Free Rad. Res., Vol. 27, pp. 143–164
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Diethyldithiocarbamate and Nitric Oxide Synergize with Oxidants and with Membrane-Damaging Agents to Injure Mammalian Cells

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Accepted by Prof. M. B. Grisham

(Received 1 October 1996; In revised form 12 March 1997)

The effect of diethyldithiocarbamate (DDC) and sodium nitroprusside (SNP) on the killing of endothelial cells and on the release of arachidonate by mixtures of oxidants and membrane-damaging agents was studied in a tissue culture model employing bovine aortic endothelial cells labeled either with ^{51}Chromium or ^{3}arachidonic acid. While exposure to low, subtoxic concentrations of oxidants (reagent H_2O_2, glucose-oxidase generated peroxide, xanthine xanthine oxidase, AAPH-generated peroxyl radical, menadione-generated oxidants) did not result either in cell death or in the loss of membrane-associated arachidonic acid, the addition of subtoxic amounts of a variety of membrane-damaging agents (streptolysin S, PLA_2, histone, taurocholate, wheatgerm agglutinin) resulted in a synergistic cell death. However, no significant amounts of arachidonate were released unless proteinases were also present. The addition to these reaction mixtures of subtoxic amounts of DDC (an SOD inhibitor and a copper chelator) not only very markedly enhanced cell death but also resulted in the release of large amounts of arachidonate (in the complete absence of added proteinases). Furthermore, the inclusion in DDC-containing reaction mixtures of subtoxic amounts of SNP, a generator of NO, further enhanced, in a synergistic manner, both cell killing and the release of arachidonate. Cell killing and the release of arachidonate induced by the DDC and SNP-containing mixtures of agonists were strongly inhibited by catalase, glutathione, N-acetyl cysteine, vitamin A, and by a nonpenetrating PLA_2 inhibitor as well as by tetracyclines. A partial inhibition of cell killing was also obtained by 1,10-phenanthroline and by antimycin. It is suggested that DDC might amplify cell damage by forming intracellular, loosely-bound complexes with copper and probably also by depleting antioxidant thiols. It is also suggested that "cocktails" containing oxidants, membrane-damaging agents, DDC, and SNP might be beneficial for killing of tumor cells *in vivo* and for the assessment of the toxicity of xenobiotics *in vitro*.

INTRODUCTION

Recent studies from our laboratories have suggested that killing of mammalian cells in infectious and inflammatory sites might involve a coordinated, synergistic, interaction among oxidants, membrane-damaging agents (microbial hemolysins, fatty acids, phospholipases, cationic proteins, bile salts, xenobiotics) and protein-

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ases.[1–10] It has also been demonstrated that while combinations of membrane-damaging agents are sufficient to kill a variety of mammalian cells in culture, the release of substantial amounts of membrane-associated arachidonic acid and metabolites necessitates the presence of a proteinase (trypsin, elastase, chymotrypsin).[5,8] Our studies have also shown that while sub-toxic, micromolar concentrations of H_2O_2 failed to kill either endothelial cells, fibroblasts or epithelial cells, these concentrations became highly cytotoxic if combined with sub-toxic concentrations of a variety of structurally-unrelated membrane perforators and a variety of proteinases,[2,8,9] is a three-component system.

The synergistic toxic effects induced by mixtures of peroxide and membrane-damaging agents could be significantly inhibited by a non-penetrating inhibitor of PLA_2[11] as well as by variety of antioxidants and by proteinase inhibitors (reviewed in[9]).

Other studies [8] have also shown that nitric oxide (NO), generated by sodium nitroprusside (SNP), enhanced in a synergistic manner the killing of epithelial cells in culture, induced by mixtures of H_2O_2 and the membrane-perforating agent, streptolysin S of group A hemolytic streptococci killing was further enhanced by diethyldithiocarbamate (DDC),[12] a potent inhibitor of superoxide dismutase (SOD) and a chelator of copper.[13] On the other hand, killing of gastric epithelial cells by peroxide was enhanced by DDC but in the absence of added membrane-damaging agents.[14] All these studies further stress the role of synergistic interactions among proinflammatory agonists as a basis for the understanding how cells are injured at inflamed sites.[9]

The present communication shows that both the killing of a variety of mammalian cells in culture and the release of arachidonic acid induced by mixtures of oxidants and a variety of membrane-damaging agents, as well as by lectins, were both markedly enhanced by DDC, by the NO generator SNP, by cyanide (CN) and to a much higher degree, if all these agents were present simultaneously. However, the release of substantial amounts of arachidonate did not necessitate the additional presence of proteinases. Both cell killing and the release of arachidonate were strongly inhibited by antioxidants, by metal chelators and by tetracycline as well as by a non-penetrating inhibitor or phospholipase A_2.[11] The possible mechanisms by which combinations of oxidants, membrane-perforators, DDC, SNP, and CN enhanced cellular damage and the relevance of these findings to the elucidation of the mechanisms of cellular injury in infection and inflammation are reviewed and discussed.

MATERIALS AND METHODS

Mammalian Cells and Cytotoxic Assays

Rat aortic endothelial cells (EC), human foreskin fibroblasts, Chinese hamster ovary cells (CHO), and monkey kidney epithelial cells (BGM) were cultivated in 24-well tissue culture plates in D-MEM medium supplemented with glutamine, penicillin, streptomycin and 10% fetal calf serum (FCS) as described in detail.[1–6,8] Trypsinized cells were labeled with either ^{51}sodium chromate (1 μCi/ml) or with ^{3}H arachidonic acid (1μCi/ml). In some experiments, cell suspensions were double-labeled and then seeded into the tissue culture plates. When the cultures became confluent, the monolayers were washed several times with serum-free medium and 1 ml aliquots of serum-free medium were added to the wells. The cells were then treated for various periods with a variety of agonists (see below). Following incubation, the supernates were removed, centrifuged for 2 minutes at 2000 rpm and 500 μl aliquotes were used to determine the amounts of soluble radioactivity released. Chromium release was measured in a gamma-counter, and the release of arachidonate was performed in a beta-counter using Sintivac as a scintillation fluid. The percent-

age of radioactivity released was determined after subtracting the radioactivity values released spontaneously from untreated controls. The total radioactivity content of the cells was determined following lysis with 1 ml of Triton X-100.[5,8]

Membrane-Damaging Agents, Oxidants and Metabolic Inhibitors

The following agents were tested for their capacity to kill cells and to release arachidonic acid.

A) Membrane-Damaging Agents

Streptolysin S (SLS) and O (SLO) from group A streptococci, Phospholipase A_2 from porcine pancreas, taurocholic acid, nuclear histone (type II-A) rich in both lysine and arginine, wheat-germ agglutinin (WGA), phytohemagglutinin (PHA), and concanavalin A (CON-A). SLS and SLO activities were assayed by hemolysis of human RBC.

B) Oxidants

Reagent H_2O_2, and glucose oxidase (GO), a generator of H_2O_2, was employed at 0.2 U/ml (180,000 units/Gr). This amount of GO yielded approximately 1 millimol of peroxidase after 1 hour when measured in D-MEM by the Thurman reaction.[15] Xanthine (HX) + xanthine oxidase (XO) (generators of superoxide and H_2O_2) were used. Superoxide was measured as described.[16] Azobis-diamidinopropane dihydrochloride (AAPH) (a generator of peroxyl radical, (ROOJ),[17] and sodium nitroprusside (SNP) (a generator of NO) was also used. One mM of SNP yielded approximately 10 μM of NO per 1 hour as measured by the Griess reagent.[18] NO generation was quantified by adding SNP to monolayers either of EC or of BGM cells in D-MEM medium. SNP is known to spontaneously yield NO at 37°C, and to be further oxidized to NO_2, which can be quantified by the Griess reagent at 550 nm.[18] Because this reagent is highly acidic the possibility that SNP might be decomposed, had to considered. Since we found that SNP was not decomposed by the reagent, the incubation of SNP at 37°C either in saline, HBSS, or in DMEM medium reflected the true generation of NO. Under the conditions of the assays, 1 millimol or SNP yielded a net amount of approximately 10 micromol of NO_2 per hour. Similar amounts of NO were generated when SNP was added to untreated monolayers of BGM cells in DMEM medium. Finally, menadione (a generator of oxidants) was used.

C) Anti-Oxidants, Chelators, Antimetabolites and Inhibitors

Bovine liver catalase, superoxide dismutase (SOD), glutathione (GSH) N-acetylcysteine, antimycin, a phospholipase A2 inhibitor, phosphatidylethanolamine-carboxymethyl cellulose (CME),[11] carboxymethyl cellulose, an inactive component of CME were used. The metal chelators, deferoxamine mesylate, 1,10-phenanthroline, 1,2,-bis (2-aminophenoxy) ethane, N, N, N-tetraacetic acid (BAPTA). Vitamin A was obtained from Hoffman LaRoche, (Basel, Switzerland). Tumor necrosis factor (TNF) and interferon gamma were obtained from Dr. S. L. Kunkel, Department of Pathology, The University of Michigan.

RESULTS

The Combined Effect of Oxidants, SLS, DDC and SNP on Endothelial Cells

Previous studies from our laboratories[8] have shown that while millimolar amounts of H_2O_2 were needed to kill BGM cells in culture, micromolar, subtoxic amounts of peroxide became highly cytotoxic if combined with subtoxic amounts of a large variety of membrane-damaging agents and with subtoxic amounts of

proteases, SNP, a generator of NO (see 8). DDC (an inhibitor of SOD and a copper chelator)[13] were also found to enhance the killing of cells either by H_2O_2[14] or by mixtures of peroxide and SLS.[12] DDC was also reported to hemolyze red blood cells in the presence of copper.[19] Therefore, we tested the combined effects of peroxide, SLS, (a distinct membrane-perforator), SNP and DDC on EC. Figure 1 shows that no cell damage was induced either by increasing amounts of GO generated H_2O_2 by SLS by GO + SLS, or by mixtures of GO with DDC. On the other hand, mixtures of GO, SLS, SNP and DDC were highly cytolytic. Cell killing was strongly inhibited by N-acetyl cysteine but neither by SOD nor by the OH inhibitor, dimethylthiourea (DMTU). H_2O_2 generated by GO was effectively replaced by a mixture of hypoxanthine (HX) and xanthine oxidase (XO) which produced both superoxide and peroxide (not shown), or by menadione (Fig. 2). Both DDC and SNP also very markedly enhanced cell killing by mixtures of SLS and AAPH-generated ROO (Fig. 3). Figure 4 shows the dependency on SLS concentrations on cell killing induced by combinations among GO, AAPH, DDC and SNP (a 5-component system), and Figures 5 and 6 show the dependency, on DDC and SNP concentrations respectively, on EC killing when combined with GO-generated H_2O_2 and SLS. In each case a distinct synergistic cell damage was induced.

The susceptibility of EC to the synergistic cell killing by combinations of oxidants, SLS, DDC and SNP depended on the density of the monolayers employed. The denser the monolayer, the greater was the amount of SLS needed to cause cell death. Thus, while cell cultures which had just reached confluency (about 150,000 cells per well) could be readily killed by combinations of SLS (5–10 units ml) and low concentrations of peroxide (50–100 μM), denser cultures (3–4 days-old containing approximately 300,000 cells/well), which were totally refractory to very large amounts of H_2O_2 (1–2 mM), could nevertheless be killed by SLS (10 Units/ml) if both DDC and SNP were also present.

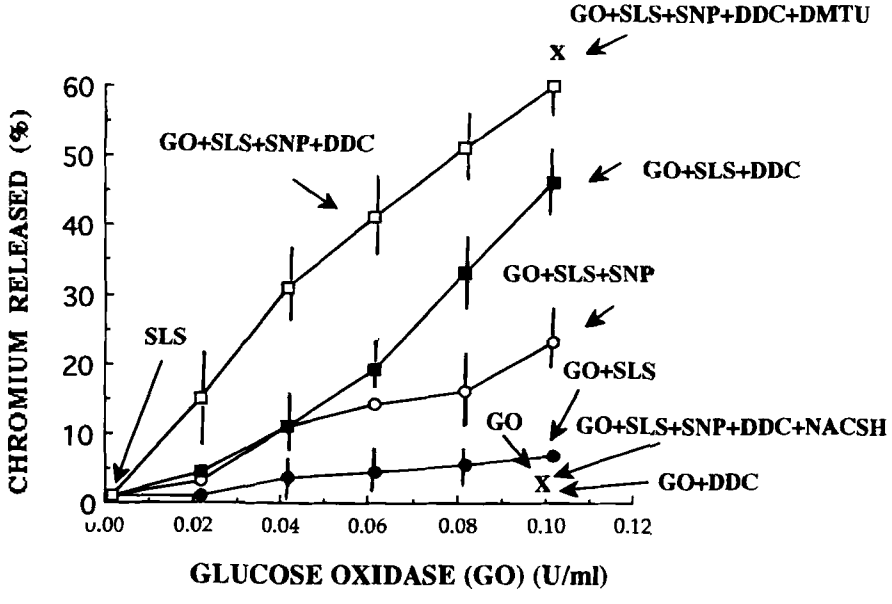

FIGURE 1 The combined effect of glucose-oxidase-generated, H_2O_2 SLS and DDC on EC. Monolayers of chromium-labeled EC were treated for 1 hour with increasing amounts of glucose oxidase (GO) (0.02–012 Units/ml), with streptolysin S (SLS) 5 HU/ml), DDC (1 mM), with SNP (91 mN) and with combinations among the four agents. N-acetyl cysteine (NACSH) at 1 mM, and dimethylthiourea (DMTU) at 1 mM, were employed as inhibitors. The data represent mean values + SD of 3 separate experiments.

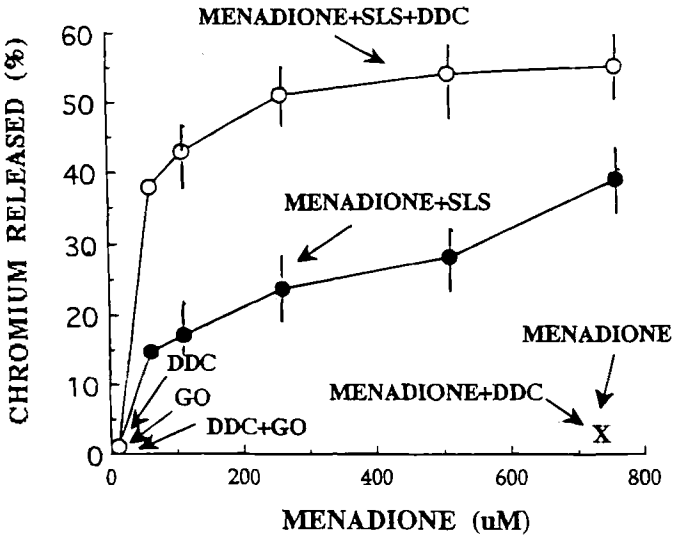

FIGURE 2 The combined effect of menadione, SLS and DDC on EC. Monolayers of chromium-labeled EC were exposed for 60 minutes to increasing concentrations of menadione, and to combinations among menadione, SLS (20 HU/ml) and DDC (2 mM). The data represent means + SD of 4 different experiments.

Since NO generated by SNP might be oxidized to NO_2, we also tested its effect on the killing of EC by mixtures of GO and SLS. It was found that $NaNO_2$, at 10 μM (the amount that might be generated if all the NO generated by 1 mM SNP was converted to NO_2) had no enhancing toxic effect when tested together with peroxide and SLS. The assumption that NO was probably the toxic agent was corroborated by showing that red blood cells, known scavenger of NO, totally depressed the SNP effect.

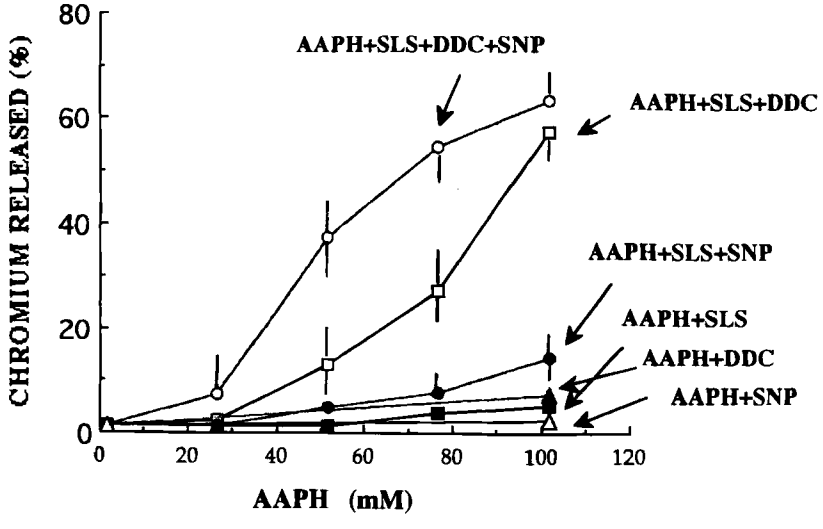

FIGURE 3 The combined effect of AAPH-generated peroxyl radical, SLS, DDC, and NP on EC. Chromium-labeled EC were exposed for 60 minutes to increasing concentration of AAPH, and to combinations among AAPH, SLS (20 HU/ml), DDC (1 mM) and NP (1 mM). The data represent mean values + SD of 3 separate experiments.

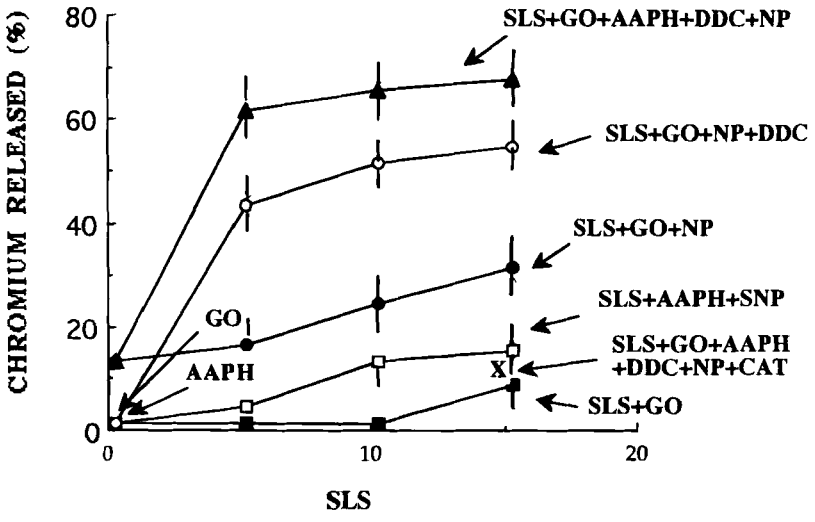

FIGURE 4 The combined effects of SLS, GO, AAPH, DDC and NP on EC. Monolayers of chromium-labeled EC were exposed for 60 minutes to increasing concentrations of SLS and to fixed amounts of GO (0.05 Units/ml), AAPH (75 mM), DDC (1 mM) and NP (1 mM). Note the distinct synergistic killing effects among the various components and the inhibition of cell killing by catalase (CAT). The data are means + S.D of 3 different experiments.

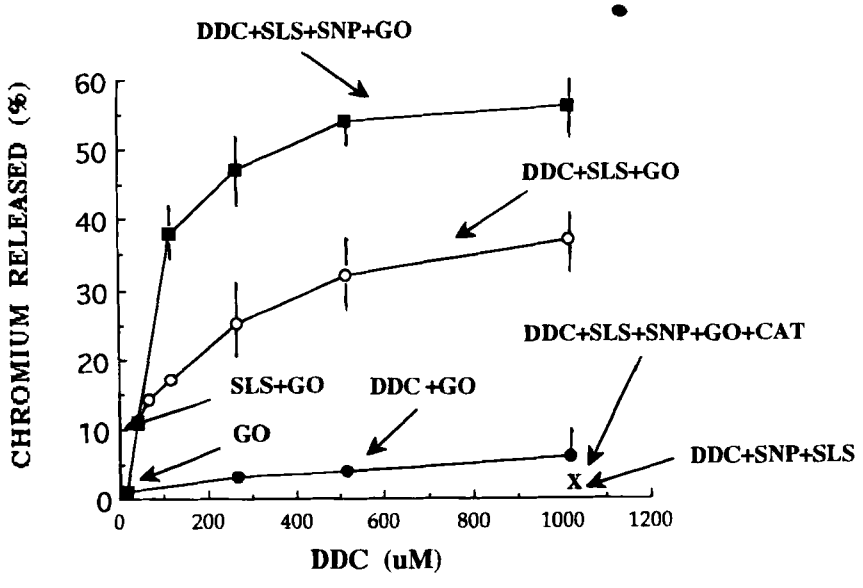

FIGURE 5 The dependency on DDC concentration of the killing of EC by mixtures of SLS, NP and GO. Monolayers of chromium-labeled EC were exposed for 60 minutes to increasing concentrations of DDC, and to combinations among DDC, SLS (10 HU/ml), GO (0.05 U/ml) and SNP (1 mM), and to mixtures among all the reagents and catalase (CAT) (500 U/ml). The data represent means + SD of 3 separate experiments.

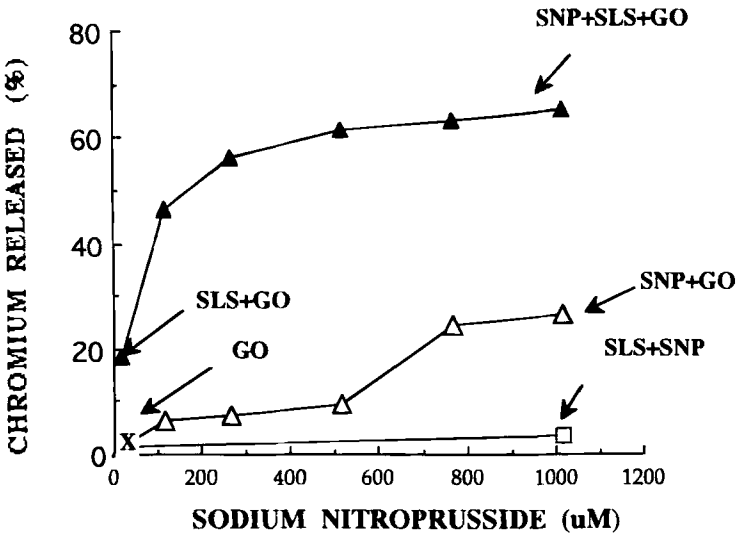

FIGURE 6 The dependency on SNP concentrations on the killing of EC by mixtures of SLS and GO. Chromium-labeled EC were exposed to increasing amounts of SNP alone and in the presence of GO (0.050 U/ml) and SLS (10 HU/ml). The data are from a typical experiment.

In other experiments, EC were pretreated for 15 minutes either with SNP, DDC or with mixtures of the two agents, washed, and then exposed to mixtures of SLS and peroxide. It was found that a distinct synergistic cell killing still took place. This suggested that both SNP and DDC exerted an irreversible sublethal injury which could be amplified by SLS + H_2O_2.

Unexpectedly, however, combinations either among SLS, GO (or HX + XO, not shown), SLS, GO, DDC or among SLS, GO, NP and DDC also induced the release of significant amounts of arachidonate (Fig. 7). These results differ from our earlier observations[5,8] showing that significant amounts of arachidonate could be released from cells only if a membrane perforator and an oxidant were combined with a protease.

Both cell killing and the release of arachidonate by combinations among the four agonists were significantly inhibited by catalase, by glutathione, by N-acetylcysteine, by the PLA_2 inhibitor, CME (see below) as well as by trypan blue (an SLS inhibitor) (not shown). In experiments in which SNP was also present, the inclusion of human RBC totally suppressed the SNP effects, presumably by scavenging NO.[8]

Role of Reducing Agents in SNP-induced killing of EC

The ability of SNP to generate NO is significantly enhanced by reducing agents (cysteine, glutathione). It was found, that the amounts of NO (measured as NO_2) generated when SNP was added to EC monolayers were approximately the same as those generated in empty wells. It was surprising, therefore, that neither cysteine nor glutathione, which significantly increased the generation SNP-induced NO, failed to further potentiate either the killing of EC or the release of arachidonate following treatment with mixtures of SNP, GO SLS and DDC (not shown). These findings suggested that intracellular reducing agents might have increased the formation of NO from SNP, which enhanced cell damage. To test this assumption, we exposed 1×10^6 washed cells for one hour to 1 mM of SNP. The supernates were removed, and the cells were lysed by Triton X-100. The amounts of NO_2 generated

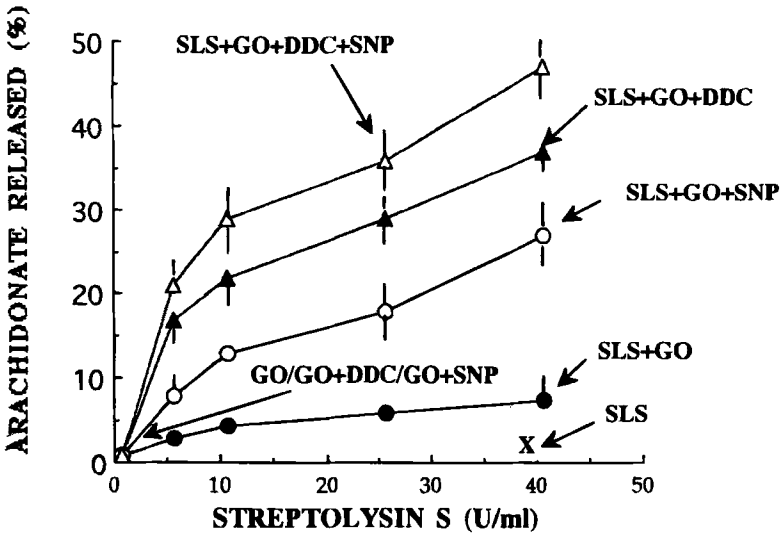

FIGURE 7 The dependency on SLS concentrations on the release of arachidonate by mixtures of GO, DDC and NP EC were exposed to increasing amounts of SLS and to fixed amounts of GO, DDC and SNP.

extracellularly and intracellularly were measured. It was found that the amounts of NO_2 generated intracellularly were about 2–3 fold higher than those detected extracellularly. This might explain why SNP could enhance cellular damage when combined with SLS and peroxide in the absence of added reductants (see Fig. 6).

The Effect of CN and Iron on BGM Cells

Since SNP is an iron and cyanide-containing substance which might generate not only NO, but also CN and iron. It was important to examine the effects of CN and Fe^{+2} on the killing of BGM cells and on the release of arachidonate following treatment with peroxide, and SLS. Figure 8 shows that CN was not cytotoxic when combined with peroxide. On the other hand, CN very significantly increased both the release of arachidonate and chromium (not shown) when combined with peroxide and SLS. However, Fe^{+2} (1–100μm) had no significant effect on the killing of EC by mixtures of H_2O_2, SLS and SNP. Higher concentrations of iron (200–1000 μM) inhibited cell killing in a dose dependent manner. It is important to note that injury by the various mixtures of agonists was absolutely dependent on the presence of SLS (a membrane perforating agent—see below).

Since DDC very significantly enhanced cell killing by mixtures of peroxide, SLS and SNP, we also tested the effect of DDC on cell killing and the release of arachidonate induced by mixtures of H_2O_2, SLS, SNP and CN (a 5-component system). It was found (not shown) that a significant further increase of cell killing took place when all five components were present simultaneously. This was totally inhibited by catalase.

Collaboration Among Additional Membrane-Perforators, Oxidants, DDC and SNP

Previous reports[3,5,6,8,10] showed that a variety of membrane-damaging agents SLO, cationic proteins, phospholipases, lysophosphatides, fatty acids, taurocholate, ethanol, lindane) acted synergistically with oxidants to kill a variety of mammalian cells. It was also reported[14] that either EC or BGM cells treated by DDC, became more susceptible to killing by peroxide. This, however, was neither associated with the modulation of other oxidant defenses, nor with the potentiation of cell

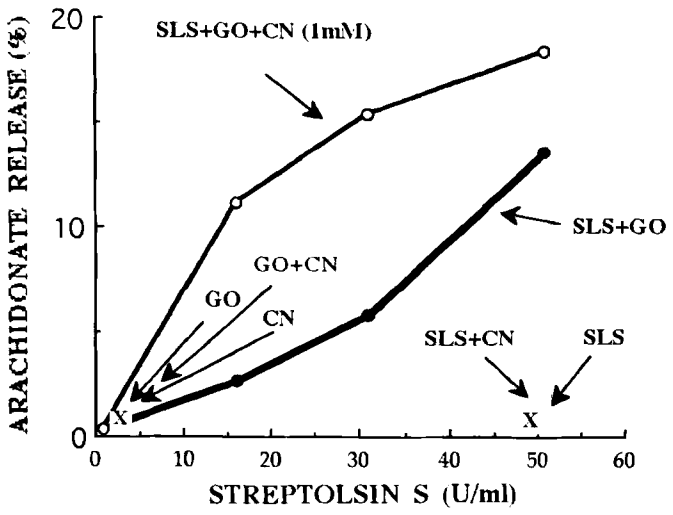

FIGURE 8 The effect of cyanide (CN) on the killing of BGM cells. Monolayers of BGM cells were exposed for 60 minutes to mixtures of increasing concentrations of potassium cyanide with fixed amounts of SLS (10 U/ml) and GO (0.05 u/ml). Note the enhancement of cell killing by CN. The results are from a typical experiment.

injury by the non-oxidant toxic agents, taurocholate, and calcium ionophor. We, therefore, tested the combined toxic effects of a mixture of oxidants, a series of additional membrane perforating agents, DDC and SNP. It was found that subtoxic amounts either of taurocholate (Fig. 9), histone (Fig. 10), phospholipase A_2 (Fig. 11), and either SLO, methanol, acetone or gamma lindane (not shown) also synergized with DDC, SNP and with oxidants generated by xanthine-xanthine oxi-

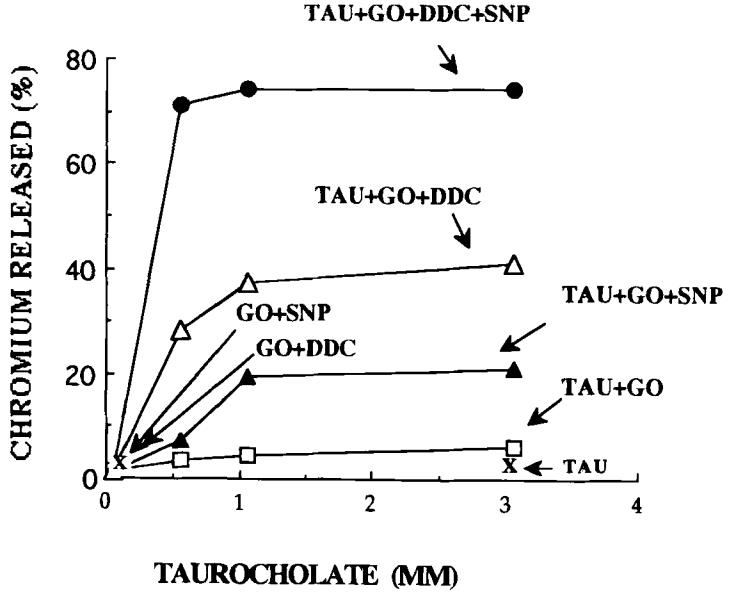

FIGURE 9 The combined effect of sodium taurocholate, GO, DDC and SNP on EC. Monolayers of chromium-labeled EC were exposed for 60 minutes to increasing concentrations of sodium taurocholate, and to combinations among taurocholate, GO (0.050 U/ml), DDC (1 mM) and SNP (1 mM). The data represent means + SD of 3 separate experiments.

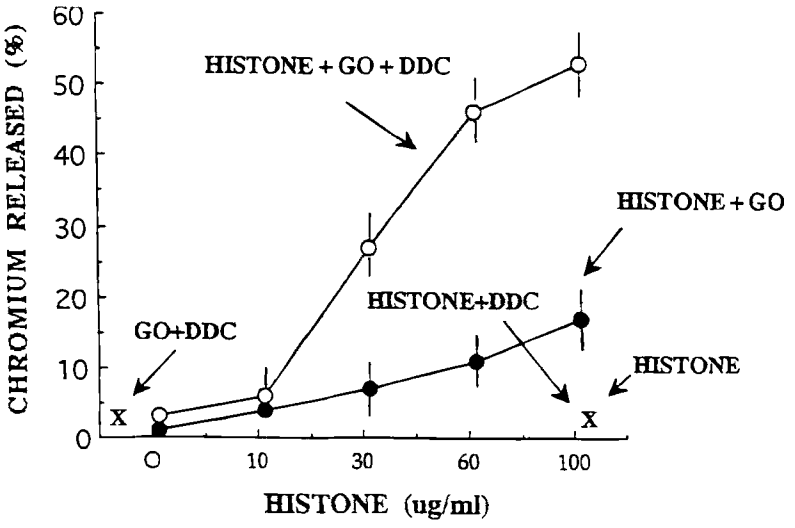

FIGURE 10 The combined effect of histone, GO and DDC on EC. Monolayers of chromium-labeled EC were exposed for 60 minutes to increasing concentrations of nuclear histone and to combinations among histone, GO (0.05 u/ml) and DDC (1 mm). The data represent means + SD for 3 separate experiments.

dase to kill EC and BGM cells and to release significant amounts of arachidonic acid. We also found that the same mixtures of agonists also killed additional cell types, e.g. fibroblasts, Chinese hamster ovary cells and Ehrlich ascites tumor cells (not shown). It thus appears that a large variety of structurally-unrelated membrane-damaging agents could potentiate, in a synergistic manner, the killing of cells if combined with oxidants, DDC and with SNP. It is also apparent that the presence of DDC and SNP in these reaction mixtures eliminated the need to include exoge-

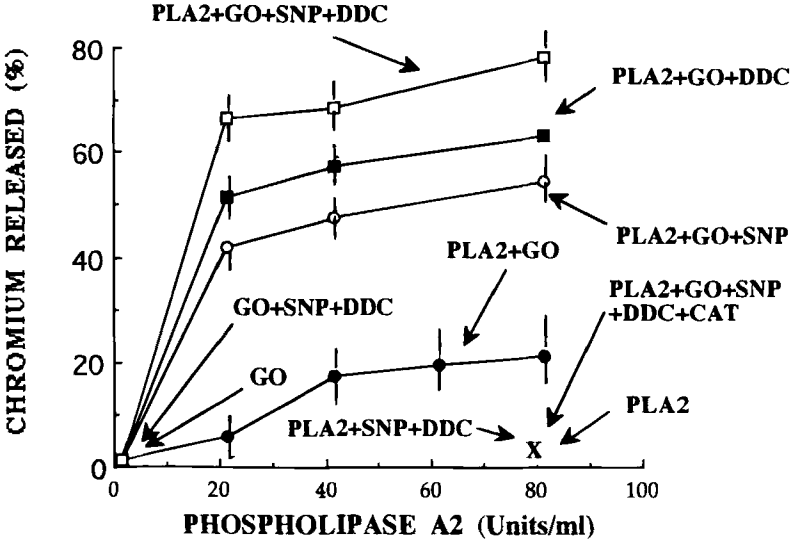

FIGURE 11 The combined effect of phospholipase A_2, SLS, GO, DDC and NP on EC. Chromium-labeled EC were exposed for 60 minutes to increasing concentrations of phospholipase A_2 and to combinations among PLA_2, GO (0.05 U/ml), DDC (1 mm) and NP (1 mM). The data represent means + SD of 3 separate experiments.

nous proteinases shown to be essential for the release of membrane-associated lipids when oxidants were combined with membrane-perforators (see 5,6,8,9).

The Role of Lectins in Cell Killing

Previous studies[16] have shown that lectins e.g. phytohemagglutinin, (PHA), or concanavalin A (Con-A) markedly enhanced the generation of chemiluminescence induced in human neutrophils following stimulation by mixtures of poly-L-arginine and the chemotactic peptide, formylated methionyl-leucyl-phenylalanine. Lectins also enhanced the release of arachidonic acid and metabolites from macrophages[20] and lymphocytes,[21] presumably by activation of membrane PLA_2 and to modulation of the cell surfaces. It was therefore of interest to test whether lectins might be also added to the list of membrane perturbators capable of collaborating with oxidants and with DDC to kill EC and to release arachidonate.

Exposure of EC to sublytic amounts of wheatgerm agglutinin (WGA) (50–150 μg/ml) alone neither killed the cells (Fig. 12A) nor resulted in the release of arachidonate (Fig. 12B). On the other hand, combinations among increasing concentrations of WGA with fixed, subtoxic, amounts of GO, SLS and DDC resulted in a progressive loss of chromium and of arachidonate. Both cell killing and the release of arachidonate were further increased if SNP was also added (not shown). Cells exposed to WGA for 30 minutes contained numerous vacuoles which surround the nucleus (pinocytotic vesicles). The "priming" effect of WGA on cell killing, the release of arachidonate and the initiation of vacuolization were totally lost if N-acetylglucosamine (1–2 mM) was added together with the cocktail of agonists (not shown). This suggested that the competitive sugar probably inhibited the binding and internalization of WGA by the cells. Under similar experimental conditions, both PHA and Con-A also primed EC for enhanced killing and the release of arachidonate when stimulated with a mixture of peroxide, SLS and DDC, but to a lower extent (not shown). Also, neither PHA or Con-A induced the formation of pinocytotic vesicles.

THE MODULATION OF CELL KILLING AND THE RELEASE OF ARACHIDONATE BY INHIBITORS

The Role of PLA_2 Inhibitor

The solubilization of membrane lipids from cells was shown to involve the simultaneous action of mixtures of oxidants, membrane-perforating agents and proteinases.[5] Since the presence of DDC and SNP eliminated, to a significant extent, the need to include proteinase in order to induce the release of arachidonate (see above), it was of interest to study the possible role played by membrane-PLA_2 in cell killing and in the solubilization of arachidonate. It was recently shown that phosphatidylethanolamine bound to carboxymethylcellulose (CME), acted as a potent nonpenetrating inhibitor of PLA_2.[11] We therefore tested this inhibitor in a series of experiments employing mixtures of cytolytic agonists. Figure 13 shows that EC pretreated for 10 minutes with increasing amounts of CME became highly refractory to killing by a mixture of either GO + SLS + DDC, or by a mixture of GO + SLS + SNP. CME also totally inhibited the solubilization of arachidonic acid induced by all these agents (not shown). On the other hand, CMC (carboxymethylcellulose) was ineffective. These results suggest that both cell killing and the release of arachidonate by mixtures of oxidants, membrane perforators, DDC and SNP, might involve activation of membrane-associated PLA_2.

Role of Chelators

Since DDC, a strong chelator of copper and an inhibitor of SOD, markedly enhanced cell killing induced by SLS + peroxide and since both copper

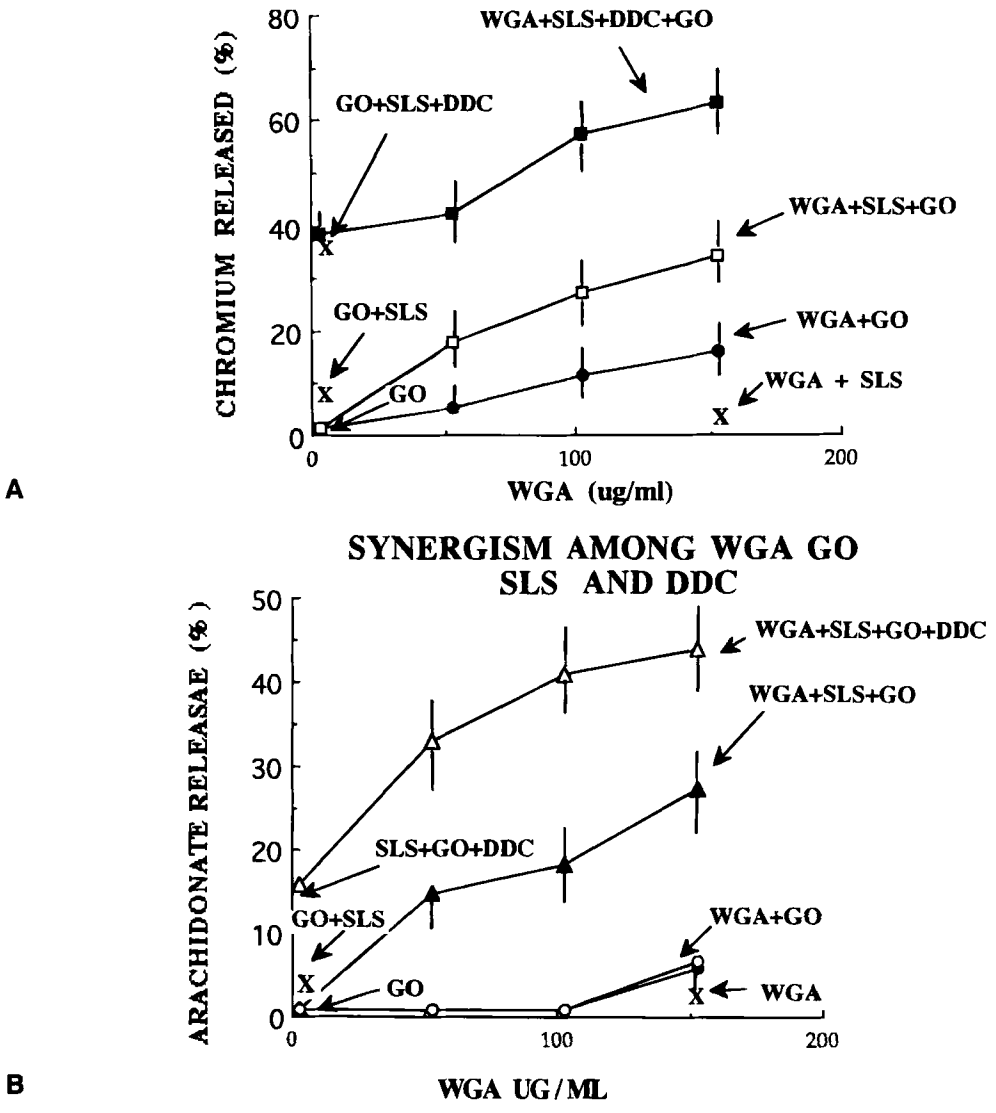

FIGURE 12 **A.** The effect of wheatgerm agglutinin (WGA) on the killing of EC by mixtures of GO, SLS and DDC. Chromium-labeled cells were exposed for 60 minutes to increasing amounts of WGA and to fixed amounts of GO (0.05 U/ml), SLS (10 U/ml), and to DDC (1 mM). The data represent means of + SD of 4 separate experiments. **B.** The effect of WGA on the release of arachidonate from EC treated by combinations among GO, SLS and DDC (see figure 11A). Arachidonate-labeled EC were exposed for 60 minutes to increasing amounts of WGA and to fixed amounts of GO, SLS and DDC (see above).

and iron might be involved in the generation of the highly toxic hydroxyl radical[23] we tested the possible role played by intracellular metals in cell toxicity. EC were preexposed for 2 hours to 1,10-phenanthroline and were then treated with a mixture of SLS, GO and DDC. Figure 14 shows that subtoxic amounts of 1,10-phenanthroline significantly inhibited killing of EC either by SLS + GO or by SLS + GO + DDC. Similarly, 1,10-phenanthroline also significantly inhibited killing of EC by ethanol + GO + DDC (not shown). This suggested that chelation of intracellular divalent metals might have reduce the formation of metal-catalyzed reactive oxygen species (hydroxyl radical). On the other hand, 1,10-phenanthroline had no significant inhibitory

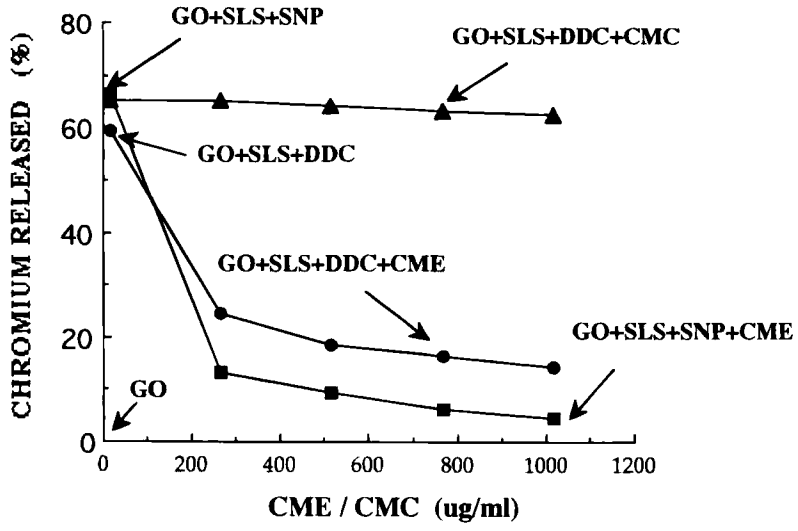

FIGURE 13 The effect of CME and CMC on the killing of EC. Chromium-labeled EC were exposed for 60 minutes to increasing amounts of GO (0.05 U/ml), SLS (10 HU/ml) and DDC (1 mM). The data represent means of 4 separate experiments.

effect on the killing of EC by combinations among GO + SLS + DDC + SNP (not shown), suggesting that non-metal catalyzed processes might be also involved.

Pretreatment of EC with BAPTA, a chelator of intracellular calcium, very significantly enhanced killing by mixtures of GO + SLS (not shown). The enhanced cell killing was only partially reversed by the addition of Ca^{2+}.

The results with the various metal chelators are intriguing. It cannot be explained at present why DDC, a potent chelator of copper acted as a dis-

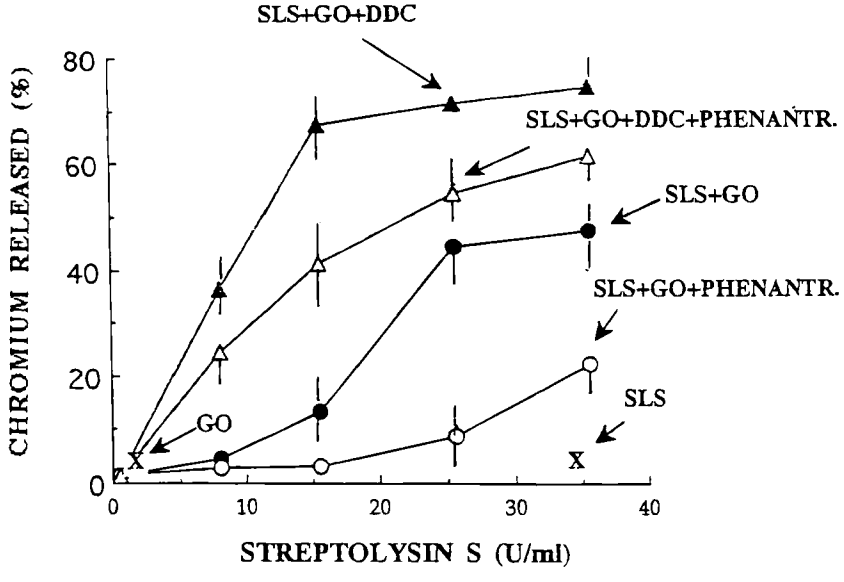

FIGURE 14 The effect of 1,10-phenanthroline (PHENANTR) on the killing of EC. Chromium-labeled cells were exposed for 2 hours to antimycin (10 μM) and then treated by mixtures of increasing amounts of SLS and fixed amounts of GO, and DDC (concentrations as above).

tinct pro-oxidant while other chelators acted in the opposite way (see Discussion).

The Role of Antimycin

Mitochondrial respiration generates oxygen-derived species.[23] These might contribute to intracellular injury. Inhibition of mitochondrial function by antimycin might also alter cellular responses to injurious agents. To test this assumption, EC were exposed for 1–2 hours to antimycin then washed and exposed to a variety of agonists. As little of 5 mM of antimycin very significantly inhibited killing of EC either by mixtures of SLS and GO or by SLS + GO + DDC (Fig. 15).

Role of Tetracycline

In addition to its antibiotic properties, tetracyclines have also been shown to inhibit metalloproteinases.[25,26] Both tetracycline and its non-antibiotic forms have recently been used clinically to combat periodontal disease in humans,[25] presumably due to their anti-proteinase activities. Since proteinases were found to increase the release of arachidonate in the presence of membrane-perforators and oxidants,[2,5,6,8] it was of interest to test the effect of tetracycline on the killing either of EC or of BGM cells and the release of arachidonate by mixtures of peroxide, SLS, DDC and SNP. Figure 16 shows that tetracycline very significantly inhibited cell killing and the release of arachidonate induced either by mixtures GO, SLS, and SNP or by SLS, SNP and DDC. However, a much lesser inhibition of cell killing took place if cells pretreated by tetracycline were washed prior to the addition of the mixtures of agonists. It is also of interest that a strong inhibition of SLS-induced hemolysis took place if the hemolysin was preincubated with tetracycline prior to the addition of RBC suggesting that it also possesses an anti-hemolytic activity. On the other hand, no such inhibition took place if all the reagents were added simultaneously. The results suggest that tetracycline inhibited cell killing either by interfering with SLS activity or by inhibiting the synergy among the reagents.

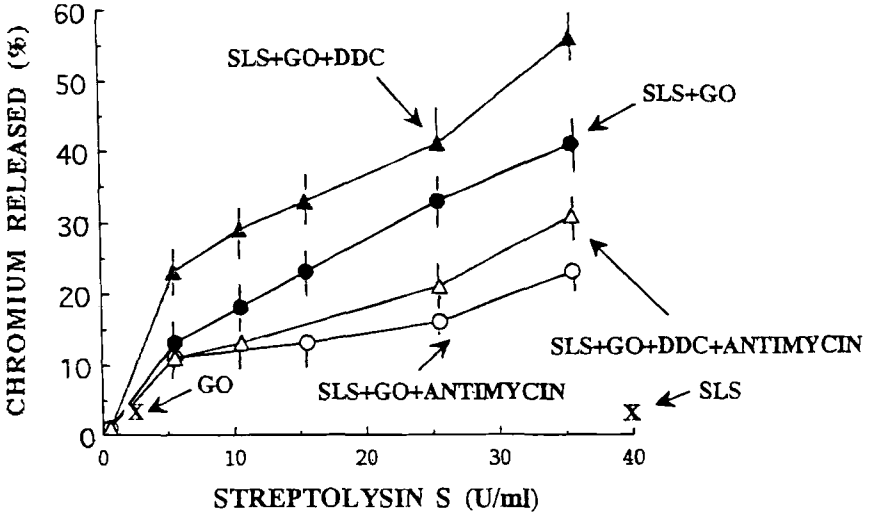

FIGURE 15 The effect of antimycin on the killing of endothelial cells. Endothelial cells were exposed to antimycin and increasing concentrations of SLS and to fixed amounts of GO, SLS and DDC (concentrations as above). The data represent means + SD of 3 separate experiments.

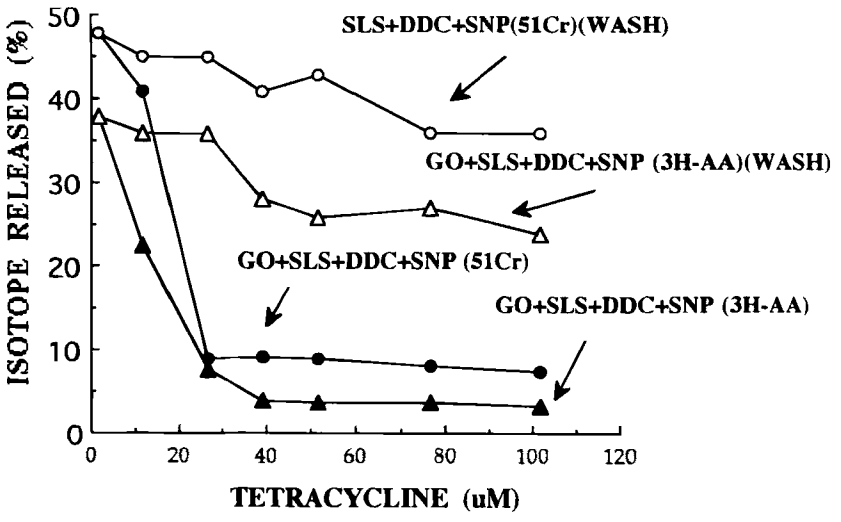

FIGURE 16 The effect of tetracycline on EC killing and on the release of arachidonate. EC double-labeled with chromium (^{51}Cr) and with arachidonate (^{3}H-AA) were exposed for 10 minute to increasing amounts of tetracycline followed by the addition of GO (0.05 U/ml), SLS (10 HU/ml), DDC (1 mM) or SNP (1 mM). The data represent means + SD of 5 separate experiments.

DISCUSSION

The data presented here further supports the assumption that both cell killing and the release of membrane lipids involves synergism among a multiplicity of proinflammatory agonists likely to be present in infectious and inflammatory sites. This basic phenomenon has been described in great detail in previous publications from our laboratories.[1–10] The novelty of the present communication is the demonstration that either DDC (a chelator of copper and an inhibitor of SOD),[13] SNP (a generator of NO, CN and iron see 27) or combinations of NP and DDC very markedly enhanced the killing of a variety of mammalian cells and the release of arachidonate induced by a mixture of subtoxic amounts of oxidants and a series of structurally-unrelated membrane-damaging agents (Figs. 1–5, 7,9,10,11). Both cell killing and the release of membrane-associated lipids were also enhanced by the calcium chelator BAPTA (not shown), by lectins (Fig. 12A and B) as well as by mixtures of TNF and γ-interferon, (not shown). On the other hand, both cell killing and the release of arachidonate were strongly inhibited either by antioxidants, by a nonpenetrating inhibitor of PLA_2 (Fig. 13), by 1,10-phenanthroline (Fig. 14), by the mitochondrial poison antimycin (Fig. 15) or by tetracycline (Fig. 16). Our findings differ, however, from those showing that DDC alone enhanced the toxicity of peroxide to gastric epithelial cells.[14] This might be due either to the employment of different cell lines or a different experimental design.

The membrane-damaging agents employed in our current study were structurally-unrelated and were derived either from bacteria (streptolysin S), or from higher plants (lectins), or from animal origin (Phospholipase A_2, taurocholate, histone). These agents mimic some of the proinflammatory agents which are produced by activated phagocytes (see 9). Thus, it is tempting to speculate that any of the membrane-damaging or perforating agent which are generated in infectious and inflammatory sites might act in concert with a variety of oxidants to enhance cell killing and to release membrane lipids from cells especially if proteinases,[2] DDC and NO are also present.

To explain how the mixtures of agonists described in the present communication enhanced cellular damage and induced the release of arachi-

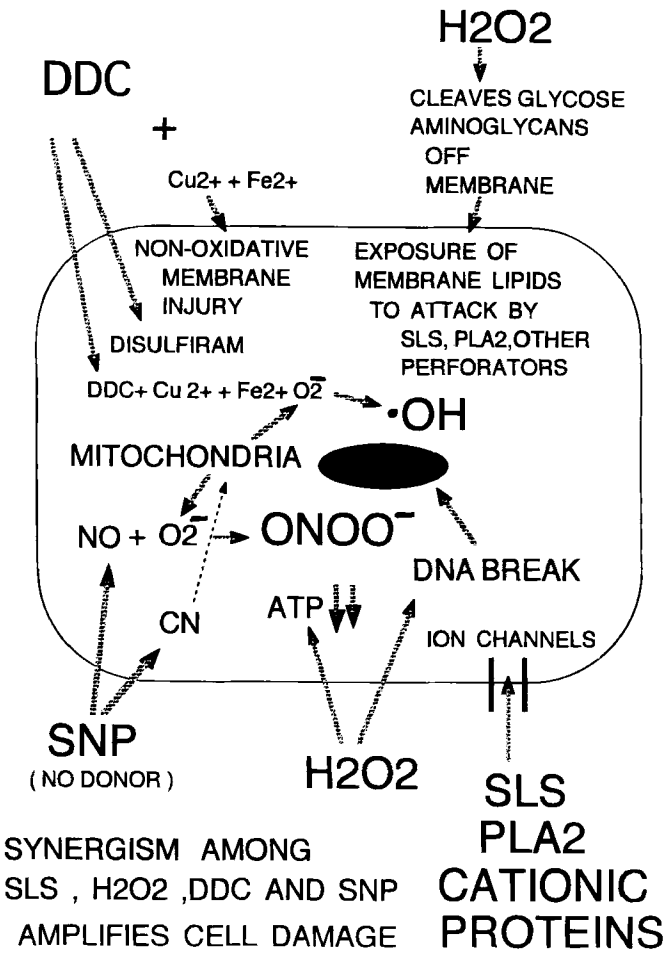

FIGURE 17 A proposed mechanism of action of the combined effects of peroxide, NO, membrane-damaging agents, DDC and metals on cells.

donate from mammalian cells, a brief review of the relevant literature dealing with the possible mechanisms by which the various agonists function, is presented.

The mechanisms by which oxidants damage cell structures had been discussed in detail.[9,28–34] Oxidants such as H_2O_2 which freely diffuse into the cell interior, lowered ATP,[30] inhibited glycolysis and repair of cellular damage, caused DNA strand break[33] and oxidized membrane lipids.[23] Peroxyl radical (ROO), formation initiates a chain reaction in membrane lipid,[17] resulting in damage to membrane lipids. The metal-catalyzed generation of hydroxyl radical has been described in great and its role in cellular injury had been reviewed.[23,31] NO,[27] besides its effect as an endothelial relaxing factor, might interact with superoxide to generate the highly toxic peroxynitrite.[35,36] NO generated by SNP was shown to cooperate with H_2O_2 to enhance killing of Fu5 hepatoma cells,[37] and of pancreatic islet cells.[38] There is also evidence for the chemical release of the cyanide anion from SNP and H_2O_2.[39] Menadione, a known generator of reactive oxygen species,[23] also collaborated with DDC and SLS to kill EC (Fig. 2).

Membrane perforators such as microbial hemolysins (SLS and SLO)[9] and phospholipases

interact with membrane phospholipids and cholesterol[40] to alter membrane permeability by forming either pores or ion channels. Phospholipases can induce the formation of the highly cytotoxic lysophosphatides, which were found to synergize with oxidants to kill EC[5] and also to prime neutrophils to generate large amounts of superoxide.[41] Subtoxic injury (perforation) of the plasma membranes by these agents might, perhaps, also allow ionized oxygen radicals, proteinases and the additional hydrolases to freely diffuse into the cell interior to exert their effect on cytoplasmic and nuclear elements.[9] A recent study has emphasized the antioxidant activities of DDC.[51] This publication examined in great detail its scavenging properties for reactive oxygen species, its reducing properties, its iron chelating properties and its protective effects on oxidant-induced damage to brain tissue, proteins, and on human LDL and DNA which could explain the apparent beneficial effect on DDC against oxidative stress-related diseases that have been observed in experimental and clinical studies. Recent studies[53] (see below) have suggested that the mechanisms by which a mixture of oxidants and SLS injure cells might involve the removal by oxidants of glycosaminoglycans from cell surfaces exposing the membrane phospholipids to the action of membrane perforators.

The mechanisms by which DDC (a potent inhibitor of superoxide dismutase, and a chelator of copper[12] and also a strong hemolytic agent when employed together with copper,[19] functions to markedly enhance cell killing when combined with oxidants and with membrane-perforating agents (Fig. 1–12), are complex and still not fully understood. DDC has been shown to act both as a pro-oxidant and as an antioxidant agent.[42]

DDC as a Pro-Oxidant Agent

Since our previous reports[1–10] showed that killing of EC could be readily induced by mixtures of low, sub-toxic concentrations of membrane-perturbators and oxidants, the role of DDC and SNP in cell killing, as described in the present report, is that of distinct amplifiers. One way by which DDC might enhance cell killing is by inhibiting SOD. This might facilitate the interaction of superoxide, of mitochondrial origin, with NO to form the toxic peroxinitrite. DDC can also loosely chelate copper and recruit this metal from stores.[13] Copper might than catalyze the formation of hydroxyl radical.[23] Sublytic cell damage, which might then occur, might be amplified by membrane perforators. This assumption is supported by the observation that DDC enhanced the accumulation of intracellular copper in cerebral astrocytes. This, in turn, was accompanied by a steep depletion of the distinct antioxidant, glutathione.[43] Also, menadione toxicity (Fig. 2), which was enhanced by DDC, was reversed by N-acetlycysteine, suggesting that oxidants depleted GSH and thus lowered the antioxidant capacities of the cells.[44] DDC was also shown to enhance mitomycin[45] and bleomycin[46] toxicities which are both mediated by the generations of ROS. A further evidence for the pro-oxidant action of DDC came from recent observations (Ginsburg, Vissers and Winterbourn—unpublished) that DDC enhanced the killing of fibroblasts and monkey kidney epithelial cells and the release of arachidonate by mixtures of hypochlorous acid and streptolysin S. This was strongly inhibited either by GSH, N-acetylcysteine or penicillamine. These findings also suggest a possible involvement of copper and perhaps also of superoxide in hypochlorite toxicity.

Paradoxically, however, chelation of copper by DDC which might have been expected to result in an antioxidant effect (by preventing OH formation) resulted in an opposite phenomenon (See Figs. 1–5, 7,11). Also, DMTU, a distinct inhibitor of OH, failed to protect cells against DDC-enhanced cytotoxicity (Fig. 1). These findings suggest perhaps that OH might not be involved in DDC-induced toxicity.

Contrary to the findings that DDC enhanced cellular damage by acting as a pro-oxidant agent,

one report[47] showed that myocytes which had been treated with DDC and which had lost 70–90% of their intracellular SOD did not become more susceptible to peroxide. Also, another study[19] showed that hemolysis of RBC and injury to nucleated cells induced by DDC+ copper did not involve the participation of ROS, suggesting that non-oxidative mechanisms might also be involved in DDC toxicity. Another way by which DDC enhanced cell damage is by its conversion to the more toxic dimer, disul-firam.[48] Our findings (Fig. 14) on the partial inhibition by 1,10-phenanthroline of cell killing by mixtures of GO, DDC and SLS, are also in line with the observations[14] that chelation of iron diminished DDC-enhanced sensitization of these cells against peroxide. This suggested that metal-catalyzed formation of certain still undefined reactive oxygen species might be involved. The oxidation of DDC by the added peroxide and its reversal by thiols indicated that DDC can engage in a cyclic reaction with peroxide and GSH. [49]

DDC as an Antioxidant Agent

Several reports suggested that DDC might also possess anti-oxidant properties.[43,50] It was recently shown[42] that DDC was specifically active in scavenging the reactive oxygen species, superoxide and H_2O_2. These oxidations yielded mainly the hydrated form of disulfiram.

Thus, DDC might have diverse properties, as a pro-oxidant, and as an antioxidant which depend on the cell species employed, the conditions of cell treatment and on the nature of the oxidant employed. However, DDC in our model, acted as a distinct pro-oxidant, capable not only of amplifying cell killing but also of facilitating the release of large amounts of membrane-associated arachidonate in the complete absence of added proteinases.[9]

Our findings on the inhibition of DDC-induced cytotoxicity by the nonpenetrating, PLA_2 inhibitor, CME (Fig. 13) suggest that cell damage might also involve the activation of membrane-associated phospholipases. These might generate lysophosphatides, known to the highly cytolytic.

The ability of SNP-generated NO to further enhance cell killing when combined with membrane perforators, oxidants and DDC suggests the possible involvement of peroxinitrite.[35,36] This toxic agent might be formed by the interaction of NO with superoxide generated either by mitochondria or by the xanthine-xanthine oxidase system. Recent studies[8,51] have also shown that NO acts synergistically with ROS to damage endothelial cells. The exposure of EC to combinations of ROS (peroxide, superoxide, proxyl radical, NO, peroxynitrite), and membrane perforators in conjunction with DDC might drastically lower the antioxidant defenses and thus contribute to cell destruction.

Since SNP might generate not only NO but also free cyanide[39] and iron, the possibility that these agents might amplify cell damage, was also considered. Figure 8 shows that while CN very markedly enhanced cell killing and the release of arachidonate by mixtures of SLS and GO, it had neither inhibitory nor stimulatory effect when SNP was also present. The mechanisms by which CN acted both as an amplifier and as a depressor of cell damage are still not known.

The possible mechanisms by which membrane-damaging agents, peroxide, DDC, SNP and CN act in concert to kill cells and to release membrane lipids are summarized in Table I.

The mechanisms by which lectins enhanced cell killing in the presence of SLS, oxidants and DDC are also not fully known. Lectins have been shown to agglutinate red blood cells, to enhance the release of PLA_2 from sponge cells and to release arachidonic acid from leukocytes.[22] This suggests that lectins might be added to the long list of membrane-perturbing agents capable of synergizing with oxidants and with DDC to kill cells (See Reference 9).

The inhibition of cell killing and arachidonate release by tetracycline (Fig. 16), an agent known for its antiproteinase activity,[25,26] suggests that intracellular proteinases might also be involved

TABLE I A Proposed mechanism of cellular injury induced by combinations among oxidants, DDC, SNP and membrane-damaging agents

AGENT	PROPOSED MECHANISMS OF ACTION
OXIDANTS:	Deplete ATP, inhibit glycolysis, induce DNA strand breaks, expose proteins to proteolysis, peroxidize lipids, induce signal transduction, activate PLA_2, generate lysophosphatides.
DDC:	Inhibit SOD and facilitate dismutation of superoxide to hydrogen peroxide, chelate transition metals and recruit copper from stores, collaborates with copper to induce membrane damage in a nonoxidate fashion. Forms disulfiram.
SNP	Generates NO, CN and iron and facilitates the formation of peroxinitrite, which might also poison mitochondria.
MEMBRANE-DAMAGING AGENTS	Alter membrane phospholipids and induce enhanced cell permeability. Induce ion channels, release membrane-associated fatty acids directly and act synergistically with oxidants and with proteinases to enhance membrane permeability and the release membrane-associated fatty acids.
*COMBINED EFFECTS OF OXIDANTS, DDC, SNP AND OF MEMBRANE-*DAMAGING AGENTS	Exposure of cells to DDC might result in the recruitment by this chelating agent of intracellular Copper and iron from stores and in the inhibition of SOD. These metals might then become available for the intracellular formation of hydroxyl radical via the Fenton reaction where superoxide generated by mitohondria might interact with H2O2. DDC can also injury membranes by collaborating either with Cu2+ (19) with Cu2+, Co2+ or with Cu2++ +Fe2 (Ginsburg and Varani unpublished observations) in a non oxidative fashion. DDC might also dimerize to the more toxic, disulfiram. Superoxide might also interact with NO generated by SNP to form the highly toxic ONOO (peroxinitrite). The presence of a mixture of SNP, DDC, H2)2 and a membrane perforating agents might also enhance the release of membrane-associated fatty acids which have been shown to collaborate with oxidants to kill cells and to further release membrane lipids (8). SNP might also general CN which was shown (Figure 8) to enhance cell damage. Oxidants might also act either directly to oxidize membrane lipids, to prevent DNA damage repair and to release glycose aminoglycans from cell surfaces exposing the membrane phospholipids to an attack either by membrane perforators (SLS, PLA2) (see 53) or by DDC-metal complexes (unpublished results). Alternatively, exposure of cells to membrane-perforators might overcome the antioxidant capacity of cells by facilitating the penetration of ionized radicals.

in DDC-induced cell killing. However, tetracyclines are also known to be chelators of metals. This might explain their protective effects against killing induced by metal-DDC complexes.

The ability of antimycin (a mitochondrial poison), to suppress cell killing by mixtures of DDC, SLS and GO (Fig. 15) suggests that ROS which might be generated by mitochondria might also play a role in cell killing. The mechanisms of this reaction are, however, still not fully understood.

The ability of mixtures of oxidants, membrane-perforators and DDC to markedly enhance the release of membrane lipids is an important observation. Fatty acids released from cells in inflamed sites might diffuse out and attack bystander cells in the presence of oxidants and DDC as suggested (See Reference 8).

Taken together, our current investigation further strengthens the assumption[9] that killing of mammalian cells in inflamed sites involve complex interactions among oxidants, membrane-damaging agents, proteinases, metals, metal chelators, cytokines and a variety of metabolic inhibitors and antioxidants.[9]

Mixtures of oxidants, DDC, SNP and membrane-damaging agents might also serve to destroy both bacteria and perhaps also tumor cells, *in vivo*. Our studies also suggest that the synergism concept of cellular injury might also

serve to assess toxicity of drugs and xenobiotics. Recent studies[10] have shown that DDC also very markedly enhances the killing of a variety of mammalian cells following treatment by mixtures of peroxide and the xenobiotic, gamma lindane. This pesticide might, therefore, also be added to the list of agents capable of killing cells in the present of oxidants and DDC. DDC might perhaps also be used to assess the relative toxicity of a variety of drugs using *in vitro* assays. This might partly replace the more costly animal experimentations used to assess drug toxicity and drug safety. Our studies also suggest that since cell killing in inflammatory and infectious conditions might involve synergistic interactions among a multiplicity of noxious agents, protection against cell damage might also involve the employment of "cocktails" of inhibitors.

Acknowledgements

Supported by a research grant from the late Dr. S. M. ROBBINS of Cleveland, Ohio, and by grants NIH-GH29707, and NIH-HL 31963.

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