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The production of DNA strand breaks in human leukocytes by superoxide anion may involve a metabolic process

(hydrogen peroxide/phorbol ester/calcium ionophore/fluoride/deoxyglucose)

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ABSTRACT **H_2O_2 is known to be capable of inducing strand-break damage in intracellular DNA, but whether O_2^- also can do so in the absence of H_2O_2 is uncertain. The difficulty in distinguishing the effects of the two is that, under physiological conditions, dismutation of O_2^- to H_2O_2 can readily occur. When human leukocytes are stimulated with phorbol 12-myristate 13-acetate (PMA), they release O_2^-, and within a few minutes strand breakage in intracellular DNA can be observed. We have attempted to determine whether the O_2^- produced is itself capable of causing DNA damage or whether H_2O_2 alone, or in combination with O_2^-, is responsible for the observed damage. Addition of catalase (up to 250 μg/ml) to remove H_2O_2 prevented no more than about 50% of the DNA damage. The majority of the remaining damage could be blocked, in a dose-dependent manner, by superoxide dismutase (SOD) or a SOD-mimetic copper complex, identifying a fraction of damage to intracellular DNA dependent upon extracellular O_2^-. We studied this O_2^--specific fraction through the use of three metabolic poisons (fluoride, 2-deoxyglucose, and A23187). These agents largely blocked DNA damage, while affecting extracellular O_2^- levels only slightly. For comparison, H_2O_2-induced DNA damage was studied with glucose oxidase to generate a flux of H_2O_2. The first two metabolic poisons had little effect, whereas A23187 did inhibit H_2O_2-induced DNA damage. We conclude that O_2^--induced damage occurs through a mechanism that differs, at least in part, from the H_2O_2 damage pathway and that the former may involve one or more metabolic steps.**

The superoxide anion (O_2^-) is a free radical species formed by the one-electron reduction of dioxygen (O_2). It has become the subject of considerable interest in biology and medicine as awareness has grown of its ubiquitous nature in aerobic cells exposed to oxygen from sources such as autooxidations, enzymic oxidations, subcellular organelles, and the respiratory burst of phagocytic cells (1). Although it is a free radical, the reactivity of O_2^- is in general quite low (2–4). Probably because of this, many workers consider that it is significant in biological systems principally because of its ability, in the presence of redox-active metals, to give rise to a much more reactive form of oxygen, the hydroxyl radical (OH·) (e.g., see ref. 5). Unsaturated fatty acids in membranes have been studied as important targets for oxygen radicals, including O_2^- (e.g., refs. 3 and 5). Recently there also has been considerable interest in the possibility that O_2^- may play a role in carcinogenesis and tumor promotion (6–10).

Our own work in tumor promotion has focused upon cellular DNA as a target for oxygen radicals (11–13). We have shown that human leukocytes, stimulated to produce O_2^- by exposure to phorbol 12-myristate 13-acetate (PMA) and other tumor promoters, suffer DNA strand breakage. It is clear that O_2^- was required, since much of the damage was abolished by the addition of superoxide dismutase (SOD), which converts O_2^- to H_2O_2 (11). However, the mechanism by which DNA damage occurs has not yet been established. In the present experiments, we have concentrated our efforts on elucidating the mechanism of O_2^--induced damage and distinguishing it from H_2O_2-induced damage. This was accomplished by carrying out experiments in the presence of catalase, such that the majority of DNA damage remaining was inhibitable by SOD. Our results provide evidence that O_2^- induces damage by a pathway that is at least partially different from the H_2O_2 damage pathway. Metabolic steps or intermediates appear to be involved because certain metabolic inhibitors can markedly lower the level of strand breakage observed.

MATERIALS AND METHODS

Preparation of Human Leukocytes. Leukocytes from normal human volunteers were isolated from freshly drawn blood, collected in 10-ml Vacutainer tubes (Becton Dickinson, Canada) containing EDTA (3.6 mM) as anticoagulant. The majority of erythrocytes were first removed by lysis with ammonium chloride (3 vol of 0.15 M NH_4Cl/0.01 M Tris·HCl, pH 7.4, to 1 vol of blood) at 15°C for 10–15 min. The remaining cells were collected by centrifugation (10 min at 0°C for 600 × *g*) and suspended in 1 vol of balanced salt solution (137 mM NaCl/5 mM KCl/0.8 mM $MgSO_4$/8.5 mM sodium phosphate, pH 7.4; glucose, if present, was at 5 mM) at 0°C. Residual contaminating erythrocytes were removed by hypotonic lysis as follows. Two volumes of cold water (0°C) was added briskly to the cell suspension, which was immediately mixed several times by inversion. After 60 sec, 0.22 vol of 1.5 M NaCl was added to restore isotonicity; the leukocytes were collected by centrifugation, washed twice with balanced salt solution, and suspended at about 1.0×10^6 cells per ml. Ten milliliters of suspension was distributed to 15-ml siliconized glass centrifuge tubes as required for each test.

Fluorometric Analysis of DNA Unwinding (FADU): Assay for DNA Strand Breakage. DNA damage analysis was carried out using a fluorometric technique that measures the rate of unwinding of cellular DNA on exposure to alkaline conditions; the technique has been described in detail previously (14). In brief, 10 ml of cell suspension was treated as described in the individual experiments and then chilled to 0°C; the cells were collected by centrifugation, washed, and distributed equally to a set of 12 tubes. Cells were lysed with a urea/detergent solution. An alkaline solution was added, and DNA strand unwinding was allowed to occur for a 60-min period at 15°C. After this treatment, the samples were neutralized, and the amount of residual double-strand DNA was estimated by using a fluorescent dye, ethidium bromide.

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Abbreviations: SOD, superoxide dismutase; PMA, phorbol 12-myristate 13-acetate.

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This allows calculation of the rate of DNA unwinding, which in turn can be approximately converted to the number of strand breaks per cell by reference to the effect produced by γ-rays. In this paper, results are expressed in units of Q_d, which have been defined precisely elsewhere (15). Q_d is a measure of the DNA damage induced by a given treatment, where one Q_d unit corresponds to about 100 strand breaks per cell (15). As in other alkaline unwinding or sedimentation procedures, single-strand breaks, double-strand breaks, and alkali-labile lesions are detected but not distinguishable.

Measurement of Extracellular Superoxide Anion. Measurement of O_2^- required a different procedure than was used previously by us (12). In the present experiments we were interested in following the time course of extracellular O_2^- release and correlating this with the time course of DNA damage. Therefore, it was important to treat cells under conditions as similar as possible for the two assays. However, the inclusion of a high concentration of cytochrome *c* (as dictated by the O_2^- measurement) would necessarily perturb the measurement of DNA damage by "scavenging" the O_2^- and thereby protect the cells. Therefore, a "10-min pulse" method was used, as described in the legend to Fig. 1.

Reagents. PMA, catalase, and 2-deoxyglucose were supplied by Sigma. SOD was obtained as Palosein (Orgotein) from Diagnostic Data (Mountain View, CA). Hydrogen peroxide-inactivated SOD was prepared by treating SOD in 10 mM Na_2CO_3 (pH 10.5) with 10 mM H_2O_2 at room temperature for 45 min (16); excess H_2O_2 was removed with catalase before addition to cells. Calcium ionophore (A23187) and cytochrome *c* were supplied by Calbiochem–Behring. The complex of indomethacin with Cu(II) was prepared as described by Weser *et al.* (17).

RESULTS

The neutrophil is a particularly favorable system for studying the effect on cellular DNA of high external concentrations of O_2^-, relatively free of H_2O_2, because it contains a specialized NADPH oxidase system in its plasma membrane, which releases O_2^- into the external medium on stimulation with agents such as PMA. Added SOD affords appreciable protection of cellular DNA under these conditions (ref. 11; Table 1). By contrast, there was no protection of cellular DNA by added SOD but complete protection by catalase when O_2^- was generated in free solution by chemical or enzymatic means, such as when O_2^- was introduced by xanthine/xanthine oxidase (13), potassium superoxide, or dialuric acid (data not shown). This is presumably because O_2^- generated at some distance from a cell can undergo dismutation before it reaches a "target" cell, and the resultant DNA strand-break damage is almost exclusively due to H_2O_2.

Catalase gave partial protection against DNA damage in PMA-stimulated neutrophils. The data of Table 1 indicate that about 50% of the observed damage could be prevented by the addition of catalase at 10 μg/ml with no additional protection at concentrations up to 250 μg/ml. In the present experiments, we were interested specifically in discriminating between DNA damage caused by O_2^- and that caused by H_2O_2. The data of Tables 1 and 2 indicate that, in the presence of catalase at either 10 or 200 μg/ml, the majority of the residual DNA damage was prevented by addition of high concentrations of bovine SOD or by Cu(II)–indomethacin, a SOD-mimetic, low molecular weight, copper(II) complex. The effect of SOD was specific for O_2^- since peroxide-inactivated SOD gave no protection. These data indicate that PMA-stimulated human leukocytes, in the presence of catalase at 10 μg/ml, can be used for studying O_2^--induced DNA damage in human cells.

A typical time course of DNA strand-break damage in the presence of catalase at 10 μg/ml following addition of PMA to a suspension of human leukocytes is shown in Fig. 1*A*. Also shown is the time course of O_2^- production (as measured by the 10-min pulse method). DNA damage occurred with a slight lag, not evident in O_2^- production. We chose to examine in more detail the mechanism of DNA damage by determining if the amount of DNA breakage could be uncoupled from the level of external O_2^-.

Three metabolic poisons were found to inhibit DNA damage without having corresponding effects on O_2^- levels (Figs. 1 and 2). The most dramatic dissociation of O_2^- and DNA damage levels was seen when using a calcium ionophore, A23187 (Fig. 1*B*). It was tested at 3 μM in our standard balanced salt medium, which lacks calcium salts. This metabolic poison blocked DNA damage but reproducibly increased the amount of PMA-induced O_2^- detected outside the cell. DNA damage was inhibited nearly as effectively by 0.3 μM A23187. The addition of calcium salts up to 0.5 mM or 3 μM ferrous sulfate had little effect on the inhibition of DNA damage by 0.3 μM or 3 μM A23187, respectively (data not shown). The second agent was sodium

Table 1. Effects of SOD and catalase on PMA-induced DNA damage in human leukocytes

Treatment		Q_d				
Enzyme	μg/ml	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5
Catalase	0	86		57		70
	10	45		28		
	50	43		35		
	100	44		29		
	150	47		—		
	200	50		32		
	250	44		29		
Catalase (10 μg/ml) + SOD	0		50	(28)	34	40
	25		40	40	31	42
	50		26	25	30	39
	100		22	21	21	36
	200		7	9	20	—
	250		—	7	—	19
Catalase (200 μg/ml) + SOD	0				(34)	(40)
	25				35	37
	50				30	34
	100				21	31
	200				14	22
Catalase (10 μg/ml) + Peroxidase-inactivated SOD	250					42

Human leukocytes were isolated and incubated for 40 min with PMA in the presence or absence of the indicated enzymes and then analyzed for DNA strand breakage as described. Each experiment was carried out with a blood sample from a different donor. Numbers in parentheses are values given elsewhere in the same experiment but repeated for clarity.

Table 2. Effect of copper–indomethacin complex and catalase on PMA-induced DNA damage in human leukocytes

Cu(II)–indomethacin complex, μM	Q_d with addition of catalase	
	10 μg/ml	200 μg/ml
0	40	(40)
2	24	32
4	24	31
9	17	29

Conditions were as in Table 1. Blood from a single donor was used.

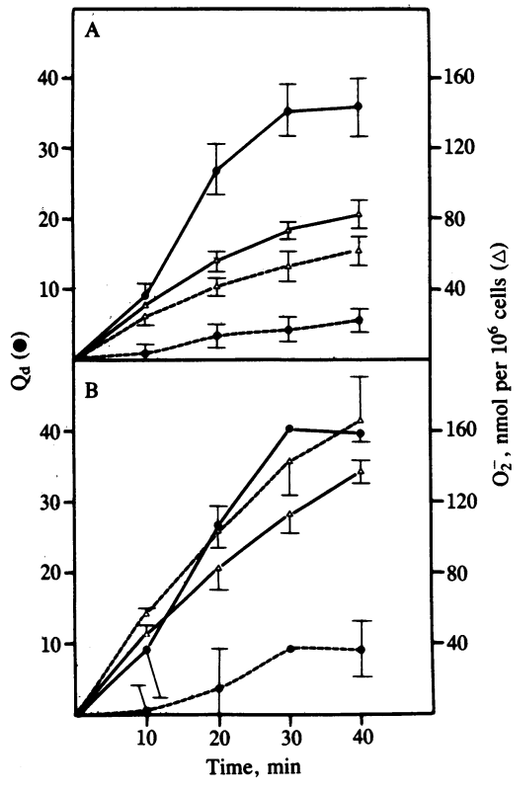

FIG. 1. Effect of sodium fluoride and of A23187 on DNA damage and O_2^- generation in PMA-treated human leukocytes. Cells were isolated at 0°C in balanced salt solution containing glucose as described and catalase at 10 μg/ml, 10 nM PMA, and either 10 mM sodium fluoride (*A*) or 3 μM A23187 (*B*) was added. Controls lacking PMA and the metabolic poisons were included. At time zero, tubes were transferred to a 41°C water bath for 1 min (to hasten temperature equilibration) and then to a 37°C bath. At indicated times, samples for analysis of DNA strand-break damage were removed and chilled rapidly. DNA strand breaks (in units of Q_d) were assayed as described. O_2^- was followed by a "10-min pulse" method as follows. Ten minutes prior to the end of each incubation period in the time course, 0.25 ml of cell suspension was removed from the same tube of cells to be used for strand-break analysis and transferred to four 1.5-ml polypropylene tubes containing sufficient cytochrome *c* and catalase (and SOD in two of the tubes) to give final concentrations, respectively, of 145 μM, 10 μg/ml, and 45 μg/ml. At the end of the specified incubation time, the tubes were quickly chilled in ice water and held until the end of the experiment. At that time, all samples were centrifuged for 1 min, the supernatants were withdrawn, and A_{550} was measured and converted to O_2^- concentration by using $\Delta\epsilon_{molar} = 21{,}000$. Results are expressed as cumulative amount of O_2^- generated (nmol per 10^6 cells) by summing consecutive 10-min "pulses." Error bars indicate SEM for four donors (*A*) or two donors (*B*). ●, DNA strand breaks induced by PMA in the absence (—) or presence (----) of the metabolic poison; △, O_2^- induced by PMA in the absence (—) or presence (----) of the metabolic poison.

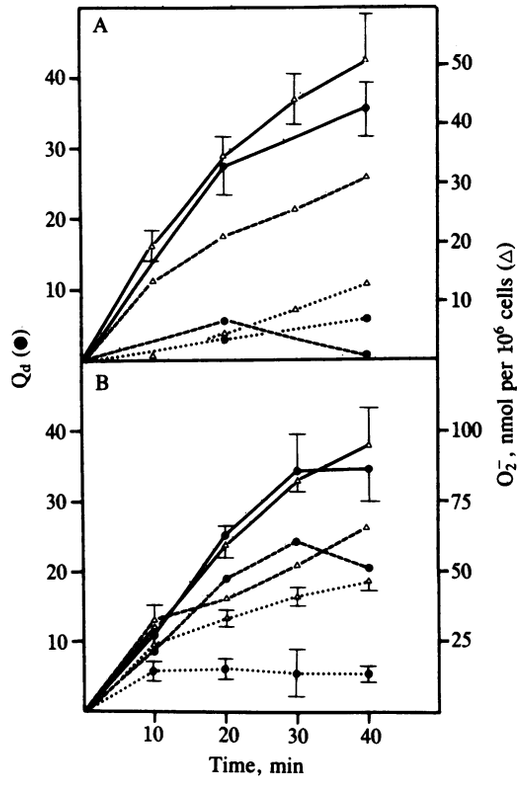

FIG. 2. Effect of 2-deoxyglucose in the presence of fructose or glucose on DNA damage (●) and O_2^- generation (△) in PMA-treated human leukocytes. Conditions were as in Fig. 1 unless otherwise specified. (*A*) Addition of fructose (50 mM; no glucose) and 0 (—) (4), 0.25 mM (----) (1), or 1.0 mM (····) (1) 2-deoxyglucose. (*B*) Addition of glucose and 0 (—) (3), 5 mM (----) (1), or 10 mM (····) (2) 2-deoxyglucose. Numbers in parentheses indicate the numbers of donors tested.

fluoride, which at a concentration of 10 mM caused a modest inhibition of PMA-stimulated O_2^- production but a nearly complete inhibition of DNA damage (Fig. 1*A*). At 20 mM fluoride, O_2^- production was more markedly inhibited (data not shown). The effect of potassium fluoride (10 mM) was not distinguishably different from that of sodium fluoride (data not shown). The third metabolic poison tested was 2-deoxyglucose. It was tested under two conditions. First, fructose was substituted for glucose (Fig. 2*A*). A high concentration of fructose (50 mM) was necessary because it is not readily phosphorylated by hexokinase. At this concentration, fructose seemed to enter the cells and be phosphorylated, since an increase in both O_2^- levels and DNA damage was observed as compared with 5 mM fructose or the complete absence of sugar (data not shown). The addition of 0.25 mM 2-deoxyglucose partially lowered O_2^- levels but blocked DNA damage almost completely. Second, a high concentration of 2-deoxyglucose was used in the presence of 5 mM glucose (Fig. 2*B*). The results were qualitatively similar to that seen in Fig. 2*A*. The effects on levels of extracellular O_2^- and intracellular DNA damage could clearly be dissociated.

For comparison and to demonstrate that O_2^- was acting other than as a precursor to H_2O_2, these same inhibitors were tested for their effects on the DNA damage caused by a flux of pure H_2O_2 generated by glucose oxidase (Fig. 3). Time-dependent DNA damage was introduced by glucose oxidase treatment; as expected, the damage was completely prevented by the addition of catalase, indicating that H_2O_2 was responsible. However, the response to inhibitors was different from that observed with O_2^--induced damage. In the case of H_2O_2-induced damage, fluoride ion actually increased the level of DNA damage observed. This may be due to the fact that there is a small inhibition of cellular catalase by fluoride ion under these conditions, as measured with a Clark electrode (data not shown). 2-Deoxyglucose caused a small decrease in DNA damage, much less than seen in O_2^--induced damage. A slight inhibitory effect of 2-deoxyglucose on glucose oxidase (as measured by oxygen uptake) may partially explain the effect on DNA damage. On the other hand, A23187 largely blocked H_2O_2-induced damage, almost as effectively as it blocked O_2^--induced damage. This was also true for H_2O_2 generated by the autooxidation of cysteamine (0.25 mM) (data not shown), indicating that it was not simply due to an unexpected inhibitory effect on the enzyme.

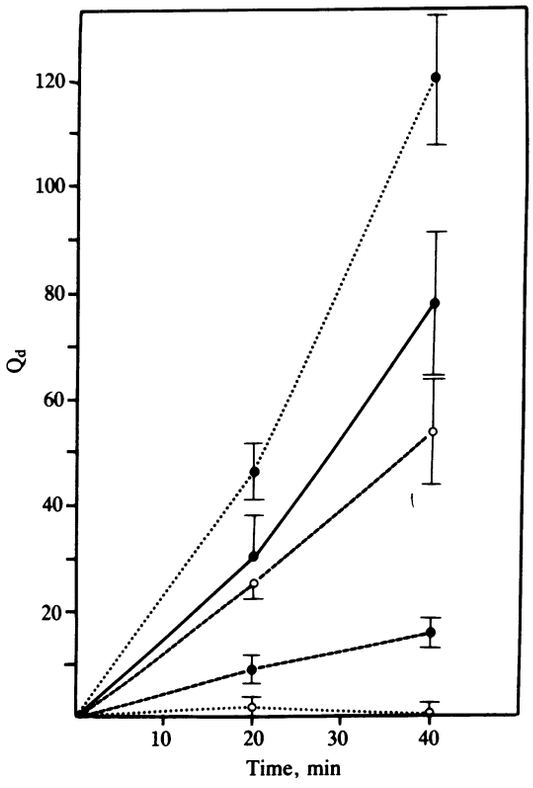

FIG. 3. Effect of metabolic poisons and catalase on DNA damage in human leukocytes induced by glucose oxidase. Cells were isolated as described in balanced salt solution containing glucose. The specified inhibitors and glucose oxidase (0.1 unit/ml) were added, and the treatment was started by warming cells quickly to 37°C. Results obtained from experiments with three donors are shown. Inhibitors added: none (-●-), catalase at 10 μg/ml (··○··), 3 μM A23187 (--●--), 10 mM 2-deoxyglucose (--○--), and 10 mM sodium fluoride (··●··).

Myeloperoxidase, present in neutrophils and possibly released in small quantities during cell isolation, was likely not involved since a similar inhibition by A23187 of glucose oxidase-induced damage was seen in the presence of 1 mM cyanide (data not shown). H_2O_2-induced DNA damage was also markedly inhibited by an iron chelator, *o*-phenanthroline (at 20 μM), in confirmation of the observation of Mello Filho and Meneghini (18) (Table 3). By contrast, O_2^--induced damage was markedly stimulated by *o*-phenanthroline.

Table 3. Effect of *o*-phenanthroline (*o*-Phen) on DNA damage (Q_d) induced in human leukocytes by PMA or glucose oxidase

Time of incubation, min	PMA-induced damage		Glucose oxidase-induced damage	
	Without *o*-Phen	With *o*-Phen	Without *o*-Phen	With *o*-Phen
10	5.3 (2.5)	42.3 (13.5)		
20	20.0 (6.7)	59.7 (8.2)	23.9 (0.7)	1.6 (0.3)
30	28.1 (10.8)	66.4 (9.0)		
40	38.0 (5.7)	65.5 (8.3)	63.7 (1.2)	6.7 (1.5)

Conditions of the experiment were as in Fig. 1 for PMA-induced damage and Fig. 3 for glucose oxidase-induced damage (except that 0.05 unit of glucose oxidase per ml was used). *o*-Phenanthroline concentration was 20 μM. There was no detectable DNA damage induced by phenanthroline alone. DNA damage is shown in Q_d units [average ± SEM (in parentheses; $n = 3$) for PMA-induced damage and average ± range (in parentheses; $n = 2$) for glucose oxidase-induced damage].

DISCUSSION

These data provide clear evidence that O_2^--induced DNA damage in cells can be blocked by the addition of metabolic poisons under conditions where high extracellular levels of O_2^- can be demonstrated. Taken together, the observations presented in this report (Table 4) indicate that the observed DNA strand-break damage is unlikely to be due to simple diffusion of O_2^- to the nucleus of the cell, dismutation to H_2O_2, and subsequent iron-catalyzed production of OH·. Such a Fenton-type reaction may be responsible for some or all of H_2O_2-induced damage because it is inhibited by *o*-phenanthroline, a lipophilic iron chelator (ref. 18; Table 3). However, the opposite effect was seen in the case of O_2^--induced damage (there was a marked increase in damage), providing evidence that O_2^- and H_2O_2 act in separate ways to cause strand breaks in DNA. An additional line of evidence that indicates that OH· is not responsible for PMA-induced strand breaks is their inefficient repair (unpublished data). By comparison, radiation-induced breaks (mostly attributable to OH·) are efficiently repaired in these cells. Thus, other mechanisms for strand breakage by O_2^- in intact mammalian cells must be sought.

Of the three poisons shown to inhibit O_2^--induced damage, 2-deoxyglucose probably has the narrowest spectrum of potential targets. We had tested it earlier (11) but at that time mistakenly believed that its ability to block DNA breakage was simply due to its ability to inhibit O_2^- production by blocking the hexose monophosphate shunt and decreasing cellular NADPH levels (19). Although 2-deoxyglucose does act in this way, the situation is likely more complicated. 2-Deoxyglucose is actually a substrate for the first enzyme in the shunt (20, 21), albeit a poor one, and so NADPH levels decrease only slowly. At the same time, it blocks DNA damage much more rapidly. Thus, it was possible to manipulate conditions such that O_2^- levels were only partially decreased, while DNA damage was nearly completely blocked. One possible explanation for the inhibition of DNA damage by 2-deoxyglucose is that an intermediate in sugar metabolism is involved. Indeed, fructose 6-phosphate has been shown to catalyze strand breaks in DNA *in vitro*, in a reaction involving oxygen radicals (22). Another observation is that cellular ATP levels drop rapidly after addition of PMA to cells and drop even further if combined with 2-deoxyglucose (ref. 19 and unpublished data). Therefore, it is possible that an ATP-requiring enzymatic step is essential for O_2^--induced damage to occur.

It is of interest that both of the other poisons (A23187 and fluoride ion) that were effective in blocking O_2^--induced damage have been reported also to be stimulators of the respiratory burst, although neither significantly stimulated

Table 4. Summary of effects of three metabolic poisons and phenanthroline

Treatment	DNA damage	O_2^- production
PMA-stimulated leukocytes		
NaF	Marked decrease	Slight decrease
A23187	Marked decrease	Slight increase
2-Deoxyglucose	Marked decrease	Partial decrease
Phenanthroline	Marked increase	NT
Glucose oxidase-treated leukocytes		
NaF	Moderate increase	
A23187	Marked decrease	
2-Deoxyglucose	Slight decrease	
Phenanthroline	Marked decrease	
Catalase	Complete inhibition	

NT, not tested.

O_2^- production under the conditions of our experiments (data not shown). An increase in O_2^- production was seen when PMA was combined with A23187; this may be related to the synergism between PMA and A23187 seen in guinea pig neutrophils (23). The observations that A23187 at 0.3 μM was almost as effective an inhibitor of DNA damage as at 3 μM and that inhibition was independent of extracellular calcium levels (up to 0.5 mM calcium) suggest that its inhibitory action may be independent of its ionophore action. Its ability to cause selective perturbation of the plasma membrane may be relevant to its inhibitory action (24). Fluoride ion is believed to act as a poison because it complexes intracellularly with calcium and magnesium ions, although the mechanism by which it blocks O_2^--induced damage is unclear. Preliminary experiments have indicated that, like 2-deoxyglucose, both A23187 and fluoride can cause a rapid drop in cellular ATP levels.

Our experimental result showing A23187 inhibition of H_2O_2-induced damage was somewhat unexpected. It may mean that metabolic processes requiring calcium ion and inhibitable by A23187 are involved in H_2O_2 damage. However, it is worth noting that this "calcium" ionophore actually has an affinity for Fe(II) [but not Fe(III)] that is orders of magnitude higher than for Ca(II) (25), although there is no indication that this property is related to the observed inhibition of H_2O_2-induced damage. It is also uncertain whether the inhibition by A23187 of H_2O_2 and of O_2^--induced DNA damage occur by similar mechanisms.

Our results provide strong evidence that H_2O_2 and O_2^- cause DNA damage by at least partially different pathways. The pattern of inhibition of DNA damage by fluoride, 2-deoxyglucose, and *o*-phenanthroline serves to distinguish the two. The critical target(s) at which the three metabolic poisons act to inhibit O_2^--induced damage is not known and is not necessarily the same for all three. Experiments in progress indicate that both the "initiation" and the "propagation" of DNA damage is blocked by these agents; that is, when added midway during the "burst," the accumulation of damage ceases rapidly. If a rapid drop in ATP proves to be a common event, then ATP-requiring enzymes may be involved. A topoisomerase that requires ATP has been implicated in DNA strand breakage by antitumor drugs such as adriamycin (26), but some direct interaction between drug and enzyme seems to be necessary (27). Adriamycin, particularly at higher concentrations, causes DNA strand breaks by a mechanism involving reactive oxygen species (28), although it does not cause strand breaks in human leukocytes (data not shown) presumably because the necessary activating enzymes are absent.

The mechanism of DNA strand breakage by O_2^- is of interest in connection with its possible involvement in tumor promotion (6–8, 10). We have been interested in trying to decide whether O_2^- acts in a rather indiscriminate manner to damage cellular components [such as might be expected to occur in the "prooxidant" state postulated by Cerutti (6)] or whether there is activation of a specific pathway that might, for example, cause alterations in gene expression. In particular, there are a number of studies of cultured cells (other than phagocytes) that indicate that biological endpoints such as transformation, chromosome breakage, sister chromatid exchanges, or growth stimulation can be modified by SOD (29–35). The implication is that low levels of extracellular O_2^- can be generated in nonphagocytes and are responsible for the observed effects. We suggest that our studies of DNA strand breakage in PMA-stimulated human leukocytes under conditions where we can measure extracellular O_2^- levels may prove useful in understanding events occurring in cultured cells. It may be that, because O_2^- is a ubiquitous species in aerobic organisms, a specific (transmembrane messenger ?) function for it within the cell has evolved.

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