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KILLING OF ENDOTHELIAL CELLS AND RELEASE OF ARACHIDONIC ACID
Synergistic Effects among Hydrogen Peroxide, Membrane-Damaging Agents, Cationic Substances, and Proteinases and Their Modulation by Inhibitors[1]

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Abstract—[51]Chromium-labeled rat pulmonary artery endothelial cells (EC) cultivated in MEM medium were killed, in a synergistic manner, by mixtures of subtoxic amounts of glucose oxidase-generated H_2O_2 and subtoxic amounts of the following agents: the cationic substances, nuclear histone, defensins, lysozyme, poly-L-arginine, spermine, pancreatic ribonuclease, polymyxin B, chlorhexidine, cetyltrimethyl ammonium bromide, as well as by the membrane-damaging agents phospholipases A_2 (PLA_2) and C (PLC), lysolecithin (LL), and by streptolysin S (SLS) of group A streptococci. Cytotoxicity induced by such mixtures was further enhanced by subtoxic amounts either of trypsin or of elastase. Glucose-oxidase cationized by complexing to poly-L-histidine proved an excellent deliverer of membrane-directed H_2O_2 capable of enhancing EC killing by other agonists. EC treated with rabbit anti-streptococcal IgG were also killed, in a synergistic manner, by H_2O_2, suggesting the presence in the IgG preparation of cross-reactive antibodies. Killing of EC by the various mixtures of agonists was strongly inhibited by scavengers of hydrogen peroxide (catalase, dimethylthiourea, $MnCl_2$), by soybean trypsin inhibitor, by polyanions, as well as by putative inhibitors of phospholipases. Strong inhibition of cell killing was also observed with tannic acid and by extracts of tea, but less so by serum. On the other

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hand, neither deferoxamine, HClO, TNF, nor GTPγS had any modulating effects on the synergistic cell killing. EC exposed either to 6-deoxyglucose, puromycin, or triflupromazin became highly susceptible to killing by mixtures of hydrogen peroxide with several of the membrane-damaging agents. While maximal synergistic EC killing was achieved by mixtures of H_2O_2 with either PLA_2, PLC, LL, or with SLS, a very substantial release of [^{3}H]arachidonic acid (AA), PGE_2, and 6-keto-PGF occurred only if a proteinase was also added to the mixture of agonists. The release of AA from EC was markedly inhibited either by scavengers of H_2O_2, by proteinase inhibitors, by cationic agents,by HClO, by tannic acid, and by quinacrin. We suggest that cellular injury induced in inflammatory and infectious sites might be the result of synergistic effects among leukocyte-derived oxidants, lysosomal hydrolases, cytotoxic cationic polypeptides, proteinases, and microbial toxins, which might be present in exudates. These "cocktails" not only kill cells, but also solubilize AA and several of its metabolites. However, AA release by the various agonists can be also achieved following attack by leukocyte-derived agonists on dead cells. It is proposed that treatment by "cocktails" of adequate antagonists might be beneficial to protect against cellular injury in vivo.

INTRODUCTION

The mechanisms by which microbial products, complement components, activated neutrophils, and macrophages injure and kill mammalian cells in infectious and inflammatory sites has been the focus of intensive investigations in the last decade (1–13). The idea that target cell killing by some of these agents might be the result of "multiple synergistic hits" induced by the combined action of microbial hemolysins, antibodies, complement components, and proteinases came from our earlier studies with both normal and malignant cells (14–16). We have recently also investigated the synergistic killing effects of combinations among hydrogen peroxide (H_2O_2), cationic polyelectrolytes, phospholipases A_2 and C, a streptococcal hemolysin, and lipoteichoic acid on a variety of cells in culture (17–21). Our working hypothesis (21) is that sublethal injuries induced in mammalian cells by membrane-damaging agents might be greatly amplified by exposure to oxidants and to proteinases.

The present model further expands our previous observations (17–21) and describes the killing of endothelial cells (EC) in culture and the parallel release of arachidonic acid and some of its metabolites from cells treated by combinations of oxidants. phospholipases, cationic agents, a streptococcal hemolysin, and proteinases. These agents were chosen because they represent products that are likely to be present in inflammatory and infectious sites. To further study the possible mechanisms by which these simulated leukocyte- and bacterial-derived "cocktails" of agonists kill EC and also degrade their membrane lipids, a variety of modulating agents (antioxidants, polyanions, proteinase inhibitors, putative inhibitors of phospholipases, antimetabolites) were tested. The possible relevance of this in vitro tissue culture model to the events that occur when

activated leukocytes induce cell and tissue injury in inflammation will be briefly discussed.

MATERIALS AND METHODS

Endothelial Cells (EC)

Rat pulmonary artery endothelial cells (EC) were obtained according to the procedure of Jaffe (22) and Ryan and White (23) employing a minimal essential medium of Eagle with Earl's salts (MEM) supplemented with glutamine, penicillin, and streptomycin as described in detail (17). Upon isolation, the cells exhibited the typical cobblestone morphology of endothelial cells. The cell monolayers were grown in 24-well plates at 37°C with 5% CO_2. All experiments were conducted on cells passaged less than 34 times since isolation.

Labeling of EC with Chromium[51]. Trypsinized endothelial cells were suspended in MEM medium supplemented with 10% FCS containing 1 μCi/ml of [^{51}Cr]$NaCrO_4$. The cells were then seeded into 24-well dishes (1 ml/well). Following incubation for 24-36 h confluent monolayers were obtained. These were washed three times in serum-free MEM medium to remove unbound radioactivity. One milliliter of serum-free medium was then added to each well and the plates were then used for cytotoxicity assays (see below).

Labeling of EC with [^{3}H]Arachidonic Acid (AA). Trypsinized endothelial cells were suspended in MEM medium supplemented with 10% FCS and 20 μl of [^{3}H]arachidonic acid (76.0 Ci/mmol) per 100 ml of cell suspension was then added. The cells were seeded into 24-well plates and grown to confluency.

Cationization of Glucose Oxidase (GO) and Catalase

Solutions containing either 1 mg/ml of GO (100 units/ml) or 1 mg/ml of catalase (100,000 units/mg) were rendered cationic by complexing with 1 mg/ml of poly-L-histidine HCl (mol wt 23,000) (PHSTD) (24). Briefly 1 ml of the enzymes was mixed with 1 mg/ml of PHSTD in saline, pH 6.0. The mixtures were incubated for 5 min at room temperature to allow complex formation. This was followed by the addition of 5 vol of saline buffered with 3 mM HEPES buffer, pH 7.4. The opalescent solution that developed was centrifuged for 5 min at 2000 rpm, and the precipitate was resuspended in 1 ml saline. Such cationized enzymes retained greater than 95% of their original activities as determined by their ability either to generate hydrogen peroxide in the presence of glucose or to destroy its activity (24). The cationized enzymes avidly bound to endothelial cells (see below).

Cytotoxicity Assay

Cytotoxicity induced by the various agonists was determined by the chromium release method described in detail (17, 18). At the end of the incubation period specified in the different experiments, the cultures were monitored microscopically for altered cell morphology. The amounts of chromium

solubilized from the cells were determined following centrifugation of the culture supernates or 5 min at 2000 rpm in a refrigerated centrifuge. Total radioactivity associated with the cells was determined after lysing control monolayers with 1 ml of 1% Triton X-100. In all experiments, the amount of radioactivity released in response to treatment was compared with the amounts released in cultures incubated with medium alone (spontaneous release). Radioactivity measurements were performed in a gamma counter. The release of [^{3}H]AA was determined in a scintillation counter employing Scintiverse BD (Fisher) as a scintillation fluid, and the results were calculated as percentage release of radioactivity compared to the total release obtained by treating control wells by Triton X-100. It should be noted that the release of more than 40% of the total chromium label from EC usually resulted in a near total inhibition of cell replication upon transfer to fresh medium (10, 17).

Measurement of Cyclooxygenase and Lipoxygenase Products

Supernatant fluids obtained from wells treated by the various agents were tested for the content of PGE_2, 6-keto-PGF, and thromboxane B_2 by radioimmunoassay performed by the University of Michigan Ligand Core Laboratory and detailed in the publication of Sporn et al. (25). Sensitivities of the assays were 0.5 pg/100 μl of supernate for thromboxane B_2 and 2 pg/100 μl for PGE_2 and 6-keto-PGF.

Agents Employed to Kill Cells and Release AA and Its Metabolites

Hydrogen Peroxide (H_2O_2). One milliliter of MEM medium (containing 1 mg/ml of glucose) was incubated with 0.2 units/ml of glucose oxidase (100,000 units/g), resulting in the generation of approximately 1 mM of H_2O_2 after 15 min. Because of glucose limitation, the amounts of peroxide generated remained nearly the same even after 2 h of incubation. This amount of GO-generated peroxide did not induce the release of any significant amounts of chromium. The method of Thurman et al. (26) was used to quantify the peroxide.

The following agents were employed to synergize with H_2O_2 to kill EC: (A) phospholipase A_2 from bee venom (0.1–25 units/ml); (B) lysophosphatidylcholine (lysolecithin, LL) (1–30 μg/ml); (C) phospholipase C from *Clostridium welchii* (0.01–0.15 units/ml); (D) streptolysin S (SLS) from group A streptococci (1000 units/ml) (27); (E) lipoteichoic acid (LTA) from group A streptococci (1–100 μg/ml) (19); (F) rabbit anti-streptococcal IgG possessing 39,000 agglutinating units/mg as assayed by passive hemagglutination of human red blood coated by LTA (19); (G) crystalline trypsin (10,000–13,000 BAEE units/mg) and pancreatic porcine elastase (40–80 units/mg protein); (H) crystalline egg-white lysozyme (56,000 units/mg) and milk lysozyme (100,000–200,000 units/mg); (I) cationic agents nuclear histone (type II-A), poly-L-arginine HCl (PARG) (mol wt 40,000), porcine pancreatic ribonuclease, polymyxin B, chlorhexidine gluconate, cetyltrimethyl ammonium bromide (CETAB), spermine, histamine HCl, defensins NP-1 and NP-5 (kindly donated by Dr. M. Friedkin from the Weitzman Institute, Rehovot, Israel); (J) hypochlorous acid (HClO), generated by the addition of sodium hypochlorite to distilled water at pH 6.0; (K) tumor necrosis factor (TNF) (form Genetec); (L) soybean trypsin inhibitor (SBTI).

The following agents were employed to modulate the killing of EC: catalase, dimethylthiourea (DMTU), deferoxamine mesylate, 6-deoxyglucose, puromycin, trifluprомazine (TFP), guanosine 5-*O*-(3-thiotriphosphate) (GTPγS), tannic acid, extracts of tea, quinacrine heparin, polyanthole sulfonate, and $MnCl_2$. Most of the agents were purchased from Sigma Chemical Co., St. Louis,

Missouri. Elastase was from Worthington Biochemical Corporation, Freehold, New Jersey. $MnCl_2 \cdot 4H_2O$ was from Mallinckrodt Chemical Works, St. Louis, Missouri.

RESULTS

Cell Killing

Combined Effect of Cationic Agents and H_2O_2 on EC. Bactericidal cationic agents are present in large amounts in granules of PMNs (leukocyte cationic proteins, defensins, myeloperoxidase, lysozyme), in eosinophils (major cationic proteins), in platelets, in plasma, (β-lysins), in saliva (histidine-rich proteins), in tears, and in milk (lysozyme) as well as in semen (spermine, spermidine) (reviewed in 28). Furthermore, large amounts of histones might also be present in inflamed sites as a result of cell death. The likelihood that membrane-damaging cationic agents (4, 6, 28) might collaborate with oxidants to kill EC was further investigated (see 17).

In a representative 2-h assay, Figures 1 and 2 show the time-course and dose-dependent release of chromium, respectively, from monolayers of EC treated with different cationic agents combined with GO-generated H_2O_2. The data suggest that subtoxic concentrations of most of the agents synergized with subtoxic concentrations of H_2O_2 kill the cells (17, 21). Under similar experimental conditions only high concentrations either of egg-white or milk lysozyme (1 mg protein/ml) showed low-level, but significant synergy with H_2O_2 (not

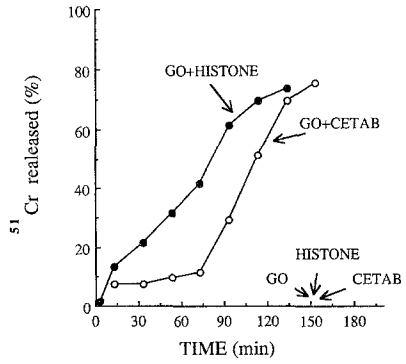

Fig. 1. Time-course of chromium release from cells treated with hydrogen peroxide and cationic agents. Monolayers of endothelial cells were incubated with histone (100 μg/ml), glucose oxidase (GO; 0.2 units/ml with CETAB (10 μg/ml), or mixtures of GO and the cationic agents, and the release of chromium from the cells was determined at intervals. A representative of a typical experiment.

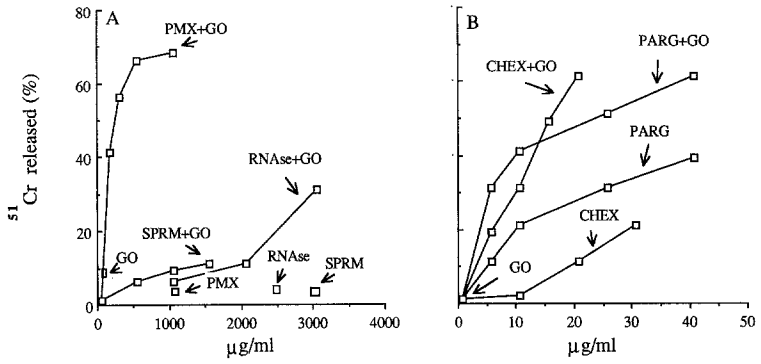

Fig. 2. Combined effect of cationic agents and hydrogen peroxide on chromium release from EC. Monolayers of EC were treated for 2 h at 37°C with increasing concentrations of cationic agents in the absence or presence of glucose oxidase (GO; 0.2 units/ml). (A) PMX, polymyxin B sulfate: SPRM, spermine HCl, RNAse, pancreatic ribonuclease. (B) PARG, poly-L-arginine; CHEX, chlorhexidine gluconate. The date are the averages of three experiments.

shown). On the other hand, histamine was totally inactive (not shown). Preliminary experiments have also shown that two defensins (at 50 μg/ml) also synergized with H_2O_2 to kill EC (50–60% chromium release). In all cases, either catalase, DMTU, Mn^{2+} (see ref. 29), or the anionic polyelectrolytes heparin and polyanethole sulfonate totally inhibited cell killing induced by combinations of cationic agents and H_2O_2 (not shown).

To determine whether or not the simultaneous presence of both H_2O_2 and the cationic agents was necessary to induce cell damage, the following experiments were performed: Cell monolayers were preincubated for 45 min either with histone or with GO. The monolayers were then washed to remove unbound agent and either GO or histone was then added. Table 1 shows that cells exposed to histone and then washed were still damaged upon addition of GO. No such phenomenon occurred, however, when the cells were first exposed to GO, washed, and then challenged with histone, suggesting that only histone avidly bound to the targets and then synergized with H_2O_2 to kill the cells. These results suggest that both peroxide and histone must be present simultaneously in order to exert a synergistic toxic effect on the cells. The presence of either catalase (100 units/ml but not heat-inactivated catalase), DMTU (100 μM) or Mn^{2+} (1 mM) (29) during the incubation period totally inhibited cytotoxicity. It was also found that EC that had been precoated either by PHSTD–catalase complex (24) or by catalase cationized by the method of Dannon et al. (30) and washed were totally refractory to the synergistic toxic effect of added GO + histone (see ref. 24).

Effect of Cationized GO on EC Killing. The synergistic killing induced by combinations of cationic agents with hydrogen peroxide also led us to explore

the possibility that an enhancement of peroxide delivery to cell surfaces might serve as a tool to enhance cell killing. For this purpose GO was complexed to PHSTD by the same method employed to cationize catalase (24). Table 2 shows that cells pretreated with cationized GO and then washed were readily killed upon the addition of histone suggesting that cationization allowed GO to adhere to the cell surfaces and to deliver H_2O_2 directly to them. To prove that cationized GO might also be effective as a tissue-damaging agent in vivo, we instilled GO–PHSTD complexes into the trachea of anesthetized rats. In preliminary experi-

Table 1. Effect of Histone and Glucose Oxidase (GO) on EC Killing[a]

^{51}Cr-labeled EC pretreated with	Followed by	Followed by	Percent Cr released
None	None	Histone (100 μg/ml)	5
GO (0.2 units/ml)	None	None	6
GO	Washing	Histone	7
GO	None	Histone	56
Histone	None	GO	57
Histone	Washing	GO	52
GO + histone	None	None	55

[a]Monolayers of EC were preincubated for 45 min at 37°C either with MEM alone, GO (which yielded approximately 750 nmol H_2O_2 after 45 min of incubation), or histone. Some of the wells were washed to remove unbound agent. This was immediately followed by the addition either of medium, GO, or histone. Following an additional 1 h of incubation, the supernatant fluids were removed and the radioactivity solubilized was determined. The data are the average of three experiments.

Table 2. Effect of Cationized Glucose Oxidase (GO) and Histone on EC Killing[a]

^{51}Cr-labeled EC pretreated with	Followed by	Followed by	Percent Cr released
GO (0.2 units/ml)	None	None	5
GO	Washing	Histone (100 μg/ml)	5
GO	None	Histone	60
GO-PHSTD complex (2 units/ml)	None	None	5
GO-PHSTD complex	None	Histone	60
GO-PHSTD complex	Washing	Histone	60
Histone	None	None	6

[a]EC monolayers were incubated for 45 min at 37°C either with GO or with GO that had been complexed with poly-L-histidine (PHSTD) (Materials and Methods). The monolayers were either left intact or washed to remove unbound agent. This was immediately followed by the addition either of medium or medium + histone. Sixty minutes later the release of soluble radioactivity was determined in the supernatant fluids. The data represent averages of three different experiments.

ments (to be published in detail), we found that cationized GO was at least 10-fold more toxic to the lung than regular GO. This was determined by measuring the index of injury following the leakage into the lungs of systemically administered iodine-labeled BSA. Such enhanced lung injury could, however, be totally inhibited by administering a mixture of glycine and $MnCl_2$ (see ref. 29), which scavenged H_2O_2.

Combined Effects of Cationic Agents, H_2O_2, and Proteinases on EC. Previous studies from our laboratory have described the synergistic killing of EC by combinations between proteinases and H_2O_2 (18) and between certain membrane-active agents and H_2O_2 (17, 21). It was therefore also of interest to test the combined effect of membrane-active agents, H_2O_2, and a proteinase on EC.

As a model, either trypsin or pancreatic elastase were used as representative proteinases, CETAB as a cationic agent (because of its resistance to protease activity) (see below), and GO as a generator of H_2O_2. Figure 3 shows that trypsin strongly synergized with GO and with subtoxic concentrations of CETAB to release large amounts of the chromium label from the targets. Similar results were obtained with elastase (not shown). Thus a triple synergy is more efficient than a double synergy in cell killing. The killing effect due to the triple synergy was markedly inhibited either by catalase, Mn^{2+}, heparin, or SBTI (not shown). Experiments with histone, however, gave inconsistent results as this cationic agent was hydrolyzed by trypsin. To circumvent this problem, we first preincubated EC with histone to allow binding and "priming" of the cells (see Table 1) and then added GO and trypsin. We found that histone binding primed the

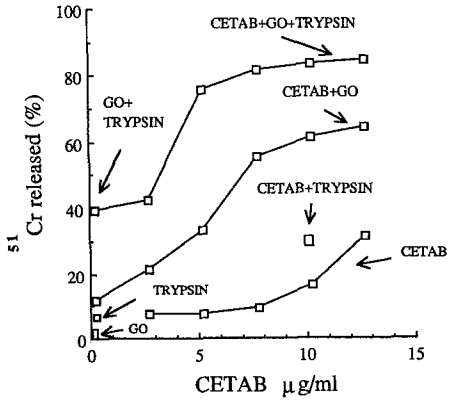

Fig. 3. The combined effects of CETAB hydrogen peroxide and trypsin on endothelial cells. Endothelial cells were exposed for 90 min at 37°C to increasing concentrations of CETAB, to fixed amounts of GO (0.2 units/ml), or trypsin (100 μg/ml), to combinations of CETAB, GO, and trypsin and to GO and trypsin. A representation of a typical experiment.

cells to cytotoxicity following the addition of GO, but no significant further enhancement of cell killing occurred upon addition of trypsin, suggesting that the protease might have hydrolyzed the cell-bound histone.

Combined Effect of H_2O_2, PLC, Lysolecithin, and Trypsin on EC. Inflammatory sites might be rich in exocytosed lysosmal products of leukocytes. Thus, target cells might either become simultaneously or sequentially exposed to a multiplicity of hydrolases, cationic proteins, and oxidants (17, 18, 21). It was of interest to test whether, as in the case of histone, other agents might also irreversibly bind to EC and kill to them in a synergistic manner. Figure 4 shows that EC preexposed to sublytic concentrations of PLC (a representative membrane-damaging agent) and then washed to remove unbound enzyme were still killed in a synergistic manner following the addition of H_2O_2. The same degree of killing was also observed if PLC and GO were simultaneously present throughout the incubation period. Similarly, cells incubated either with PLA_2 or with SLS (both highly hemolytic and cytolytic) and then washed and exposed to GO were also killed in a synergistic manner (not shown), suggesting that these membrane-active agents bound to the membrane and continued to exert their synergistic action with H_2O_2. Experiments performed with PLA_2, however, necessitated the addition of fatty acid-free albumin to remove the cleaved fatty acid from the membranes of the EC.

Experiments on the combined effect of LL and GO on EC killing (see Figure 12 below) showed that cells exposed to LL for 60 min, followed by a

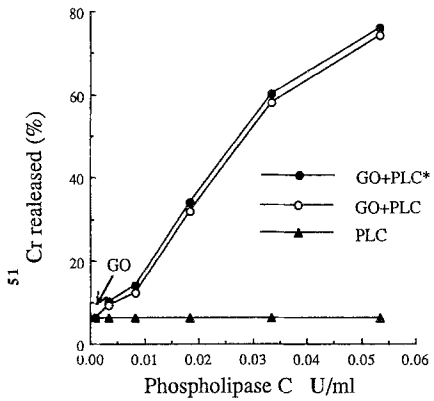

Fig. 4. The combined effect of PLC and GO on chromium release from EC. Monolayers of EC were treated for 30 min at 37°C with increasing concentrations of PLC. Some of the monolayers were washed to remove unbound PLC (*). GO (0.2 units/ml) was then added to all the wells, and the release of radioactivity was determined after a further incubation for 60 min. Note that EC, exposed to PLC and washed, were killed to the same degree as cells incubated with mixtures of PLC and GO, suggesting that PLC firmly bound to EC and synergized with H_2O_2. The data are the averages of two experiments.

wash to remove unbound agent and by the addition of GO, were also killed but to a much lower degree (about 25% release of chromium), suggesting that LL must be simultaneously present with H_2O_2 to exert its maximal toxic effects (see also Figure 14 below).

EC treated either by mixtures of PLC and peroxide or by SLS and peroxide developed, within 15–30 min, large blebs (pseudopodlike structures), usually in one pole of the cell. Many of these structures were later found as free-floating lipid like droplets (liposomes?). These morphological changes were essentially similar to those described either in tumor cells (16) or in epithelial cells exposed to similar agonists (21). It is of interest that detachment of the liposomelike structures never occurred following exposure of EC either to SLS, PLC, or peroxide alone.

Combined Effects of LTA, Anti-LTA, and H_2O_2 on EC. Previous studies have shown that target cells that had been sensitized by LTA derived from group A streptococci were killed following the addition of anti-LTA antibodies + complement (31–33). In other studies, we have shown that human PMNs sensitized by LTA and then treated by anti-LTA globulin generated superoxide, H_2O_2, and chemiluminescence and released a variety of lysosomal enzymes (19). The possibility that the presence of LTA–anti-LTA complexes on the surface of EC might render them more susceptible to the killing effects of H_2O_2 was investigated. Monolayers of EC were exposed to LTA (10–100 μg/ml) for 30 min to allow cell binding. While LTA-coated EC exposed either to GO or to anti-LTA IgG (200 μg protein/ml) were not injured, a very marked release of radioactivity (50–60% of total) occurred when the LTA-sensitized EC were exposed to mixtures of anti-LTA–IgG and GO. Under similar conditions, nonimmune rabbit IgG (500 μg protein/ml) failed to cause such an effect. Paradoxically, however, and unexpectedly, EC incubated for 2 h with mixtures of anti-LTA IgG and H_2O_2 were also killed (50–60% chromium release). The possibility that the cytolytic effect observed was due to the presence in the anti-LTA IgG of antibodies cross-reacting with some epitopes present on the surface of EC was considered. Cross-reactivity between microbial antigens and those present in mammalian cells has been extensively studied (see Discussion). To address this point, we incubated the anti-streptococcal IgG (1 mg protein/ml) with streptococci (1 ml of a 10.OD at 550) for 2 h at 37°C and for an additional 24 h at 4°C to adsorb potential cross-reactive antibodies. The streptococci were then removed by high-speed centrifugation, and the supernatant fluid was employed to kill EC in the presence of GO. We found that the adsorbed IgG lost 60–70% of its cytotoxicity when combined with sublytic amounts of GO-generated H_2O_2. In other experiments we first exposed EC to anti-LTA IgG (1 mg protein/ml) for 2 h, then washed the cells and treated them with sublytic amounts of peroxide. A significant cytolytic reaction (25–35% net chromium release) was also observed. These experiments suggested that we might be dealing with a novel

phenomenon that noncytotoxic antibodies, which might recognize surface components of EC, became cytolytic in the presence of H_2O_2 (see Discussion).

Modulation of EC Killing by Various Agents

To further study the mechanisms by which combinations among oxidants, membrane-damaging agents, and proteinases kill EC and release membrane lipids from these cells (see below), we tested the modulating effects of serum proteins, certain metabolic inhibitors, an agent affecting intracellular calcium, an analog of G-proteins, a transition metal chelator, a cytokine, and an agent that coats and denatures cell surfaces (see below).

EC were preincubated with the various agents for various times and then challenged either with GO-generated H_2O_2 alone or with combinations of peroxide and membrane-damaging agents.

Effect of 6-Deoxyglucose and Puromycin. EC preincubated for 3 h either with 30 mM 6-deoxyglucose (Figure 5) or with 1 mM puromycin (not shown) to deplete energy sources and to affect protein synthesis did not become more susceptible to killing by hydrogen peroxide alone but were killed in a synergistic manner (60% chromium release) by mixtures of subtoxic amounts of peroxide

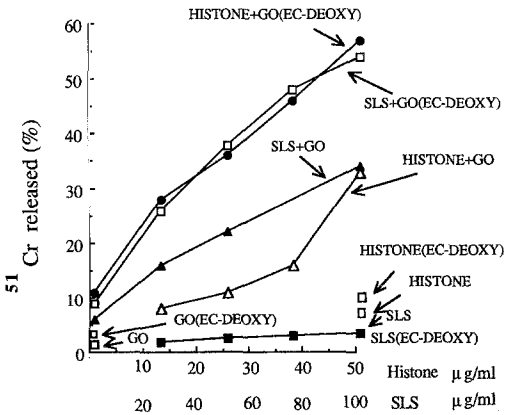

Fig. 5. Effect of 6-deoxyglucose on killing of endothelial cells by mixtures of H_2O_2 with histone or SLS. EC were exposed for 3 h at 37°C either to 5 mg/ml 6-deoxyglucose (EC-DEOXY) or to medium alone. The cells were then treated with increasing amounts either of histone or streptolysin S (SLS) and in the absence or presence of glucose oxidase (0.2 units/ml). Sixty minutes later, the release of chromium from the cells was determined. Note that while neither GO-generated hydrogen peroxide SLS, nor histone significantly enhanced the killing of deoxyglucose-treated (DEOXY) cells, combinations of GO + SLS or GO + histone were highly cytolytic for the deoxyglucose-treated cells. The data are the averages of two experiments.

and subtoxic amounts of either PLC or of SLS. Our results, however, differ from those showing that 6-deoxyglucose inhibited cell killing by defensins (34, 35). It is also of interest that EC that had been preincubated with 6-deoxyglucose did not possess diminished capacities to scavenge H_2O_2 (not shown), suggesting that the antimetabolite probably did not diminish their cell-associated catalase or glutathione peroxidase.

Effect of GTPγS. Killing of EC by mixtures of poly-L-arginine (PARG) and hydrogen peroxide (Figure 2B) was unaffected by pretreatment with GTPγS. This agent was found to inhibit killing of targets by PARG (36), presumably because this polycation interacted with G-proteins. The possible reason for the observations made by Elferink and Deirkauf (36) might be due to the binding of GTPγS (an anion) by PARG (a distinct polycation) but probably not to interference with G-proteins.

Effect of Deferoxamine. The possibility that EC killing by mixtures of H_2O_2 and the various membrane-damaging agents involved the generation of hydroxyl radical via the metal-mediated Haber-Weiss reaction (37), which could have killed the cells, was tested by the addition of deferoxamine (3 mM) to the reaction mixtures. No modulation of cell killing was observed, however, suggesting that OH radical might not be involved in EC killing in this model (37).

Effect of TNF. It is well accepted that TNF is cytotoxic for a variety of mammalian cells (38, 39). The possibility that subtoxic amounts of TNF might act synergistically with H_2O_2 to kill EC was also investigated. TNF (up to 500 μg/ml), which failed to cause any substantial release of chromium from EC after 2 h of incubation, synergized with H_2O_2 only to a very small extent ($6 \pm 2.0\%$ chromium release, $N = 3$). It is of interest in this respect that the toxicity of TNF to target cells was found to be greatly reduced in the presence of H_2O_2 (40).

Effect of Hypochlorous Acid. HClO is a potent cytotoxic oxidant that is generated by activated PMNs (41). We, however, found that HClO at a wide range of subtoxic concentrations did not modulate EC killing induced by mixtures of H_2O_2 with either PLC or with SLS.

Effect of Trifluopromazin (TFP). TFP was found to modulate superoxide generation induced in PMNs (42, 43). The possibility that interference with calmodulin (44) might also alter the susceptibility of EC to killing by mixtures of hydrogen peroxide and membrane-damaging agents was therefore investigated. Figure 6 shows that pretreatment with TFP very markedly enhanced the killing of EC either by H_2O_2 or by mixtures of hydrogen peroxide and PLC

Effect of Tannic Acid. Previous publications have shown that tannic acid modulated several leukocyte functions (45–47) and bacteriolysis (48). The denaturing activities of tannic acid and of extracts of tea, known to be rich in this acid, was also investigated in the EC model. We found that coating of EC either by noncytotoxic amounts of tannic acid (10^{-5}–10^{-4} M) or by 10–100 μl of

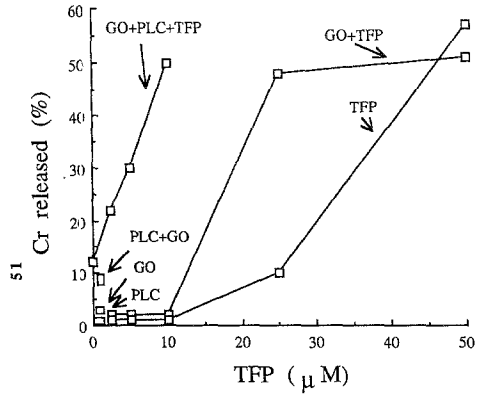

Fig. 6. Effect of trifluprom azine (TFP) on killing EC in the presence of PLC and GO. EC monolayers were exposed for 120 min at 37°C to increasing concentrations of TFP. The cells were then further exposed for an additional 60 minutes either to GO (0.2 units/ml), PLC (0.05 units/ml), or mixtures of PLC and GO. The data are the averages of three experiments.

extracts of black tea (1 g of tea brewed with 10 ml of boiling water), followed by a cell wash, caused 95% inhibition of chromium release from EC exposed to mixtures of H_2O_2 either with PLC or with SLS (not shown). The same amounts either of tannic acid or tea also very markedly inhibited lysis of human RBC induced either by SLS or by PLC. Our findings suggest, therefore, that coating by tannic acid, which alters cell surfaces, might also affect other cell types (e.g., epithelial cells in the gut), protecting them against microbial toxins and leukocyte hydrolases.

Effect of Serum Proteins and Inhibitors. The possibility that serum proteins might modulate EC killing by mixtures of oxidants and membrane-damaging agents was also investigated. Figures 7 and 8 show that larger amounts of either CETAB or histone were necessary to kill EC in the presence of H_2O_2 and 10% fetal calf serum. Inhibition by serum proteins might be due to albumin (a polyanion), which bound the cationic agents employed. On the other hand, serum at the same concentration caused only about 25% inhibition of EC killing induced by mixtures of H_2O_2 with either SLS or PLC (not shown). In another EC model we also investigated the effect of higher amounts of serum on EC killing induced by a "super killer cocktail" of agonists (H_2O_2, HClO, histone, PLC, LL, and trypsin) that might mimic the mixture of agents likely to be generated by PMNs in inflamed sites. Such a cocktail had a killing capacity of at least five times that induced by mixtures of peroxide with any of the single agonists tested. Figure 9 shows that even in the presence of 40% serum, no substantial inhibition of EC killing by the superkiller cocktail occurred unless a mixture of antagonists (catalase, taurine, heparin, SBTI, and phosphatidylcholine—an inhibitor of PLC and LL) was also added. These results suggest that

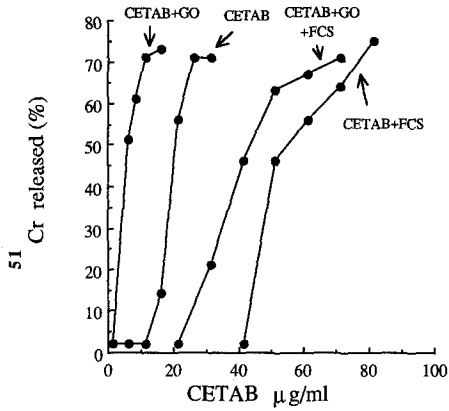

Fig. 7. Effect of fetal calf serum (FCS) on killing of endothelial cells by mixtures of CETAB and hydrogen peroxide. EC monolayers were exposed either to increasing concentrations of CETAB or CETAB + GO (0.2 units/ml). Note that in the presence of 10% fetal calf serum larger amounts of CETAB are needed to kill the cells, both in the absence and presence of GO. The data represent results of a typical experiment.

cell injury in inflammatory sites might also be inhibited by such a mixture of antagonists administered either systemically or injected locally.

Release of Arachidonic Acid and Metabolites from EC

It is well established that an alteration in the integrity of cell membranes occurs when certain microbial products (hemolysins, phospholipases, pore-forming toxins) as well as snake and bee toxins interact with cells (2, 3, 21). The activation of membrane-associated PLA_2 by a variety of agonists might also result in the release of AA and its metabolites, as well as in the formation of membrane-associated lyso compounds (20). To study whether the killing of EC by the synergistic effects of oxidants and membrane-damaging agents was also accompanied by the solubilization of AA and certain of its metabolites, we employed parallel tissue culture plates labeled either with chromium or tritiated AA. We treated the cells with a variety of agonists and determined the solubilization of chromium and of AA.

Figure 10 shows the time course of chromium and of AA release from EC treated by combinations of H_2O_2, PLC, and trypsin. While maximal killing of EC was induced by mixtures of peroxide and PLC, maximal solubilization of AA necessitated the additional presence of the proteinase. Similar effects were also found when elastase replaced trypsin (not shown). A very marked decrease in AA release was observed when either catalase, DMTU, or SBTI were included

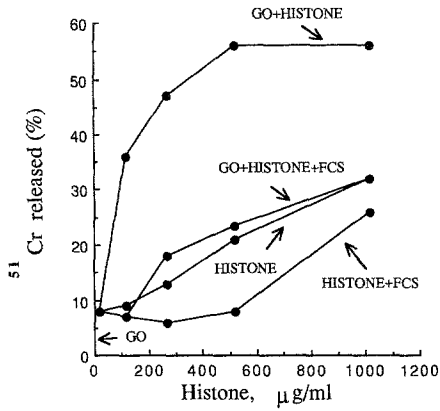

Fig. 8. Effect of serum on killing of endothelial cells by mixtures of GO + histone. EC were exposed for 90 min at 37°C either to increasing amounts of histone or combinations of GO (0.2 units/ml) + histone + 10% fetal calf serum. Note that larger amounts of histone are needed to kill cells in the presence of 10% serum. The data are the averages of two experiments.

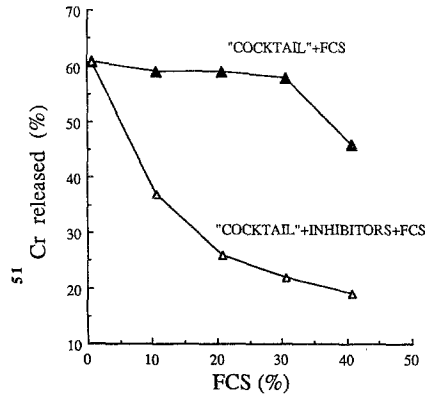

Fig. 9. Effect of serum and inhibitors on killing of EC induced by a cocktail of agonists. EC were exposed for 90 min at 37°C to a cocktail comprised of GO (0.2 units/ml), histone (100 μg/ml), phospholipase C (0.05 units/ml), hypochlorous acid (10^{-4} M), lysolecithin (5 μg/ml), and trypsin (50 μg/ml) and in the presence of increasing concentrations of fetal calf serum. Note that even 40% serum failed to significantly inhibit killing of EC by this cocktail of agonists. On the other hand, a cocktail of inhibitors comprised of catalase (100 units/ml) soybean trypsin inhibitor (200 μg/ml), phosphatidylcholine (500 μg/ml), taurine (1 mM), and heparin (85 units/ml) markedly depressed cell killing in the presence of 10–40% serum. The data are the averages of two experiments.

in the reaction mixtures (not shown). Figure 11 shows the dependency on trypsin concentration of AA release in the presence of PLC and H_2O_2, again showing that all three agents had to be present simultaneously to achieve maximal solubilization of AA.

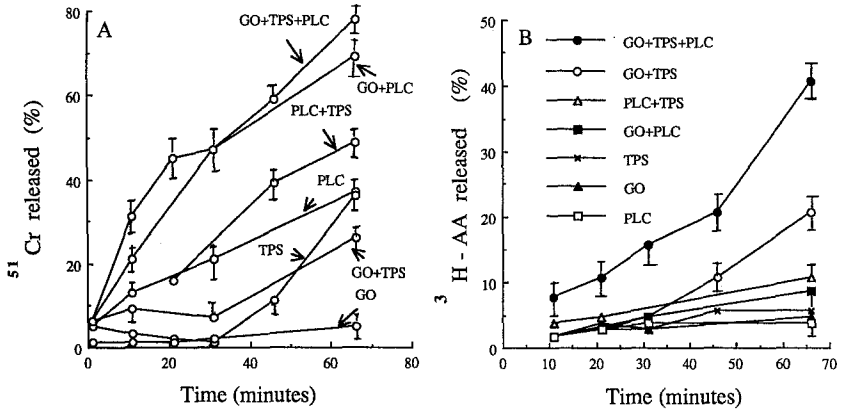

Fig. 10. Time course of the release of chromium (A) and arachidonic acid (AA) (B) from endothelial cells treated with a mixture of agonists. Parallel wells of endothelial cells labeled either with ^{51}Cr or with [^{3}H]arachidonic acid were treated for 90 min at 37°C either with GO (0.2 units/ml), PLC (0.01 units/ml), trypsin (25 μg/ml), or by combinations of GO and PLC, PLC and trypsin, and GO + PLC + trypsin; (A) chromium release; (B) arachidonic acid release. The data are mean value ± *SD* of five experiments.

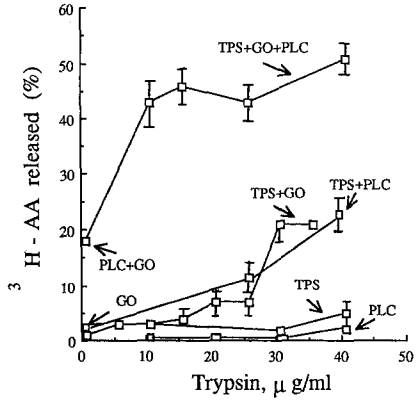

Fig. 11. Dependency on trypsin concentration of the release of arachidonic acid from cells treated by GO and PLC. EC labeled with [^{3}H]arachidonic acid were treated for 90 min at 37°C with increasing concentrations of trypsin and with combinations of GO (0.2 units/ml), PLC (0.005 units/ml) and trypsin. The data represent mean values ± *SD* of five separate experiments.

Figure 12 further shows that while H_2O_2 mixed with LL induced a high release of chromium, the additional presence of a proteinase was needed to induce maximal solubilization of AA. In every case the presence either of catalase, DMTU, Mn^{2+}, or SBTI very markedly depressed the release of AA from EC (not shown). Under similar experimental conditions, mixtures of H_2O_2, SLS,

and trypsin also released synergistic amounts of PGE_2 (Figure 13) and mixtures of H_2O_2 either with PLC or with SLS and with trypsin released synergistic amounts of 6-keto-PGF (Figure 14). On the other hand, none of the mixtures of agonists tested had the capacity to solubilize significant amounts of thromboxane B_2 (not shown).

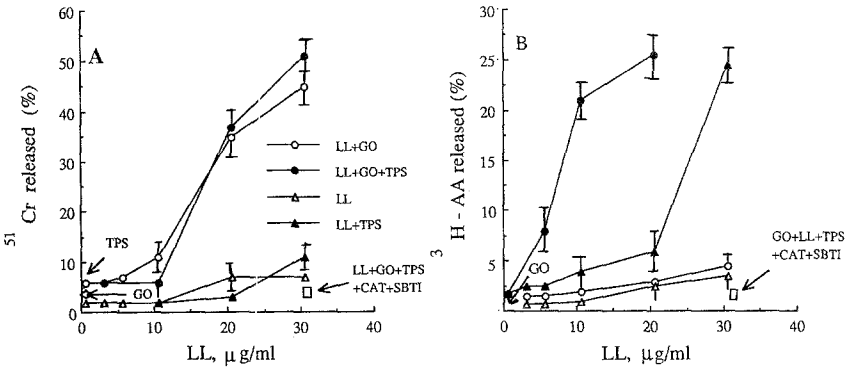

Fig. 12. Combined effects of GO, lysolecithin (LL), and trypsin (TPS) on chromium and arachidonic acid release from endothelial cells. Monolayers of chromium-labeled and [^{3}H]arachidonic acid-labeled EC were exposed for 90 min at 37 °C to increasing concentrations of LL and to mixtures of LL with GO (0.2 units/ml) and trypsin (30 μg/ml). Note that while LL + H_2O_2 was sufficient to cause maximal cell death, mixtures of H_2O_2 + LL + trypsin were necessary for maximal solubilization of arachidonic acid. The data are mean values ± *SD* of three separate experiments.

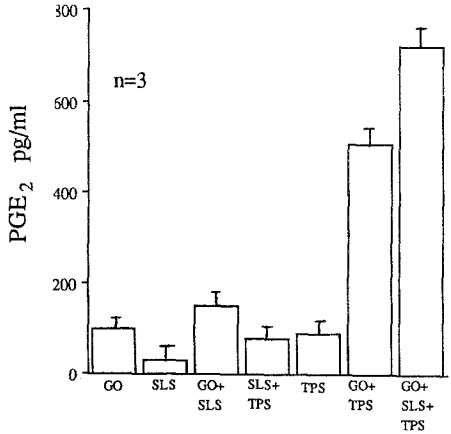

Fig. 13. Combined effects of H_2O_2, streptolysin S (SLS), and trypsin (TPS) on the release of PGE_2 from endothelial cells. Monolayers of EC were exposed to mixtures of GO (0.2 units/ml), streptolysin S (50 units/ml), and trypsin (100 μg/ml). Note that maximal release of PGE_2 necessitated the simultaneous presence of all three agents. The data represent means values ± *SD* of three separate experiments.

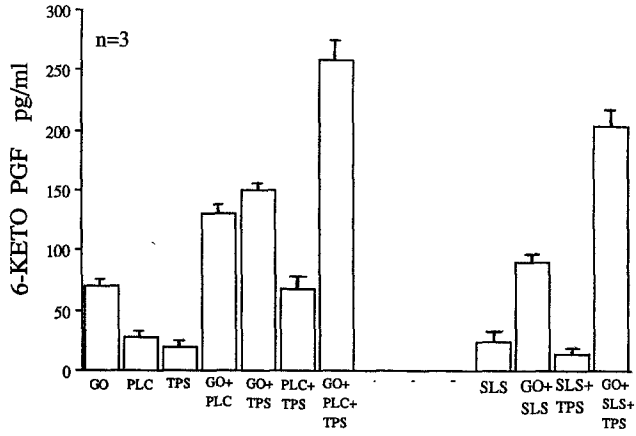

Fig. 14. Combined effects of hydrogen peroxide, PLC, SLS, and trypsin (TPS) on the release of 6-keto-PG from endothelial cells. Monolayers of endothelial cells in 6-well plates were treated for 120 min at 37°C with mixtures of GO (0.2 units/ml) with PLC (0.050), SLS (50 units/ml), and trypsin (20 μg/ml). Note that maximal release of 6-keto-PG necessitated the simultaneous presence of hydrogen peroxide, PLC, or SLS, and trypsin. The data represent mean values ± *SD* of three separate experiments.

Modulation of AA Release by Various Agents

Effect of Cationic Agents. Cationic agents (histone, PARG, CETAB, chlorhexidine) that synergized with H_2O_2 kill EC (Figures 1, 2) but failed to synergize with H_2O_2 to solubilize AA (not shown). It was, therefore, of interest to test the possibility that cationic agents might interfere with AA release from EC. Cell monolayers were exposed for 15 min to subtoxic concentrations either of PARG (5 μg/ml) or CETAB (5 μ/ml) and then washed. The cells were then exposed to mixtures of H_2O_2 with PLC and trypsin (see Figure 10). We invariably found that preexposure to cationic agents depressed AA release by 75–100% (not shown). These results suggest that cationic agents released either by leukocytes or from disintegrating cells in vivo might perhaps modulate the release of AA and its metabolites.

Effect of HClO. HClO (at 10^{-4} M), which failed to synergize with H_2O_2 to kill EC, caused 75% inhibition of AA release when mixed with peroxide, PLC, and trypsin (not shown).

Effect of Tannic Acid. Tannic acid at 10^{-5} M caused 90% + 5.0 (N = 3) inhibition of AA release from EC treated with mixtures of H_2O_2, PLC, and trypsin and 85% ± 4.0 (N = 3) inhibition of AA release with mixtures of H_2O_2 and trypsin alone. As little as 10 μl of tea depressed AA release induced by mixtures of H_2O_2 with PLC and trypsin by about 50% (not shown).

Effect of Quinacrine. The possibility that treatment by agonists that might activate a membrane-associated PLA_2 might also release AA was studied by the inclusion of quinacrine (a PLA_2 inhibitor) in the reaction mixtures. It was found that at 10^{-4} M quinacrine depressed AA release from EC treated with mixtures of peroxide, PLC, and trypsin by about 50% (not shown). Paradoxically, however, quinacrine at this concentration acted synergistically with H_2O_2 to kill EC (not shown).

DISCUSSION

The present experimental model further examines the assumption that cellular injury that accompanies infectious and inflammatory processes might be due, in part, to the synergistic action among oxidants and hydrolases secreted either by activated leukocytes, released by disintegrating host cells, or elaborated by microorganisms (17, 18, 21). Such agents alter cell viability (14, 15, 17, 18, 21) and are also instrumental in releasing membrane-associated arachidonic acid (AA) and some of its metabolites, which might further modulate the inflammatory processes.

Our studies suggest that the simultaneous presence of H_2O_2 and several of the membrane-damaging agents is absolutely essential to secure maximal killing of the targets (Figures 1–3). Histone, SLS, and PLC, at subtoxic concentrations, which bind to EC membranes and resist washing, can nevertheless continue to synergize with H_2O_2 to release radiolabeled chromium (Table 1, Figures 4 and 12), presumably because of their ability to alter membrane permeability and thus to prime the cells for a further oxidant insult (3, 17, 21, 34, 35).

Since H_2O_2 does not irreversibly bind to membranes (Tables 1 and 2), the ability to bind GO (an H_2O_2-generating enzyme) to mammalian cells by cationization with PHSTD (Table 2) might serve as a useful tool to enhance cell killing in vivo (i.e., tumor cells). Our preliminary findings on the high toxicity of cationized OG in the rat lung model is one such an example (29).

The findings that chlorhexidine, a potent cationic antiseptic widely used in dentistry to treat oral lesions, also acts synergistically with H_2O_2 to kill EC (Figure 2B) suggest that this agent should either not be applied to severely inflamed tissue or used in the oral cavities of patients undergoing irradiation of the head and neck for treatment of malignancies. The same might be also true for the antibiotic polymyxin B (Figure 2A).

The ability of lysozyme, a distinct bacteriolytic polycation (48) to synergize with hydrogen peroxide to kill EC is also important, as inflammatory exudates might contain large amounts of this enzyme, which, like other polycations present in inflammatory sites (4, 6, 28), might also amplify oxidant damage, espe-

cially in an acidic milieu that increases the cationization of the enzyme. Thus, such cationic proteins might also synergize with oxidants, with phospholipases, and with microbial hemolysins to kill target cells, as shown in the present study. It might also be speculated that since synergism between SLS and oxidants kills EC, other microbial hemolysins and perhaps also the attack complex of complement (13) might also synergize with oxidants and with proteinase to enhance cellular damage.

The findings that the synergistic killing effect induced by oxidants and membrane-active agents was totally inhibited either by scavengers of H_2O_2 (catalase, DMTU, Mn^{2+}), by polyanions, by proteinase inhibitors, or by phospholipids (Figure 9) also suggest that in in vivo models the administration of adequate "cocktails" of antagonists might ameliorate the toxic effects exerted by such potentially synergizing agents (Figure 9) (21, 29).

The ability of 6-deoxyglucose (Figure 5) and puromycin (not shown) to markedly enhance the susceptibility of EC to the synergistic killing effects of oxidants with PLC or histone but not to H_2O_2 alone suggests that the enhanced susceptibility was presumably caused by altering complex metabolic pathways that might affect the ability of the cells to repair damage caused by H_2O_2 (49). The interference with calcium balance induced by the calmodulin antagonist TFP (Figure 6) (42–44) also markedly enhanced the susceptibility of EC to killing by combinations of H_2O_2 with membrane-damaging agents. On the other hand, neither GTPγS (a putative antagonist of G proteins), shown to inhibit target cell killing by PARG (36), nor TNF, shown to possess a cytotoxic activity (38, 39), had any appreciable modulating effects on EC killing. In this respect it is of interest that H_2O_2 diminished the toxic effects of TNF on targets (40), also suggesting that a complex interplay among cytokines, oxidants, and proteinases might exist in inflamed sites.

The findings that both tannic acid and extracts of tea, known to be rich in this acid, very markedly inhibited both the killing of EC induced by mixtures of H_2O_2 and PLC or SLS, and the hemolysis of RBC caused either by PLC or by SLS, is noteworthy. It further suggests that other cell types, e.g., epithelial cells in the gastrointestinal tract, might also be shielded by tannic acid against toxins elaborated by gram-negative microorganisms as well as against leukocyte-derived oxidants and hydrolases (45–47).

The inability of deferoxamine to inhibit EC killing induced by the various "cocktails" suggests that the OH radical that could have been generated might not be involved in our model (37). Experiments to localize a possible presence of the OH radical by spin-trapping might resolve this question.

The findings that rabbit anti-streptococcal IgG acted synergistically with H_2O_2 to kill EC and the ability of streptococci to adsorb the bulk of the active agent present in IgG that synergized with hydrogen peroxide to kill EC are intriguing. It points, however, to the possible presence in the anti-streptococcal

serum of antibodies cross-reacting with EC (50–52). If, indeed, cytotoxicity was caused by such cross-reactive antibodies, it might be the first report to show that such potential cytotoxic antibodies might be amplified by a peroxide. It is of interest, however, that anti-streptococcal antibodies cross-reactive with heart or kidney have never been shown to possess cytotoxic activities either in vitro or in vivo (50–52) unless an additional insult, i.e., treatment with isoproteranol was given (53) (also a synergistic action?).

A very important observation described in our study is that, whereas two agents (H_2O_2 combined with a membrane-damaging agent) were sufficient to cause maximal cell death as measured by the chromium release assay (Figures 1–6, 10A, and 12A), a significant solubilization of AA and several of its metabolites necessitated the additional presence of a proteinase (Figures 10A, 12A, 13, and 14). It suggests that potent cyclooxygenase products, capable of modulating the inflammatory responses, can also be released from dead cells abundantly present in inflamed sites. It is also suggested that both the measurements of lipid and chromium release from cells yield better information on the mechanism of cell damage. The findings that none of the agonists tested were capable of releasing significant amounts of thromboxane B_2 is not understood and further studies on this issue are warranted (55).

The role of H_2O_2 as a low-level releaser of arachidonic acid from a variety of mammalian cells was recently reported (25, 54–56). One study (25) showed that even millimolar concentrations of peroxide failed to release any substantial amounts of labeled AA from macrophages. Another study (55) showed that H_2O_2 was capable of solubilizing some 6-keto-PGF_1 and thromboxane B_2 from targets. This was strongly inhibited by quinacrine (a PLA_2 inhibitor). Our study, however, is the first report showing that a proteinase is necessary for the solubilization of large amounts of AA from EC treated by mixtures of H_2O_2 with cytolytic agents (PLC, LL, SLS).

The cardinal role played by proteinases in AA release, as shown in the in vitro model, implies that a similar phenomenon might also occur in inflammatory sites known to contain all the necessary agonists capable of causing cell death and probably also the release of membrane lipids possessing potent pharmacological properties.

However, not all the membrane-active agents tested were capable of synergizing with H_2O_2 and with a proteinase to release AA from EC. Cationic agents, which, together with H_2O_2, killed EC (Figures 1–3), failed to release any appreciable amounts of AA. Furthermore, cationic agents (PARG, chlorhexidine, CETAB) very markedly inhibited the release of AA from the injured cells induced by mixtures of oxidants with membrane-damaging agents and with a proteinase. Inhibition by cationic agents might be due to their ability to interact electrostatically with anionic sites (sialic acid, acid phospholipids) present on cell surfaces. They might also inactivate membrane-associated enzymes that partic-

ipate in lipid release (21). Other potent inhibitors of AA release from EC are quinacrine (an inhibitor of PLA_2), HClO, which might oxidize surface components, and tannic acid and tea extracts, which are known to denature proteins and which were also shown to inhibit lysis of RBC by PLC and SLS.

Taken together, our findings suggest that sublethal injuries induced in mammalian cells, either by oxidants that interfere with cell metabolism and inhibit repair of minor cellular damage (57–61) or by membrane-damaging agents (21), can be greatly amplified by mixtures of the two types of agonists. Cellular destruction might further be augmented if proteinases and other proinflammatory agents are also present. The detailed mechanisms by which oxidants, membrane-damaging agents, and proteinases injure and kill mammalian cells has recently been reviewed in detail (12, 21, 57–62), and this issue is beyond the scope of the present communication.

Our studies with the EC model in culture might open new avenues to elucidate the mechanisms of tissue destruction in vivo. The data in Figure 9 indicate that serum proteins might not hinder the synergistic effects of a mixture of agonists likely to be present in inflamed sites. Furthermore, adequate cocktails of antagonists might prove beneficial for the prevention of the synergistic killing and lipid-releasing capacities of leukocyte products. Such cocktails of antagonists might also prove beneficial in preventing tissue damage in ischemia and reperfusion (62) as well as in a variety of other inflammatory conditions and their sequelae.

Finally, current studies from our laboratories (submitted for publication) have also shown that EC exposed to subtoxic amounts of a variety of pesticides (lindane, parathion, malathion) were also killed in a synergistic manner by subtoxic mixtures of oxidants and membrane-damaging agents. These agents also released substantial amounts of AA. It is suggested, therefore, that the synergy phenomenon described in our communications (17, 18, 21) is probably universal.

REFERENCES

1. GINSBURG, I. 1979. The role of lysosomal factors of leukocytes in the biodegradation and storage of microbial constituents in infectious granulomas. *In* Lysosomes in Biology and Applied Therapeutics, Vol. 6. J. T. Jacques and H. Shaw, editors. North Holland Publishing Company, Amsterdam. 327–406.
2. HOLDER, I. (editor). 1985. Bacterial Enzymes and Virulence. CRC Press, Boca Raton, Florida.
3. GINSBURG, I. 1985. Streptococcal enzymes and virulence. *In* Bacterial Enzymes and Virulence. I. Holder, editor. CRC Press, Boca Raton, Florida. 121–144.
4. SPITZNAGEL, J. K. 1984. Non-oxidative antimicrobial reactions of leukocytes. *Contemp. Top. Immunobiol.* **14**:283–343.

5. BECKER, E. L. 1990. The cytotoxic action of neutrophils on mammalian cells in vitro. *Prog. Allergy* **40:**183–208.
6. ELSBACH, P., and J. WEISS. 1988. Phagocytic cells: Oxygen-independent antimicrobial systems. *In* Inflammation: Basic Principles and Clinical Correlates. J. I. Gallin, M. Goldstein, and R. Snyderman, editors. Raven Press, New York. 445–470.
7. HALLIWELL, B. 1987. Oxidants and human disease: Some new concepts. *FASEB J.* **1:**358–363.
8. KLEBANOFF, S. P. 1988. Phagocytic cells: Products of oxygen metabolism. *In* Inflammation: Basic Principles and Clinical Correlates. J. I. Gallin, M. Goldstein and R. Snyderman, editors. Raven Press, New York. 391–444.
9. FARBER, J. L., E. KYLE, and J. B. COLEMAN. 1990. Biology of disease; mechanism of cell injury by activated oxygen species. *Lab. Invest.* **62:**670–679.
10. VARANI, J., S. E. G. FLIGIEL, S. L. KUNKEL, U. RYAN, and P. WARD. 1985. Pulmonary endothelial cell killing by human neutrophils: Possible involvement of hydroxyl radical. *Lab. Invest.* **53:**656–663.
11. WARD, P. A., and J. VARANI. 1990. Mechanisms of neutrophil-mediated killing of endothelial cells. *J. Leuk. Biol.* **48:**97–102.
12. LICHTENSTEIN, A. 1989. Mechanism of neutrophil-mediated oxidative and nonoxidative tumor lysis. *In* New Horizons of Tumor Immunotherapy. M. Torisu and Y. Yoshida, editors. Elsevier Science Publishers, New York. 79–100.
13. SHIN, M. L., and D. F. CARNEY. 1988. Cytotoxic action and other metabolic consequences of terminal complement proteins. *Prog. Allergy* **40:**44–81.
14. GINSBURG, I. 1959. Action of streptococcal hemolysins and proteolytic enzymes on Ehrlich ascites tumor cells. *Br. J. Exp. Pathol.* **40:**417–423.
15. GINSBURG, I., and M. RAM. 1960. Action of antibodies and plasmins in Ehrlich ascites tumor cells. *Nature* **185:**328–330.
16. GINSBURG, I., and N. GROSSOWIZ. 1960. Action of streptococcal hemolysins on Ehrlich ascites tumor cells. *J. Pathol. Bacteriol.* **80:**111–119.
17. GINSBURG, I., D. F. GIBBS, L. SCHUGER, K. J. JOHNSON, U. S. RYAN, P. A. WARD, and J. VARANI. 1989. Vascular endothelial cell killing by combinations of membrane-active agents and hydrogen peroxide. *Free Radical. Biol. Med.* **7:**369–376.
18. VARANI, J., I. GINSBURG, L. SCHUGER, D. GIBBS, J. BROMBEREG, K. J. JOHNSON, U. S. RYAN, and P. A. WARD. 1989. Endothelial cell killing by neutrophils. Synergistic interaction of oxygen products and proteases. *Am. J. Pathol.* **135:**435–438.
19. GINSBURG, I., S. E. G. FLIGIEL, P. A. WARD, and J. VARANI. 1988. Lipoteichoic acid-anti-lipoteichoic acid complexes induce superoxide generation by human neutrophils. *Inflammation* **12:**525–548.
20. GINSBURG, I., P. A. WARD, and J. VARANI. 1989. Lysophosphatides enhance superoxide responses of stimulated human neutrophils. *Inflammation* **13:**163–174.
21. GINSBURG, I., R. MISGAV, A. PINSON, J. VARANI, P. A. WARD, and R. KOHEN. 1992. Synergism among oxidants, proteinases, phospholipases, microbial hemolysins cationic proteins and cytokines. *Inflammation* **16:**519–537.
22. JAFFE, E. A. 1984. Culture and identification of large vessel endothelial cells.. *In* Biology of Endothelial Cells. E. A. Jaffe, editor. Nijhoff. The Hague.
23. RYAN, U. S., and L. WHITE. 1986. Microvascular endothelium isolation with microcarriers: Arterial venous. *J. Tissue Cult. Methods* **10:**15–25.
24. GIBBS, D. F., J. VARANI, and I. GINSBURG. 1989. Formation and use of poly-L-histidine catalase complexes: Protection of cells from hydrogen peroxide-mediated injury. *Inflammation* **13:**465–474.
25. SPORN, P. S., M. PETER-GOLDEN, and R. H. SIMON. 1988. Hydrogen peroxide-induced arachidonic acid metabolism in the rat alveolar macrophage. *Am. Rev. Respir. Dis.* **137:**49–56.

26. THURMAN, R. G., H. G. LEYLAND, and R. SCHULTZ. 1972. Hepatic microsomal ethanol oxidation hydrogen peroxide formation and the role of catalase. *Eur. J. Biochem.* **25**:420–430.
27. GINSBURG, I., and T. N. HARRIS. 1965. Oxygen-stable hemolysins of group A streptococci. IV. Studies on the mechanisms of lysis by cell-bound hemolysin of red blood cells and Ehrlich ascites tumor cells. *J. Exp. Med.* **121**:647–656.
28. GINSBURG, I. 1987. Cationic polyelectrolytes: A new look at their possible roles as opsonins, as stimulators of respiratory burst in leukocytes, in bacteriolysis and as modulators of immune-complex diseases (a review hypothesis). *Inflammation* **11**:489–515.
29. VARANI, J., I. GINSBURG, D. F. GIBBS, P. S. MUKHPADHYAY, C. SULAVIK, K. J. JOHNSON, Y. M. WEINBERG, U. S. RYAN, and P. A. WARD. 1991. Hydrogen peroxide-induced cell and tissue injury: Protective effects of Mn^{2+}. *Inflammation* **15**:291–301.
30. DANNON, D., L. GOLDSDTEIN, Y. MARINOVSKI, and E. SKUTELSKY. 1972. Use of cationized ferritin as a label of negative charges on cell surfaces. *J. Ultrastruct. Res.* **38**:500–510.
31. DISHON, T., R. FINKEL, Z. MARCUS, and I. GINSBURG. 1967. Cell-sensitizing factors of group A streptococci. *Immunology* **13**:555–564.
32. NEEMAN, N., and I. GINSBURG. 1971. Red cell sensitizing agents of group A streptococci. II. Immunological and immunopathological properties. *Isr. J. Med. Sci.* **8**:1807–1816.
33. LOPATIN, D., and R. E. KESSLER. 1985. Pretreatment with lipoteichoic acid sensitizes target cells to antibody-dependent cellular cytotoxicity in the presence of anti-lipoteichoic antibodies. *Infect. Immun.* **48**:638–643.
34. LICHTENSTEIN, A., T. GANZ, M. E. SELSTED, and R. I. LEHRER. 1986. In vitro tumor cytolysis mediated by peptide defensins of human and rabbit granulocytes. *Blood* **68**:1407–1410.
35. LICHTENSTEIN, A., T. GANZ, T. M. HUGYEN, M. E. SELSTED, and R. I. LEHRER. 1988. Mechanism of target cytolysis by peptide defensins. *J. Immunol.* **140**:2686–2693.
36. ELFERINK, J. G. R., and M. DEIRKAUF. 1989. Permeabilization and calcium-dependent activation of rabbit polymorphonuclear leukocytes by poly-L-arginine. *Inflammation* **13**:285–294.
37. VARANI, J., S. E. G. FLIGIEL, G. O. TILL, R. G. KUNKEL, U. S. RYAN, and P. A. WARD. 1985. Pulmonary endothelial cell killing by human neutrophils: Possible involvement of hydroxyl radical. *Lab. Invest.* **53**:656–663.
38. VARANI, L., M. J. BENDELOW, D. E. SEALY, S. L. KUNKEL, D. E. GANNON, U. S. RYAN, and P. A. WARD. 1988. Tumor necrosis factor enhances susceptibility of vascular endothelial cells to neutrophil-mediated killing. *Lab. Invest.* **59**:292–295.
39. STEINBECK, A. M., and J. A. ROTH. 1989. Neutrophil activation by recombinant cytokines. *Rev. Infect. Dis.* **1**:549–556.
40. BAUD, L., H. AFFRES, J. PEREZ, and R. ARDIALLOU. 1990. Reduction in tumor necrosis factor and cytotoxicity by hydrogen peroxide. *J. Immunol.* **166**:556–560.
41. SLIVKA, A., A. F. LOBUGLIO, and S. J. A. WEISS. 1980. A potential role of hypochlorous acid in granulocyte-mediated tumor cell toxicity. *Blood* **55**:347–354.
42. GINSBURG, I., R. BORINSKI, M. SADOVNIC, Y. EILAM, and K. RAINSFORD. 1987. Poly-L-histidine, a potent stimulator of superoxide generation in human blood leukocytes. *Inflammation* **11**:253–277.
43. COHEN, H. J., C. M. E. LOVANIEC, and S. E. ELLIS. 1980. Chlorpromazin inhibition of granulocyte superoxide production. *Blood* **56**:23–33.
44. HALLET, M. B., and A. K. CAMPBELL. 1984. Is intracellular Ca^{2+} the trigger for oxygen radical production by polymorphonuclear leukocytes? *Cell Calcium* **5**:1–19.
45. KREOFSKY, T. J., J. A. RUSSEL, and M. S. RHORBACH. 1990. Inhibition of alveolar macrophages spreading and phagocytosis by cotton bract tannin: A potential mechanism in the pathogenesis of byssinosis. *Am. J. Pathol.* **127**:263–274.
46. JOHNSON, C. M., M. N. HANSON, and M. S. ROHRBACH. 1986. Endothelial cell cytotoxicity of cotton bract tannin and aqueous cotton bract extracts. *Environ. Health Perspect.* **66**:97–103.

47. VUK-PAVLOVIC, Z., and N. S. RHORBACH. 1990. Modulation of inflammatory cell function by cotton bract tannin changes in capacity of alveolar macrophages and neutrophils to produce hydrogen peroxide. *Am. J. Respir. Mol. Biol.* **3**:235–243.
48. GINSBURG, I., M. LAHAV, and P. GIESBRECHT. 1982. Effect of leukocyte hydrolases on bacteria. XVI. Activation of leukocyte factors and cationic substances on autolytic wall enzymes in *Staphylococcus aureus*: Modulation by anionic polyelectrolytes in relation to the survival of bacteria in inflammatory exudates. *Inflammation* **6**:401–417.
49. SCHRAUFSTATTER, I. U., P. A. HYSLOP, J. JACKSON, and C. C. COCHRANE. 1986. Mechanisms of oxidant injury of cells. *In* Leukocyte Migration and Its Sequelae. H. Movat, editor. Satellite Symposium, 6th International Congress on Immunology. 1150–1160.
50. TRENNIN, J. J. (editor). 1967. Cross-Reacting Antigens and Neoantigens (with Implications for Autoimmunity and Cancer Immunity). Williams and Wilkins, Baltimore.
51. KAPLAN, M. H. 1969. Auto-immunity to heart and its relation to heart diseases. *Prog. Allergy* **13**:408–429.
52. GINSBURG, I. 1972. Mechanisms of cell and tissue injury induced by group A streptococci: Relation to poststreptococcal sequelae. *J. Infect. Dis.* **126**:419–454.
53. GASENFELD, E., E. ROSENMANN, M. A. DAVIES, and A. LAUFER. 1966. Isoproteranol induced myocardial lesions in the immunized and non-immunized rat II. Histopathological lesions after specific immunization. *Immunology* **10**:193–198.
54. PRASAD, M. R., and D. K. DAS. 1989. Effect of oxygen-derived free radical and oxidation of the degradation in vitro of membrane phospholipids. *Free Radic. Res. Commun.* **7**:381–388.
55. MURTHY, S. N. S., C. G. COONEY, and H. R. CLEARFIELD. 1990. Hydrogen peroxide-induced alterations in prostaglandin secretion in the rat colon in vitro. *Inflammation* **14**:645–661.
56. DUANE, P. G., K. L. RICE, D. E. CHARBONEAU, M. B. KING, and D. P. GILBOE. 1991. Relationship of oxidant-mediated cytotoxicity to phospholipid metabolism in endothelial cells. *Am. J. Respir. Cell Mol. Biol.* **4**:408–416.
57. SPRAG, R. G. 1991. DNA strand break formation following exposure of bovine pulmonary artery and aortic endothelial cells to reactive oxygen products. *Am. J. Respir. Cell. Biol.* **4**:4–10.
58. MEHROTRA, S., P. MAKKAR, and P. N. VISWANATHAN. 1991. Mitochondrial damage by oxygen species in vitro. *Free Radic. Biol. Med.* **10**:277–283.
59. VLESSIS, A. A., P. MULLER, D. BARTOS, and D. TRUNKEY. 1991. Mechanisms of peroxide-induced cellular injury in cultured adult cardiac myocytes. *FASEB J.* **5**:2600–2605.
60. HALLECK, M. M., J. H. RICHBURG, and F. C. KAUFMANN. 1992. Reversible and irreversible oxidant injury to PC12 cells by hydrogen peroxide. *Free Radic. Biol. Med.* **12**:137–144.
61. GERA, J. F., and A. LICHTENSTEIN. 1991. Human neutrophil peptide defensins induce single strand DNA breaks in target cells. *Cell. Immunol.* **138**:108–120.
62. PIPER, H. M. (editor). 1990. Pathophysiology of Severe Ischemic Myocardial Injury. Kluwer Academic Publisher, Dordrecht.