ production, and thereafter both were produced with similar kinetics. The level of DNA breaks reached a maximum at 40 min and then remained at the same level for at least another 20 min. For the remaining experiments, the 40-min time point was used as a measure of total DNA breaks.

Relationship Between Level of $O_2^{\cdot -}$ and the Number of DNA Breaks in Fluoride-Treated Cells

We have previously shown that sodium fluoride is a potent inhibitor of DNA strand breakage in PMA-stimulated leukocytes [Birnboim and Kanabus-Kaminska, 1985]. The data of Figure 2 demonstrate a dose-dependent inhibition by sodium fluoride of $O_2^{\cdot -}$ production. At very high concentrations (20 to 40 mM), over 80% inhibition of superoxide production could be achieved (data not shown). There was a lack of concordance between the number of DNA breaks and the cumulative amount of $O_2^{\cdot -}$ produced over 40 min. Inhibition of DNA strand breaks was much greater than inhibition of $O_2^{\cdot -}$ production over the range 2.5 to 10 mM sodium fluoride. At 10 mM, 95.2 ± 1.1% of DNA strand breakage was inhibited while only 52.3 ± 2.3% of $O_2^{\cdot -}$ production was suppressed. These results indicate that inhibition of strand breaks by sodium fluoride can at least partially be dissociated from the inhibition of $O_2^{\cdot -}$ production in human granulocytes.

Since sodium fluoride is known to interact with heme compounds, we tested whether fluoride might affect the ability of cytochrome c to detect $O_2^{\cdot -}$. Using auto-oxidation of 6-hydroxydopamine as a source of $O_2^{\cdot -}$, the reduction of cytochrome c was unaffected by 10 mM sodium fluoride: Δ_{550} of 0.461 ± 0.009 and 0.458 ± 0.006 (average ± SEM, n = 4), control vs. fluoride, respectively. Sodium fluoride is known to inhibit many cellular enzymes by complexing intracellular magnesium [Borel, 1945]. The data of Table I provide further evidence that depletion of intracellular magnesium can inhibit $O_2^{\cdot -}$ production by granulocytes. The ionophore A23187 can transport Mg^{++} as well as other

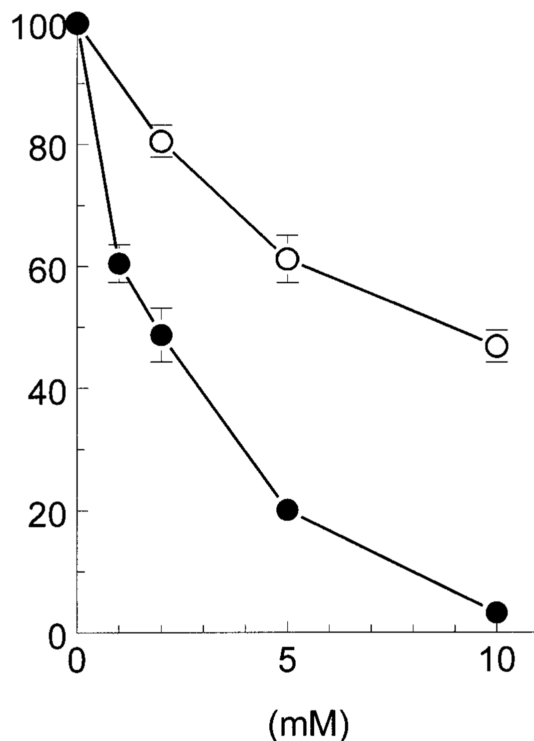

Fig. 2. Effect of sodium fluoride on DNA strand breaks and $O_2^{\cdot-}$ production in PMA-stimulated granulocytes. Cell concentration for both assays was 1 × 10^6 cells/mL and incubation time was 40 min at 37°C. DNA strand breaks (●) were determined using the FADU procedure and $O_2^{\cdot-}$ production (○) was determined by the pulse method as described in Materials and Methods. The averages ± SEM of 4–7 separate $O_2^{\cdot-}$ experiments and 5–7 DNA break experiments are shown. In the absence of inhibitor, 64.1 ± 1.6 (SEM) (n = 20) Q_d units of DNA breaks were induced in PMA-stimulated cells.

TABLE I. Depletion of Intracellular Magnesium Inhibits the Respiratory Burst*

	Superoxide production (nmol/10^6 cells/30 min)	
	0 Mg^{++}	0.8 mM Mg^{++}
1. PMA (50 nM)	74.8 ± 15.4	77.4 ± 26.4
2. PMA + A23187 (3 μM)	77.2 ± 11.5	70.8 ± 2.0
3. PMA + A23187 + EGTA (5 mM)	28.2 ± 4.6	85.8 ± 1.4
4. PMA + A23187 + EDTA (5 mM)	27.1 ± 2.1	30.9 ± 0.2

*Human granulocytes [Birnboim, 1990] were suspended at a concentration of 0.5 × 10^6 cells/mL in BSS without or with 0.8 mM $MgSO_4$ and the indicated agents were added. Aliquots (0.25 mL) were removed and transferred to tubes as described in Materials and Methods for the discontinuous method, except that incubation was carried out for 30 min instead of 10 min. Results shown are averages ± ranges for 2 independent experiments. The two values in line 3 are statistically significantly different ($P < 0.05$ in a paired 2-sided Student's *t*-test).

divalent cations across cell membranes [Pfeiffer and Deber, 1979]. Addition of A23187 and either EGTA or EDTA inhibited $O_2^{\cdot-}$ production in the absence of extracellular Mg^{++}. This inhibition was prevented by 0.8 mM Mg^{++} in the presence of EGTA but not in the presence of EDTA, presumably reflecting the much greater affinity of EDTA than EGTA for Mg^{++} [Dawson et al., 1986]. These data support the suggestion that sodium fluoride inhibits $O_2^{\cdot-}$ production by depleting intracellular Mg^{++}.

Effect of Other Inhibitors on the Levels of $O_2^{\cdot-}$ and DNA Breaks

The relationship between the amount of $O_2^{\cdot-}$ produced and the number of DNA strand breaks was further explored by examining the effect of other inhibitors of the respiratory burst to vary the amount of $O_2^{\cdot-}$ produced. Agents were chosen at concentrations that gave partial inhibition of $O_2^{\cdot-}$ production. Zinc chloride inhibited $O_2^{\cdot-}$ production and DNA breakage with very similar kinetics (Fig. 3). Staurosporine, a protein kinase C inhibitor, inhibited strand breakage to a greater extent than $O_2^{\cdot-}$ production (Fig. 4). The opposite effect was seen with DTX-1, an okadaic acid-related protein phosphatase inhibitor; it inhibited $O_2^{\cdot-}$ production preferentially over DNA breakage (Fig. 5). None of these agents alone caused a significant number of DNA strand breaks in the absence of PMA (data not shown). These results are replotted in Figure 6 to more clearly indicate that decreasing the amount of $O_2^{\cdot-}$ using pharmacological agents does not, except in the case of zinc, produce a corresponding decrease in the number of DNA strand breaks.

Nature of DNA Breaks in PMA-Stimulated Granulocytes

DNA breaks in PMA-stimulated granulocytes were measured by FADU, an alkaline unwinding method that detects both SSB and DSB. To determine what proportion of these breaks might be DSB, PFGE was carried out. H_2O_2 is known to cause exclusively SSB [Stamato and Denko, 1990]. The number of DSB detected in PMA-treated granulocytes was relatively low (Table II), corresponding to the number induced by approximately 5 Gy of ^{60}Co γ rays [Sandhu and Birnboim, 1993]. The total number of breaks (SSB + DSB) detected by

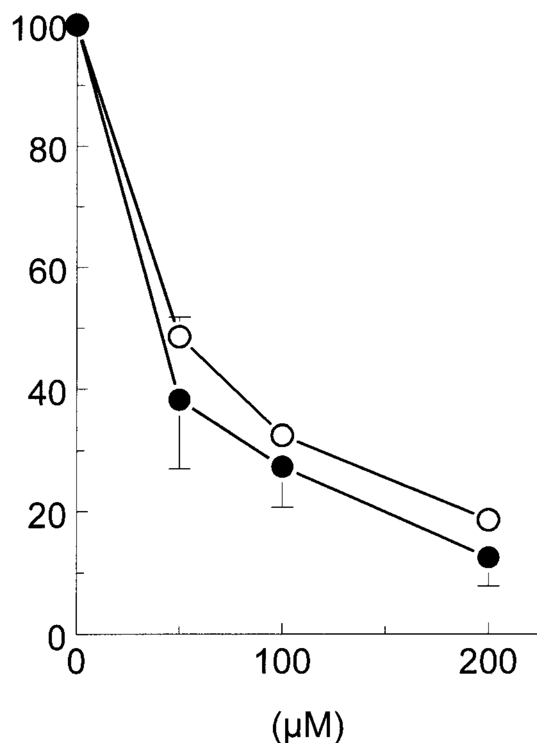

Fig. 3. Effect of zinc chloride on DNA strand breaks and $O_2^{\cdot-}$ production in PMA-stimulated granulocytes. Conditions as in Figure 2.

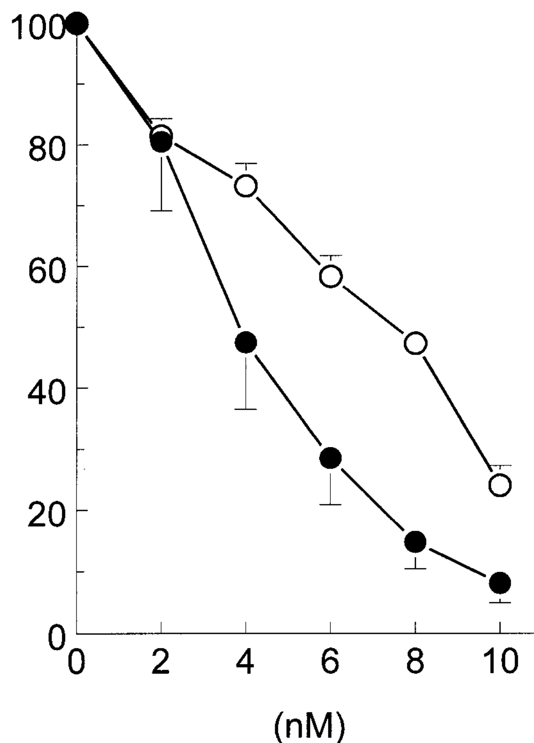

Fig. 4. Effect of staurosporine on DNA strand breaks and $O_2^{\cdot-}$ production in PMA-stimulated granulocytes. Conditions as in Figure 2.

FADU was comparable to that induced by about 6 Gy of radiation. Thus, the ratio of DSB to total breaks in PMA-stimulated granulocytes, at 40 min after PMA treatment, is similar to that induced by low LET (linear energy transfer) radiation, i.e., about 5% [Bradley and Kohn, 1979].

Granulocytes are known to undergo apoptosis, including the generation of nucleosome-sized DNA fragments [Lee et al., 1993; Afford et al., 1992; Savill et al., 1989]. When control granulocytes were incubated in culture, a "ladder" of DNA fragments was detected after 6 h (data not shown). By contrast, DNA breaks were detected by FADU within minutes after PMA treatment (Fig. 1C, dashed line). There was no change in cell viability as measured by trypan blue uptake or ethidium bromide exclusion for at least 1 h (data not shown). It would, therefore, seem unlikely that breaks detected by FADU were related to apoptosis or necrosis. Interestingly, PMA treatment actually prevented the formation of the DNA ladder seen in control cells (data not shown). The explanation may be that cells die by a necrotic mechanism after about 3 h, before the onset of apoptosis.

DISCUSSION

Although the respiratory burst of granulocytes has been extensively studied and is fairly well understood, the mechanism by which DNA breaks are induced in PMA-stimulated granulocytes remains uncertain. Since DNA breaks are not observed in granulocytes from patients with Chronic Granulomatous Disease, a genetic disease affecting the respiratory burst oxidase, it is clear that products of the respiratory burst ($O_2^{\cdot-}$, H_2O_2) are required [Birnboim and Biggar, 1982]. To investigate the mechanism further, we examined the relationship between the number of DNA strand breaks and the amount of $O_2^{\cdot-}$ produced in PMA-stimulated granulocytes. If the primary product of the respiratory burst ($O_2^{\cdot-}$) or a downstream product (H_2O_2) was directly responsible for the breaks by a free radical attack on the DNA backbone (as is seen with ionizing radiation), then a direct relationship between the two might be expected. Quantitative measurements of both DNA breaks and $O_2^{\cdot-}$ were required to test this relationship. Measurement of DNA strand breaks by the FADU method is a well-established procedure [Birnboim, 1990; McWilliams et al., 1983; Birn-

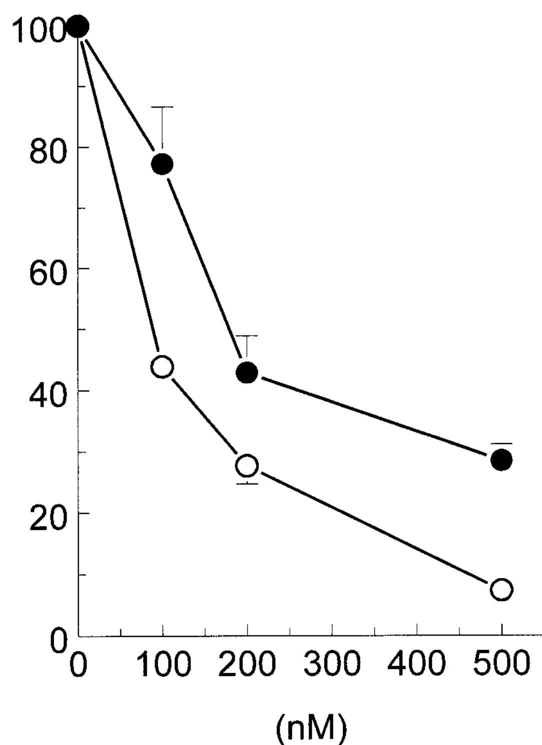

Fig. 5. Effect of DTX-1 on DNA strand breaks and $O_2^{\cdot-}$ production in PMA-stimulated granulocytes. Conditions as in Figure 2.

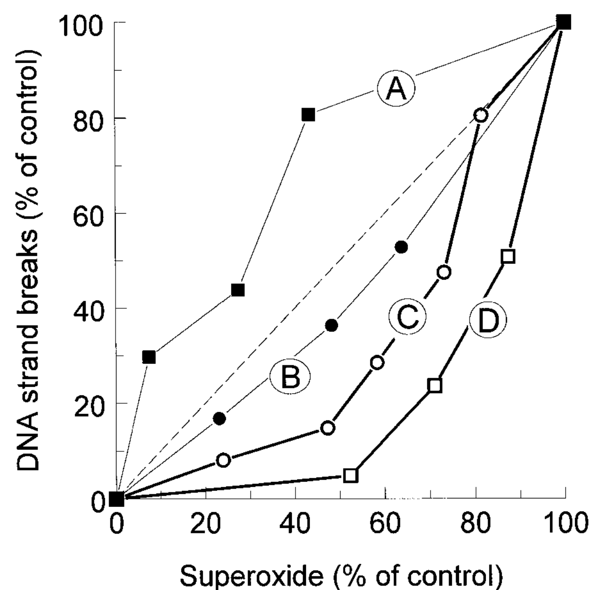

Fig. 6. Replot of the data of Figures 2–5 to allow comparison of the effect of several respiratory burst inhibitors on the relationship between DNA strand breaks and $O_2^{\cdot-}$ in PMA-stimulated granulocytes. Curve A, DTX-1; curve B, zinc; curve C, staurosporine; curve D, sodium fluoride. The dotted line represents a theoretical linear correlation.

boim and Jevcak, 1981] that gives a linear ^{60}Co γ ray dose-response curve for ≤70 Q_d units of breaks [McWilliams et al., 1983] (Birnboim, unpublished data), the maximum level observed in PMA-stimulated granulocytes. However, quantitative measurement of $O_2^{\cdot-}$ produced by granulocytes is not straightforward and it was therefore necessary to establish appropriate methods for the cell concentration range and time period needed. The widely used method of continuously monitoring the reduction of ferricytochrome c in a superoxide dismutase-inhibitable fashion was first examined [Fridovich, 1970]. Technical difficulties, including high background scattering and absorbance of cells and ferricytochrome c, were encountered when measuring large amounts of $O_2^{\cdot-}$ produced by granulocytes (i.e., >10 min and >0.25 × 10^6 cells/mL). The *discontinuous 10 min-pulse* method minimized these problems. Although there is no independent way to determine the completeness of $O_2^{\cdot-}$ trapping, the maximum amount of $O_2^{\cdot-}$ detected by the *pulse* method is as high or higher than reported by others who have studied quantitative and tem-

TABLE II. Low Level of DSB in PMA-Stimulated Human Granulocytes†

Treatment	PFGE (% DNA out of well)	FADU (Q_d units)
Control cells	6.2 ± 0.7 (n = 7)	0
PMA	10.1 ± 1.3 (n = 7)*	59.7 ± 2.3 (n = 3)
PMA + catalase	8.6 ± 0.9 (n = 6)	60.8 ± 6.0 (n = 2)
Glc. oxidase (0.025 U/mL)	5.0 (n = 1)	14.7 ± 2.2 (n = 3)
Glc. oxidase (0.050 U/mL)	5.3 (n = 1)	41.1 ± 9.3 (n = 3)

†PFGE and FADU are measures of double-strand breaks and total DNA breaks, respectively. Q_d is defined as the difference in DNA unwinding rates between treated and control cells [McWilliams et al., 1983]. Catalase, where added, was at 10 μg/mL. Glucose oxidase was used to generate a flux of H_2O_2; although the result of only a single experiment in each case is shown, it is well established that H_2O_2 causes single-stranded, not double-stranded, breaks [Stamato and Denko, 1990]. Cells were treated under identical conditions prior to carrying out the two types of analysis. All incubations were at 37°C for 40 min. Other details as in Materials and Methods.

*Significantly different from control ($P < 0.05$) (ANOVA, post-hoc Dunnett's test).

poral aspects of the respiratory burst [Nathan, 1987; Test and Weiss, 1984; Black et al., 1991a,b]. Using these methods, a simple relationship between the amount of $O_2^{\cdot-}$ detected and the number of DNA breaks produced was not seen. This led us to conclude that, at least in part, other mechanisms are involved in DNA breakage.

Four pharmacologically active agents were tested for their ability to affect DNA strand breakage relative to $O_2^{\cdot-}$ production. Sodium fluoride has the potential to act on a large number of cellular targets. It can inhibit some metalloenzymes by forming complexes with intracellular Mg^{++} and other metal ions [Borel, 1945]. For example, inhibition of enolase, a Mg^{++}-requiring enzyme of anaerobic glycolysis, will lead to a marked decrease in cellular ATP. The data of Table I provide more direct evidence that depletion of intracellular Mg^{++} can inhibit PMA-stimulated $O_2^{\cdot-}$ generation and is consistent with the possibility that sodium fluoride may also act in this way. Paradoxically, sodium fluoride alone is a weak stimulator of the respiratory burst of granulocytes [Gabler et al., 1986; Curnutte and Babior, 1975; Elferink, 1982], acting through a G protein [Hartfield and Robinson, 1990; Toper et al., 1987]. Sodium fluoride also enhances PMA-stimulated leukotriene production and stimulates adenylate cyclase by a mechanism involving G proteins [Downs et al., 1980; Howlett et al., 1979; Brom et al., 1989]. Unlike other halides, it is not redox active and is not a substrate for myeloperoxidase [Sepe and Clark, 1985; Klebanoff, 1968]. Previously, we have reported that sodium fluoride inhibits the production of DNA strand breaks in PMA-stimulated leukocytes but *stimulates* DNA breakage in cells exposed to glucose oxidase as a source of H_2O_2 [Birnboim and Kanabus-Kaminska, 1985]. This is evidence that the mechanism operative to produce strand breaks in PMA-stimulated leukocytes differs from H_2O_2-treated cells.

Other agents were also used to decrease the amount of $O_2^{\cdot-}$ produced by PMA stimulated granulocytes. Zn^{++} may interfere with the respiratory burst by blocking a proton channel that releases protons to the external face of the membrane [Simchowitz, 1985; Cross, 1990] or a Na^{+}/Ca^{++} exchanger [Simchowitz et al., 1990]. Interestingly, Zn^{++} in the same concentration range used in our study has been shown to inhibit a Ca^{++}/Mg^{++}-dependent endonuclease that cleaves DNA to oligonucleosome-size fragments at later stages of apoptosis [Brown et al., 1993; Walker et al., 1994; Sunderman, 1995]. In some cells, an endonuclease activity has been observed in early stages in apoptosis which produced large DNA fragments; this enzyme is not inhibited by Zn^{++} [Brown et al., 1993; Walker et al., 1994]. However, in renal tubular epithelial cells, an endonuclease is activated early after exposure to H_2O_2 and this enzyme is inhibited by Zn^{++} [Ueda and Shah, 1992]. Zn^{++} is also known to inhibit protein phosphatases and protein tyrosine kinases [Pang and Shafer, 1985]. Staurosporine is an inhibitor of protein kinase C, blocking the activation of the respiratory burst in granulocytes by PMA [Watson et al., 1991]. Although a very potent inhibitor of protein kinase C, staurosporine can also inhibit other protein kinases, with only slightly higher IC_{50} [Hidaka and Kobayashi, 1992]. DTX-1 (dinophysistoxin-1, 35-methylokadaic acid) is a specific inhibitor of protein phosphatases 1 and 2A [Suganuma et al., 1992]. It has previously been reported to modulate the respiratory burst of granulocytes [Djerdjouri et al., 1995; Okuyama et al., 1996; Berkow and Dodson, 1993; Ding and Badwey, 1992; Lu et al., 1992].

The accumulated evidence does not favour a model of direct oxyradical attack on DNA. None of the four agents tested are known to be free radical scavengers. Although the majority of breaks appear to be SSB (suggestive of a H_2O_2-like mechanism), the addition of catalase to eliminate extracellular H_2O_2 had no effect. The dissociation of the level of DNA strand breaks from the level of $O_2^{\cdot-}$ and the known effect of these agents on signal transduction pathways has therefore lead us to postulate that there exists a biochemical pathway producing DNA strand breaks. Support for such a pathway comes from earlier findings that breaks in PMA-treated granulocytes are not rejoined, as occurs with ionizing radiation-induced breaks in the same cells [Birnboim, 1986], and that addition of sodium fluoride rapidly stops strand breakage without stopping $O_2^{\cdot-}$ production [Birnboim, 1988]. The present findings therefore support a model of DNA breakage in PMA-treated granulocytes involving a biochemical pathway as opposed to direct oxyradical damage to the DNA backbone.

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