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INTRODUCTION

Environmental oxidative stress and anti-oxidant defenses. A variety of distinct biochemical changes in lymphocytes and in various other target cells are induced by oxidants, including hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^\bullet). These changes include alterations in enzymatic activities, lipid peroxidation and damage to DNA. H_2O_2 rapidly permeates cells and would, in most cellular environments, have a lifetime that would permit it to diffuse appreciable distances before reaction. It is therefore proposed that H_2O_2 is the dominant oxidant leading to DNA strand breaks (1). Also, oxidizing reactive species on free-radical-damaged proteins, protein hydroperoxides that can consume glutathione, have been demonstrated. The long-lived nature of the reactive moieties indicates that they may be able to diffuse and transfer damaging reactions to distant cellular sites (2).

The principal oxidants in the lower atmosphere are ozone and two by-products of ozone photodissociation, the hydroxyl radical and hydrogen peroxide (3). Ozone is a very toxic air pollutant affecting organic molecules via free radical- and lipid peroxide-mediated mechanisms (4). T lymphocytes from subjects exposed to ozone *in vivo* exhibited significantly reduced mitogenic response for several weeks following the exposure (5,6). Since ozone is a highly reactive oxidant pollutant, it is unlikely that it interacts directly with blood lymphocytes. Rather, the lymphocytotoxic effect of ozone is probably mediated by soluble products of pulmonary cells that are affected directly by the oxidant (7). Pryor et al. (8) have demonstrated that the interaction of ozone in the presence of water with unsaturated fatty acids, ozone's primary target in lung lining fluids, produces aldehydes and hydrogen peroxide. When bronchoalveolar lavage (BAL) was used, the yield of hydrogen peroxide production was 55%. Based upon those experiments with ozone at 2.8 ppm (nearing smog levels) the interaction with BAL would yield approximately 8.25 nmoles/ml H_2O_2 over two hours. The polyamine oxidase system we propose to study involves oxidative stress exerted on lymphocytes by enzymatic products, which include hydrogen peroxide and an aldehyde, at about 5 nmoles/ml H_2O_2 over forty eight hours. This exposure approximates the daily average of urban ozone profile including night time - 0.1 ppm (9). Therefore, we propose that our system can serve as a model for environmental oxidant exposure.

Ionizing radiation can be used as a means of introducing oxygenating radicals into lymphocytes in a geometrically and temporally precise way. The absorption of radiation involves splitting H_2O molecules (the most common constituent of cells) into OH^\bullet and H^\bullet radicals which are initially distributed in proportion to the radiation dose distribution (10). OH^\bullet radicals generated within a cell would generally react immediately with very little diffusion into the surrounding medium. In addition irradiation of dissolved O_2 will produce the superoxide radical, HO_2^\bullet , also following the radiation dose distribution. The superoxide radical has intermediate reactivity between that of OH^\bullet and H_2O_2 and will diffuse moderate distances before reacting. In comparison, H_2O_2 is less active and may diffuse many cell diameters before interacting with cellular macromolecules. Accelerated electrons are quite easy to control and precise levels of oxygen stress can be generated by irradiating cells and the surrounding medium. A radiation dose of 1.0 Gy will generate 2.72×10^{-8} mol/l of OH^\bullet , 0.68×10^{-8} mol/l of H_2O_2 and 0.008×10^{-8} mol/l of HO_2^\bullet in water, and similar values are expected initially in the water component of cells growing in nutrient medium. The use of ionizing radiation to administer oxidative stress enhances the control of the concentration and timing of the exposure and provides a different type of stress (mainly mediated by OH^\bullet) than that produced by enzymatically-generated H_2O_2 . Ionizing radiation is a common stressor found in the general environment, and results of these experiments will provide information on the efficacy of several anti-oxidant strategies on environmentally relevant levels of oxidative stress (11).

All respiring cells produce H_2O_2 and have various intracellular anti-oxidant mechanisms. Most of the H_2O_2 is reduced to H_2O by two enzymes: catalase and selenium-dependent glutathione peroxidase (12). In addition, intra- and extracellular organic molecules are protected from oxidation by various anti-oxidants that can also be used pharmacologically and nutritionally to control oxidative damage. Oxidants present in the gas phase of cigarette smoke cause lipid and protein peroxidation. N-acetylcysteine (NAC), that can scavenge several oxidant species including H_2O_2 (13), increased (when given orally) glutathione plasma levels and was therefore suggested to have beneficial potential in smokers (14). Vitamins C and glutathione appear to be important in protecting plasma lipids and proteins against oxidant stress, respectively (15,16).

Radioprotectors are substances which, if present during irradiation, reduce the effect of the radiation and also diminish the cellular free radical levels. Most radioprotectors are excellent donors of H^\bullet and include a number of proteins and peptides with thiol groups (RSH). Glutathione is such a compound that occurs naturally in most mammalian cells and can be added exogenously to provide an extra degree of radioprotection to that provided endogenously.

Hypothesis

The effects of oxidative stress on human lymphocytes are modulated by endogenous mechanisms. We expect some endogenous anti-oxidant mechanisms to be induced by oxidative stress.

Technical objectives

To study three models of inducing oxidative stress in lymphocytes: a) PAO activity generating extracellularly low levels of H_2O_2 for two days (mimicking exposures to environmental chemical toxicants); b) electron irradiation generating both extra- and intracellularly mainly OH^\bullet ; and c) high levels of reagent H_2O_2 generating short but acute stress.

The following question was asked during the second year of work:

To what extent, if at all, are levels of endogenous antioxidant molecules (catalase, glutathione peroxidase, glutathione, vitamins E and C) altered in human T lymphocytes subjected to oxidative stress?

BODY

Methods

T and B cells from the peripheral blood of healthy donors were studied. Cells were incubated for all assays in a serum-free medium, because fetal calf serum contains PAO activity(17). Therefore, RPMI-1640 with Nutridoma-HU supplement (Boehringer Mannheim Biochemicals, Indianapolis, IN) was used.

Lymphocyte preparation. Heparinized peripheral blood from healthy donors was used as a source of lymphocytes. Cells were purified by Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient centrifugation. The resultant mononuclear cell preparation was allowed to adhere to plastic dishes to remove macrophages and other adherent cells. Non-adherent mononuclear cells were then used either as a source of T or B cells.

For T cells, non-adherent mononuclear cells were mixed with a suspension of neuroaminidase-treated sheep erythrocytes and incubated at $37^\circ C$ for 15 min, followed by centrifugation and further incubation at $4^\circ C$ for 45 min. Thereafter, the rosetted cells were obtained by centrifugation through Ficoll-Hypaque. The erythrocytes in the cell pellet were lysed by exposure to 0.83% NH_4Cl . The rosetted cells contained more than 98% $CD3^+$ T cells, and 0.4-1% $M3^+$ monocytes as determined by flow cytometry.

For B cells, non-adherent mononuclear cells were mixed with magnetic beads carrying anti-B cell antibodies on their surface and incubated at $4^\circ C$ for 15 minutes. Dynabeads M-450 with anti-CD19 antibodies (Dynal, Lake success, NY) were used at ten million beads/ml. The rosetted $CD19^+$ B cells were isolated by magnetic force and the beads were detached using reagents and equipment from Dynal. The resultant isolated B cells were unstimulated and 99% pure, as determined by flow cytometry.

Oxidative stress. These modes were used:

a) A longitudinal low level extracellular stress (mimicking exposures to environmental chemical toxicants) - lymphocytes were preincubated for 2 days with a commercial preparation of PAO (Sigma) at 5×10^{-4} U/ml and spermidine at 5 μM . This exposure generates gradually 5 μM H_2O_2 over two days²⁵.

b) Electron radiation generating both extra- and intracellularly mainly OH^\bullet .- lymphocytes were exposed to a radiation dose of 6 Gy for 5 minutes. This dose produces nonlethal cellular responses(11) and generates oxidants per time unit at about 20 fold higher levels than mode a), but for a much shorter period of time. We used a 2.5 MeV Van de Graaff accelerator that is capable of generating electrons or

protons to a maximum energy of 2.5 MeV. Doses were continuously monitored by means of parallel plate ionization chambers coupled with a stable, vibrating reed, electrometer.

c) A short high level extracellular stress-reagent H_2O_2 was added directly at 20, 50, 100 and 200 μM for 2 hours. We have found that these levels suppress IL-2 production in human blood lymphocytes by 8, 17, 67 and 88%, respectively, without affecting cell viability(18).

The suppressive effect was studied in the preincubation mode in order to exclude any possible effects on the assay used. All reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated.

Measurements of catalase activity. Ten million cells were lysed by sonication (two 10 seconds pulses with a 10 second interval) in 0.5 ml PBS. The resultant sonicate was centrifuged at 14,000xg for 10 minutes at 4°C . Catalase activity was measured in the supernatant. Fifty μL of the supernatant were mixed with 600 μL of 15 mM H_2O_2 in a cuvette. The kinetics of the decrease in light absorbance at 240 nm (H_2O_2 decomposition) were determined for 3 minutes in a DU 640 spectrophotometer (Beckman, Fullerton, CA). A cuvette containing only PBS served as blank. A cuvette without a sample was used to ensure that H_2O_2 does not decompose spontaneously under our experimental conditions. Enzymatic activity was expressed as the rate constant of a first-order reaction (k) divided by the protein concentration. A_1 and A_2 refer to the absorbance before and after a given time interval of measurement (t), respectively. $k=(2.3/t)(\log A_1/A_2)(\text{sec}^{-1}\cdot\text{mg protein}^{-1})$ (19,20).

Glutathione peroxidase assay. Ten million cells were lysed by sonication (two 10 seconds pulses with a 10 second interval) in 0.5 ml PBS. The resultant sonicate was centrifuged at 14,000xg for 10 minutes at 4°C . Two hundred mL of cell lysate were added to an equal volume of double-strength Drabkin's reagent (1.6 mM KCN, 1.2 mM $\text{K}_3\text{Fe}(\text{CN})_6$, 23.8 mM NaHCO_3) and the mixture was agitated for several seconds. Aliquots were added to 2.66 ml 50 mM sodium phosphate/5 mM EDTA buffer, pH 7.5. A one to one dilution of Drabkin's reagent in water was used as a blank. The following were added sequentially: 0.1 ml 8.5 mM NADPH, 1 unit of glutathione reductase (type III, Sigma), 10 μL 1.125 M NaN_3 , 0.1 ml 0.15 M glutathione, and 0.1 ml 2.2 mM H_2O_2 . The changes in absorbance at 340 nm were determined over 5 minutes. Activity was expressed as nmol of H_2O_2 converted/mg protein/min (21).

Reduced Glutathione assay. Glutathione was assayed with the Bioxytech GSH-400 kit by Oxis International (Portland, OR). This kit determines glutathione specifically. Thirty five million cells were lysed in 0.5 ml of ice-cold 5% metaphosphoric acid. The lysate was centrifuged at 1000xg for 10 minutes at 4°C . Three hundred mL of the supernatant were mixed with 0.6 ml of the following: 200 mM potassium phosphate, pH 7.8, containing 0.2 mM diethylenetriaminepentaacetic acid (DTPA) and 0.025% lubrol. Fifty μL of 12 mM 4-chloro-1-methyl-7-trifluoromethyl-quinolinium methylsulfate in 0.2N HCl were added to the above mixture and the tube was vortexed. Fifty μL of 30% NaOH were added and the mixture was vortexed. Samples were incubated at room temperature for 10 minutes in the dark and absorbance was read at 400 nm. A standard curve was established with reduced glutathione at 0-100 μM .

Vitamin C assay. Fifty million cells were lysed in 1.2 ml 3% perchloric acid. The lysate was vortexed and centrifuged at 20,000xg for 20 minutes at 4°C . The concentration of Vitamin C in the supernatant was determined by high pressure liquid chromatography at Lab Corp., Special Chemistry Division, Burlington, NC.

Vitamin E assay. Fifty million cells were resuspended in 830 μL PBS. The cell suspension was mixed with 170 μL of 25% ascorbic acid. The sample was heated for 5 minutes in a 70°C water bath. One hundred and seventy μL of 10N KOH were added and the mixture was mixed and heated for 30 min. at 70°C . The sample was cooled in an ice bath. Four ml of hexane were added, the mixture was shaken

vigorously for 2 min and centrifuged at 1500 rpm for 5 minutes at 4°C. The upper layer(hexane phase)was separated and fluorescence was determined at excitation 298nm, emission 328nm. Vitamin E levels were calculated with a standard curve of 0-10 µg/ml hexane (22).

Statistical analysis of data. Data were analyzed, where appropriate, using student's T test.

Results

In our Statement Of Work for the second year we propose to answer the following question: To what extent, if at all, are levels of endogenous antioxidant molecules (catalase, glutathione peroxidase, glutathione, vitamins E and C) altered in human T lymphocytes subjected to oxidative stress?

To answer this question we established in our laboratory the appropriate assays and measured the five parameters in human peripheral blood lymphocytes subjected to different types of oxidative stress.

Catalase activity

Every oxidative stress exposure except for hydrogen peroxide at 20 µM, induced a significant rise in the cellular catalase activity of T lymphocytes above the basal level (Figure 1, Appendix A). These results suggest that hydrogen peroxide at 20 µM was the only exposure that induced a detectable rise in cellular peroxides (as reported last year by us) because it was also the only exposure that did not induce the anti-oxidant enzyme catalase. No catalase activity was detected in B lymphocytes probably due to the low numbers of cells available from the peripheral blood.

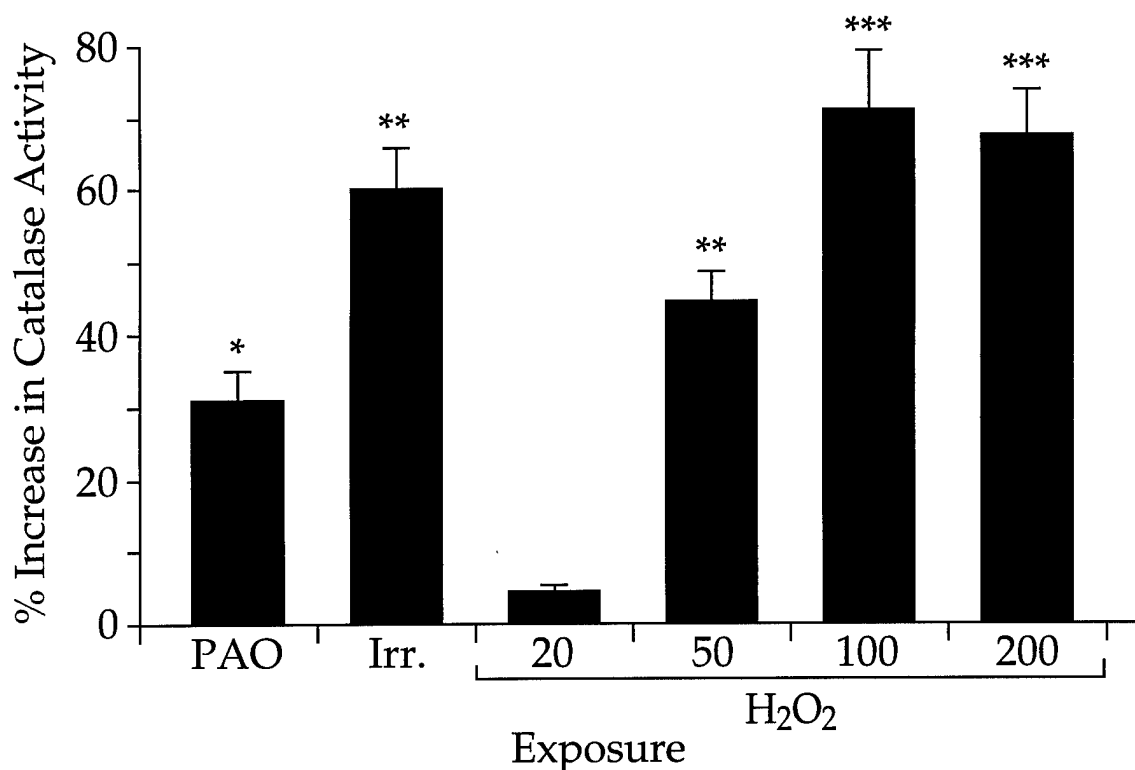


Figure 1.

Enhancement of catalase activity by oxidative stress. T cells were exposed to polyamine oxidase (PAO, for 2 days at 5×10^{-4} U/ml + spermidine at 5 μ M, and then washed and incubated for 2 hours in fresh medium), or irradiation (Irr., for 5 minutes at 6 Gy and then incubated for 2 hours in fresh medium), or hydrogen peroxide (H₂O₂ at the indicated concentration, μ M, for 2 hours and then washed and incubated for 2 hours in fresh medium). Cells were lysed and cytosolic catalase activity was measured by following kinetically the decomposition of hydrogen peroxide in a spectrophotometer, and calculated per protein concentration. Catalase activity in untreated cells was 1.2 ± 0.09 (sec⁻¹·mg protein⁻¹). Results shown are the increase in catalase activity in stressed cells over untreated cells \pm SD. The increase in catalase activity was significant at $p < 0.01^*$, 0.005^{**} , 0.0005^{***} .

Glutathione peroxidase activity

The basal levels of glutathione peroxidase in T cells were 20.1 ± 2.5 nmoles/mg protein/minute whereas the levels in B cells were approximately 4 times higher - 85.4 ± 9.7 nmoles/mg protein/minute. None of the oxidative stresses affected glutathione peroxidase activities in either the T or the B lymphocytes (Table 1). These results suggest that oxidative stress has a differential effect on catalase (enhancement) versus glutathione peroxidase (no change) activities in exposed lymphocytes.

Table 1**Glutathione peroxidase activities in lymphocytes exposed to oxidative stress**

<u>Oxidative stress</u>	<u>T cells</u>	<u>B cells</u>
None	20.1±2.5	85.4±9.7
H ₂ O ₂ 20µM	18.9±2.1	87.6±12.0
H ₂ O ₂ 50µM	17.9±4.2	79.6±10.4
H ₂ O ₂ 100µM	19.4±3.0	88.6±7.9
H ₂ O ₂ 200µM	22.8±2.9	75.9±8.4
Irradiation	18.7±2.8	80.4±6.9
Polyamine oxidase	23.8±2.7	89.7±11.8

Lymphocytes were purified, exposed and assayed as mentioned in the Methods section. Glutathione peroxidase activity is expressed as nmoles/mg protein/minute±SD, n=3.

Reduced glutathione (GSH) levels

A standard of glutathione yielded an excellent calibration curve using the Bioxytech GSH-400 kit (figure 2). However, even when 35 million peripheral blood lymphocytes were used per sample, the basal levels of reduced glutathione were 10.5±0.9 µM at optical densities of approximately 0.03. Therefore, detecting a decrease in reduced glutathione concentrations upon exposure to oxidative stress became impossible, even when the more sensitive Tietze method was employed. Consequently, it would be appropriate to assess the effect of N-acetylcysteine (which increases glutathione levels in cells) on cells exposed to oxidative stress, as originally proposed for the third year of this project, as an alternative approach towards testing the role of glutathione in the defense of lymphocytes against oxidative stress.

Reduced glutathione calibration curve

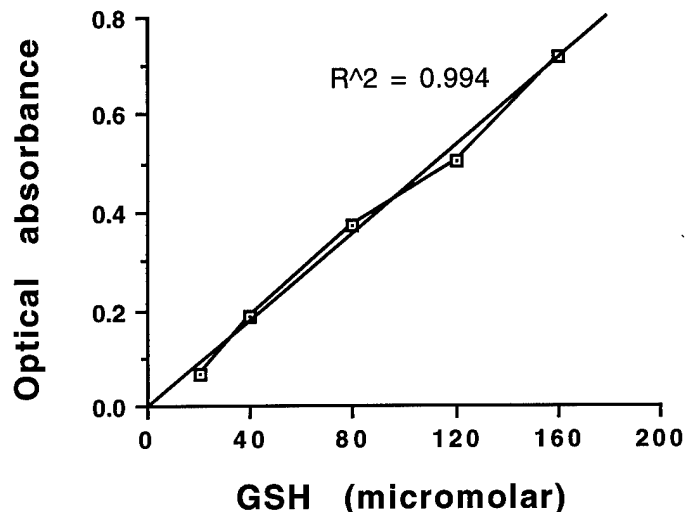


Figure 2.

Vitamin C concentrations

The Basal levels of vitamin C in peripheral blood lymphocytes were so low that we had to perform the assay for free in an outside site (Lab Corp., Burlington, NC) that specializes in anti-oxidant measurements, using very sensitive high pressure liquid chromatography methods. Still, 30-50 million cells per sample were required to detect measureable levels of vitamin C. No consumption of vitamin C was noted in cells exposed to various types of oxidative stress (table 2). These results do not support a role for exogenous vitamin C as a protective agent against oxidative stress in human peripheral blood lymphocytes.

Table 2

Vitamin C concentrations in human lymphocytes

<u>Oxidative stress</u>	<u>Batch 1 (50 million cells/sample)</u>	<u>Batch 2 (35 million cells/sample)</u>
None	0.9	0.4
H ₂ O ₂ 200μM	0.8	
Polyamine oxidase		0.5
Irradiation		0.4

T lymphocytes were purified, exposed and assayed as described in the Methods section. Levels of vitamin C are presented as mg/dL.

Vitamin E levels

As for vitamin C, the levels of vitamin E were so low that each sample consisted of approximately 60 million cells. We were able to construct a reliable standard curve (figure 3). Based on this curve we did not detect any depletion of vitamin E from cells exposed to the various types of oxidative stress employed. The basal levels of Vitamin E were $0.6 \pm 0.05 \mu\text{g}/\text{ten million cells}$. These results do not support a role for vitamin E in protecting human peripheral blood lymphocytes against oxidative stress.

Vitamin E calibration curve

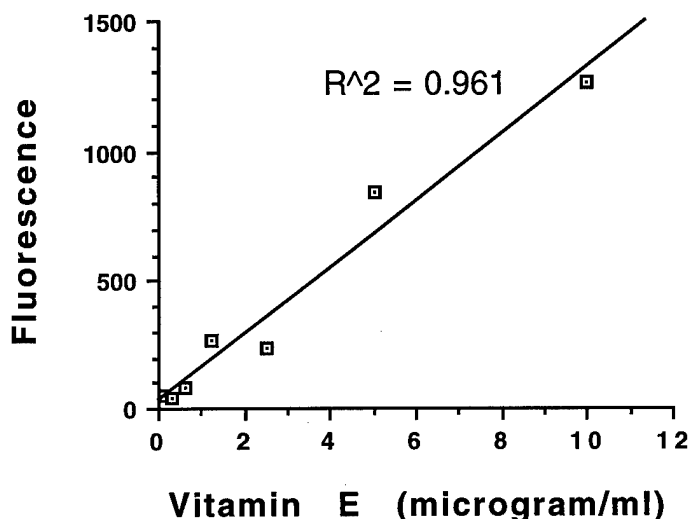


Figure 3.

Studies benefiting from the infra-structure supported by this grant

Up to here, we described the results of research that was directly funded by this grant. The infrastructure created in our laboratory (including the purification of blood cells, maintaining of an epithelial cell line and establishment of the electrophoretic mobility shift assay) allowed the graduate students of the principal investigator to perform their separate research activities. Although these activities were in no way directly supported by this grant, it is appropriate to recognize the fact that the students' research (which is ancillary to the grant) benefited indirectly from the grant. Describing these results is further justified by the general areas of the students' projects: the signal transduction cascade initiated in lung cells by the oxidant ozone; and cellular stress in transformed lymphocytes.

Ozone is a major environmental oxidant which is generated by UV radiation and high-voltage sparks. The oxygen radicals it generates are similar to those encountered by tissues during reperfusion. The lung epithelial cells respond directly to ozone. Consequently, a signaling cascade is initiated in which oxidative stress and various protein kinases transmit the signal from the plasma membrane to the nucleus. There, several transcription factors enhance the expression of a set of genes including IL-8 (Appendices B,C,D).

Apoptosis is a form of active cell death which occurs in response to severe cellular stresses including oxidative stress. There are instances where clinicians are interested in inducing this "suicide" path in cells. A prominent example is the death of cancer cells. Many anti-cancer drugs kill their target cells by inducing apoptosis. Unfortunately, cancer cells have developed an array of mechanisms to avoid death, including the activity of an efflux pump (P-glycoprotein=P-gp) that removes anti-cancer drugs from intra-cellular compartments. We have formerly found that aspirin, at plasma attainable levels, induced the expression of P-gp mRNA, protein and function, in a human T lymphoma cell line. We now report that aspirin protected lymphoma cells against induction of apoptotic death by the anti-cancer drugs adriamycin and taxol (Appendix E). These results raise the possibility that the use of aspirin may be contra-indicated during or shortly before cancer chemotherapy sessions.

Discussion

Three modes of oxidative stress: hydrogen peroxide, polyamine oxidase activity generating hydrogen peroxide gradually, and irradiation, enhanced catalase activity in human peripheral blood T lymphocytes. Glutathione peroxidase activity and reduced glutathione, vitamin C and vitamin E levels, were not modulated by oxidative stress. Only exposure to hydrogen peroxide at 20 μ M did not enhance catalase activity in T lymphocytes.

Direct measurements of lipid hydroperoxides performed in the former year of this project did not detect increased levels following oxidative stress, except for exposure of T lymphocytes to the lowest concentration of hydrogen peroxide (20 μ M). We proposed a possible explanation to these findings suggesting that the direct biochemical damage by this low stress was not enough to induce endogenous anti-oxidant mechanisms and therefore allowed for the detection of lipid peroxidation. Our current results strongly support our proposal, i.e., catalase activity was enhanced by each type of oxidative stress except for the weakest stress which in turn did cause detectable lipid peroxidation. Therefore, lipid peroxidation appears to be prevented, or at least reversed, by catalase activity. Interestingly, glutathione peroxidase which is another major hydrogen peroxide scavenger did not show enhanced activity upon exposure to oxidative stress. The reasons for this are unclear and may be related to the specific biology of peripheral blood lymphocytes.

We assessed the modulation of the endogenous levels of two anti-oxidants (vitamins C and E) by oxidative stress. It was assumed that if these anti-oxidants play an important role in the defense of the cells against oxidative stress, their levels should decrease upon exposure to oxidants. This did not turn out to be the case. Again, it seems that catalase is the most important endogenous anti-oxidant agent from among the four we measured. The fifth, reduced glutathione, occurred at levels that were below our detection ability in peripheral blood lymphocytes.

CONCLUSIONS

Assessing the effects of oxidative stress on endogenous anti-oxidant molecules revealed dramatic differences between different anti-oxidants. It seems that only catalase activity is modulated by oxidative stress, putatively being recruited to protect against the stress. On the other hand, two classical anti-oxidants (vitamins C and E) are not consumed during the stress period. This questions the importance of these agents as supplements aimed at providing protection against oxidative stress in lymphocytes.

Military occupational settings include the exposure of soldiers to smoke and combustion products which contain very high concentrations of oxidants including peroxides and peroxy radicals. Also, oxidative damage may occur secondary to biological or chemical weapon exposure. Serious trauma is another instance where the resultant ischaemia and reperfusion can cause oxidative stress and tissue antioxidant depletion.

The vast majority of studies into human lymphocyte biology and biochemistry were carried out using transformed cell lines. We, however, use ex vivo peripheral blood cells which are a physiological target of oxidants and therefore are the most appropriate model to identify defense mechanisms against toxic exposures, and the only system that allows follow-up of exposed individuals.

The results and conclusions of this project's second year provide the following two benefits:

1. Enhanced catalase activity may be developed as a marker for the ability of cells from exposed soldiers to mount an anti-oxidative response.
2. Since vitamins C and E were apparently not part of the anti-oxidative defense arsenal of the

lymphocyte, other compounds need to be identified that can protect cells (and presumably also exposed individuals) against oxidative stress. This is planned for the third year of this grant.

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**OXIDATIVE STRESS SUPPRESSES TRANSCRIPTION FACTOR
ACTIVITIES IN STIMULATED LYMPHOCYTES**

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SUMMARY

Effects of oxidative stress on stimulation-dependent signal transduction leading to interleukin-2 expression, were studied. Purified quiescent human blood T lymphocytes were subjected to: a. Acute exposure to hydrogen peroxide, b. Chronic exposure to hydrogen peroxide, and c. Acute exposure to ionizing radiation. The cells were then stimulated for 6 hours. DNA-binding activities (determined by the electrophoretic mobility shift assay) of 3 transcription factors: NF κ B, AP-1 and NFAT, were abolished in the lymphocytes by all 3 modes of oxidative stress. The lymphocytes exhibited lipid peroxidation only upon exposure to the lowest level of hydrogen peroxide used (20 μ M). All 3 modes of oxidative stress induced catalase activity in the lymphocytes. The only exception was hydrogen peroxide at 20 μ M which did not induce catalase activity.

Conclusions: 1. Suppression of specific transcription factor functions can potentially serve as a marker of exposure to oxidative stress and its effects on human lymphocytes; 2. Lipid peroxidation is only detectable in human lymphocytes upon exposure to weak oxidative stress which does not induce catalase activity; 3. Therefore, transcription factor DNA-binding activities are more sensitive to oxidative stress than lipid peroxidation.

INTRODUCTION

T lymphocytes are activated following the binding of a ligand to the antigen receptor complex. One of the early manifestations of this interaction is the transcriptional activation of the Interleukin 2 (IL-2) gene [1]. IL-2 is a pivotal lymphokine involved in B and T lymphocyte, as well as natural killer cell regulation (1). The modulation of IL-2 transcription by nuclear proteins can serve as a general readout that would be affected by any abnormality occurring earlier in the activation pathway. A transcriptional enhancer in the promoter region of the IL-2 gene responds to signals generated after activation through the T cell antigen receptor [2]. A number of positive regulatory elements have been identified in this region, including: NFAT, AP-1, NFkB, EGR-1, AP-3, Oct-1, and Sp1 [3-5]. Transcription factors binding to the first three, which are expressed in primary human T cells only upon stimulation, are plausible targets for suppression of T cell activation.

A variety of distinct biochemical changes in lymphocytes and in various other target cells are induced by the oxidants hydrogen peroxide and hydroxyl radical. These changes include alterations in enzymatic activities, lipid peroxidation and damage to DNA. Ionizing radiation can be used as a means of introducing oxygenating radicals into lymphocytes in a geometrically and temporally precise way. The absorption of radiation involves splitting H_2O molecules (the most common constituent of cells) into hydroxyl radicals and H^\bullet radicals which are initially distributed in proportion to the radiation dose distribution [6]. In addition, irradiation of dissolved O_2 will produce the superoxide radical, HO_2^\bullet , also following the radiation dose distribution. The superoxide radical has intermediate reactivity between that of strongly reactive hydroxyl radicals and relatively weakly reactive hydrogen peroxide.

We have previously described a new mechanism of IL-2 down-regulation [7]. Endogenous hydrogen peroxide produced by monocytes and endogenously

produced or exogenously added polyamines provide down-regulatory signals for IL-2 production by human peripheral blood T cells. The interaction between polyamine oxidase (PAO, EC 1.4.3.4, monoamine oxidase) and the polyamine spermidine generates products (including hydrogen peroxide) over two days that provide chronic low-level oxidative stress, suppressing IL-2 production. Furthermore, we found that PAO activity suppressed protein tyrosine phosphorylation, calcium mobilization and nuclear DNA-binding activities [8].

The objective of this study was to compare the effects of three modes of inducing oxidative stress in human lymphocytes on stimulation-induced transcription factors, in order to facilitate the development of functional markers for the exposure to, and effect of, oxidative stress in man. Oxidative stress was exerted on unstimulated cells since the vast majority of peripheral blood lymphocytes are in a quiescent state. The modes studied were: a) high levels of reagent hydrogen peroxide generating short but acute stress; b) PAO activity generating extracellularly low levels of hydrogen peroxide for two days; and c) electron irradiation generating both extra- and intracellularly mainly hydroxyl radicals.

MATERIALS AND METHODS

Cells

T cells from the peripheral blood of healthy donors were studied. Cells were incubated for all assays in a serum-free medium, because fetal calf serum contains PAO activity [7]. Therefore, RPMI-1640 with Nutridoma-HU supplement (Boehringer Mannheim Biochemicals, Indianapolis, IN) was used.

Lymphocyte preparation. Cells were purified by Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient centrifugation. The resultant mononuclear cell preparation was allowed to adhere to plastic dishes to remove macrophages and other adherent cells.

Non-adherent mononuclear cells were mixed with a suspension of neuroaminidase-treated sheep erythrocytes and incubated at 37°C for 15 min, followed by centrifugation and further incubation at 4°C for 45 min. Thereafter, the rosetted cells were obtained by centrifugation through Ficoll-Hypaque. The erythrocytes in the cell pellet were lysed by exposure to 0.83% NH_4Cl . The rosetted cells contained more than 98% CD3^+ T cells, and 0.4-1% M3^+ monocytes as determined by flow cytometry.

Oxidative stress

These modes were used:

- a) A short high level extracellular stress - reagent hydrogen peroxide was added directly at 20, 50, 100 and 200 μM for 2 hours. We have found that these levels suppress IL-2 production in human blood lymphocytes without affecting cell viability [9].

b) A longitudinal low level extracellular stress - lymphocytes were preincubated for 2 days with a commercial preparation of PAO (Sigma, a monoamine oxidase which oxidizes polyamines at a 2.5-fold higher rate than benzylamine, 7) at 5×10^{-4} U/ml and spermidine at 5 μ M. This exposure generates gradually 5 μ M hydrogen peroxide over two days and suppresses IL-2 production in response to mitogenic stimulation [7].

c) Electron radiation generating both extra- and intracellularly mainly hydroxyl radicals - lymphocytes were exposed to a radiation dose of 6 Gy for 5 minutes. This dose produces nonlethal cellular responses [10] and generates oxidants per time unit at about 20 fold higher levels than mode b), but for a much shorter period of time. We used a 2.5 MeV Van de Graaff accelerator that generated electrons to a maximum energy of 1.8 MeV. The cells were exposed in suspension to high energy x-rays generated by stopping the electron beam in a tantalum plate. Doses were continuously monitored by means of parallel plate ionization chambers coupled with a stable, vibrating reed, electrometer.

Oxidative stress was exerted and the cells were washed and rested for 2 hours before stimulation in order to exclude any possible effects on the assay used.

Measurements of transcription factor activities

For T cell stimulation we used phytohemagglutinin (PHA) (1 μ g/ml) + tetradecanoyl phorbol acetate (TPA) (5 ng/ml), for 6 h at 37°C, 5% CO₂, prior to collecting the cells for nuclear extraction.

DNA-binding determination by the electrophoretic mobility shift assay (EMSA)

Preparation of nuclear extracts. Cells were washed and nuclear extracts were prepared according to a modification of Schreiber et al. [11]. This method is suitable for small numbers of cells and therefore appropriate (based on our experience; 8,12) for studies of peripheral blood lymphocytes. Cells were washed and resuspended in

Tris buffered saline, transferred to an Eppendorf tube and repelleted. The cell pellet was resuspended in a buffer containing: 10 mM Hepes, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, 5 µg/ml Aprotinin, 5 µg/ml Antipain, 100 µM Benzamidine, 5 µg/ml Leupeptin, 5 µg/ml Pepstatin, 5 µg/ml soybean trypsin chymotrypsin inhibitor, pH 7.9. The cells were allowed to swell on ice for 15 min and NP-40 at 0.625% was added. The tube was vortexed for 10 seconds and centrifuged for 30 seconds in a microfuge. The nuclear pellet was resuspended in a buffer containing 20 mM Hepes, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and the 7 aforementioned protease inhibitors, pH 7.9. The tube was vigorously rocked on wet ice for 15 minutes on a shaking platform, and the nuclear extract was centrifuged for 5 minutes to remove insoluble nuclear matrix. The protein concentration of the supernatant was determined (Bradford method, Bio-Rad Protein Assay Kit). Aliquots were stored at -70°C.

DNA-protein interactions. DNA probes containing the binding sites from the IL-2 promoter region [3] were purchased from Genosys (The Woodlands, TX). The probe for NFAT-1 spans between nucleotides -255 and -285:

5'-GGAGGAAAACTGTTTCATACAGAAGGCGTT-3'

The probe for AP-1 spans between nucleotides -140 and -156:

5'-TTCAAAGAGTCATCAG-3'

The probe for NFκB spans between nucleotides -190 and -214:

5'-TAACAAACAGGGATTTCACCTACAT-3'

The probes were labeled with ³²P-ATP using T4 polynucleotide kinase (Promega). For the binding assay, 10,000 cpm DNA probe (~ 0.2 ng) were combined with 2 µg poly(dI-dC) (a nonspecific competitor DNA), 3 µg BSA (a protein carrier) and 10 µg nuclear extract in a final reaction volume of 20 µl. The binding reaction mixture was incubated for 15 min in a 30°C water bath. The protein-DNA complexes were detected on a 4% low-ionic-strength native polyacrylamide gel. The gel was dried under vacuum and autoradiographed.

Measurements of lipid peroxidation

Quantitative peroxide assay. A lipid compatible formulation of the PeroXOquant Quantitative Peroxide Assay (Pierce Chemical Co., Rockford, IL) was used. This assay is adapted to measure cellular hydroperoxides. To differentiate between hydrogen peroxide and peroxides of cellular molecules (such as lipid peroxides) we followed the recommendations of the manufacturer and regarded any catalase (7000U/ml)-inhibitable measurement as representing hydrogen peroxide. In the assay, peroxides convert Fe^{2+} to Fe^{3+} in a sulfuric acid solution. The Fe^{3+} complexes with the xylenol orange dye to yield a purple product with absorbance at 540-600 nm. The molar extinction coefficient of the xylenol orange- Fe^{3+} complex is $1.5 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ in 25 mM H_2SO_4 at room temperature. Five million cells were lysed by sonication (two 10 seconds pulses with a 10 seconds interval) and incubated for 15-20 minutes at room temperature in the following working solution (10 times the volume of the sonicate): 0.25mM ammonium ferrous (II) sulfate, 25mM H_2SO_4 , 4mM BHT, 125 μM xylenol orange in methanol. Results were read at 595 nm in a microtiter plate reader. For calibration and validation, a series of hydrogen peroxide solutions at concentrations between 1 μM to 1mM were prepared and assayed. Results were calculated per protein concentrations as determined by the Bradford method. Since the peroxide assay allows to measure peroxides without lipid extraction, a blank without ammonium ferrous (II) sulfate and H_2SO_4 was used to subtract endogenous iron (and other transition metals) readings [13].

Measurements of catalase activity

Ten million cells were lysed by sonication (two 10 seconds pulses with a 10 second interval) in 0.5 ml PBS. The resultant sonicate was centrifuged at 14,000 $\times g$ for 10 minutes at 4°C. Catalase activity was measured in the supernatant. Fifty μL of the

supernatant were mixed with 600 μ L of 15 mM H_2O_2 in a cuvette. The kinetics of the decrease in light absorbance at 240 nm (H_2O_2 decomposition) were determined for 3 minutes in a DU 640 spectrophotometer (Beckman, Fullerton, CA). A cuvette containing only PBS served as blank. A cuvette without a sample was used to ensure that H_2O_2 does not decompose spontaneously under our experimental conditions. Enzymatic activity was expressed as the rate constant of a first-order reaction (k) divided by the protein concentration. A_1 and A_2 refer to the absorbance before and after a given time interval of measurement (t), respectively. $k=(2.3/t)(\log A_1/A_2)$ ($\text{sec}^{-1}\cdot\text{mg protein}^{-1}$) [14,15].

Reagents

All reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated.

Statistical analysis of data

Data were analyzed, where appropriate, using student's T test.

RESULTS

Transcription factor DNA-binding studies

Since this study aims to develop markers of oxidative stress-induced suppression of cellular function, we studied the ability of DNA sequences from the IL-2 promoter to bind to proteins present in nuclei of lymphocytes that are stimulated by mitogens and are commencing proliferation. IL-2 is central to the cellular immune response and inability to express this gene would result in cellular dysfunction of T lymphocytes. Three DNA-binding activities present in activated lymphocytes were studied: NFκB, AP-1 and NFAT. The cells were subjected to oxidative stress: an enzymatic activity (PAO) generating hydrogen peroxide, irradiation and four concentrations of hydrogen peroxide administered directly to the cells. None of these stresses affected lymphocyte viability which remained at 95%, as determined by trypan blue exclusion. Figure 1 demonstrates the effect of oxidative stress on NFκB DNA-binding in T lymphocytes. While unstimulated cells (Unst.) do not express NFκB DNA binding, stimulated cells (Stim.) do express this activity and the interaction is specific as shown by its prevention in the presence of a specific competitor (an excess of unlabeled NFκB DNA, Stim.+Comp.). All the types and levels of oxidative stress we employed completely abolished the induction of NFκB DNA-binding in stimulated T lymphocytes.

As can be seen in Figures 2 and 3, protein binding activities to two other DNA sequences (AP-1 and NFAT) were only expressed in stimulated T cells and were abolished by exposing the cells to oxidative stress, similar to the results obtained with the NFκB sequence.

Lipid peroxide determination in lymphocytes exposed to oxidative stress

Since the goal of these studies was to develop markers of oxidative stress in lymphocytes, we measured lipid peroxidation as a biochemical parameter of exposure to oxidants. The basal level of lipid peroxides in T lymphocytes was 2.4 ± 0.7 nmoles lipid hydroperoxides/mg protein. Only treatment with hydrogen peroxide at 20 μ M for 2 hours induced a rise in cellular peroxides to 11.9 ± 1.8 nmoles lipid hydroperoxide/mg protein ($P < 0.0005$), while the other treatments (hydrogen peroxide at 50-200 μ M, polyamine oxidase and irradiation) did not induce any rise in the levels of lipid peroxides above basal levels. A possible explanation to these findings is that the direct biochemical damage was repaired within two hours after the exposures. The lowest concentration of hydrogen peroxide may not have been sufficient to induce appropriate levels of anti-oxidant defenses, allowing the lipid peroxidation to be detected. To investigate this possibility, levels of the major anti-oxidative enzyme - catalase, were measured in T lymphocytes exposed to oxidative stress.

Catalase determination in lymphocytes exposed to oxidative stress

Every oxidative stress exposure except for hydrogen peroxide at 20 μ M, induced a significant rise in the cellular catalase activity above the basal level (Figure 4).

DISCUSSION

Three modes of oxidative stress: hydrogen peroxide, polyamine oxidase activity generating hydrogen peroxide gradually, and irradiation, suppressed the activation-dependent DNA-binding activities of three transcription factors: NF κ B, AP-1 and NFAT, in human peripheral blood T lymphocytes. Only exposure to hydrogen peroxide at 20 μ M generated measurable lipid peroxidation products in T lymphocytes while this was the only exposure that did not induce an increase in cellular catalase activity.

We have previously reported [8] that exposure to polyamine oxidase results in suppression of transmembrane signal transduction in human peripheral blood T lymphocytes. This leads to suppression of the activation-dependent expression of transcription factors in the nucleus, and finally to inhibition of the transcription of the IL-2 gene. In the current study, we compared the effects of different types and levels of oxidative stress on nuclear signal transduction in exposed human lymphocytes. Three transcription factor DNA-binding activities were suppressed in T lymphocytes by every condition of oxidative stress employed. The fact that we did not detect a dose response dependence in the suppression of transcription factor activity by hydrogen peroxide suggests that suppressing early signaling events by oxidative stress [8], results in an all-or-none effect on distal signaling steps in the nucleus.

The DNA-binding activities of NF κ B and AP-1 are induced upon exposure to oxidants [16-18]. The apparent contradiction with our results may be resolved by recognizing that in our system (but not in the other studies mentioned), cells were incubated for 2 hours in fresh medium after the exposures, followed by stimulation for 6 hours, and only then were DNA-binding activities determined. Therefore, we are assessing the effects of oxidative stress on T cell mitogenic activation rather than the direct effect on transcription factor activities. We have previously found that

oxidative stress suppresses early signal transduction steps, protein tyrosine phosphorylation and calcium mobilization [8]. Therefore, the eventual suppression of transcription factor activities may actually reflect early effects of oxidative stress on lymphocyte transmembrane signal transduction. In this context, the decline in IL-2 production by human T lymphocytes from aged persons in response to *in vitro* stimulation, is associated with impaired activation of AP-1 and NFAT [19]. In view of the oxygen radical-related theory of aging, this is potentially an example of T cell suppression at the signal transduction level by oxidative stress *in vivo*.

We found that radiation suppressed the expression of DNA-binding activities in activated T lymphocytes. Radiation of human lymphocytes *in vitro* was previously found to suppress constitutive surface marker expression [20] and enhance micronuclei occurrence following stimulation [21].

Since NFkB and AP-1 were recently found to be the most important IL-2 cis-regulatory elements in normal T cells [22], and we have shown that the acute and chronic modes of oxidative stress used in this study suppress IL-2 production by human lymphocytes [7,9], our results strongly suggest that suppression of transcription factor function caused by inducing oxidative stress in human lymphocytes, contributes to down-regulation of IL-2 production and cellular activation. IL-2 is a major growth factor regulating T lymphocyte proliferation [23]. Therefore, suppression of nuclear signaling events that control IL-2 expression reflects not only exposure but also the detrimental effect of exposure to oxidative stress inducers.

Direct measurements of lipid hydroperoxides did not detect increased levels following oxidative stress, except for exposure of T lymphocytes to the lowest concentration of hydrogen peroxide. The same hydrogen peroxide concentration (20 μ M) was the only one from among the oxidative stress exposure employed in the current study, that did not induce a rise in catalase activity. Therefore, we suggest that when the oxidative stress induces an anti-oxidative response, i.e., catalase

activity, lipid peroxidation is not detectable. PAO did not induce lipid peroxidation, although the enzymatic activity generates only 5 μM H_2O_2 , because the intensity of the cellular stress is determined not only by the concentration of the oxidant but also by the duration of the exposure (in the case of PAO - 2 days). Accordingly, PAO exposure induced an increase in cellular catalase activity.

On the other hand, all 3 modes of oxidative stress resulted in suppression of cellular function that was clearly evident even 8-26 hours after the exposures, as judged by transcription factor activities. Our results suggest that a functional parameter (nuclear signal transduction) is much more sensitive than a structural parameter (lipid peroxidation) as a marker of oxidative damage to human blood lymphocytes.

These results suggest that transcription factor functions can potentially be used as markers of blood lymphocyte exposure to oxidants (including hydrogen peroxide and hydroxyl radicals) which generate either acute or chronic stresses. Our studies were conducted with human peripheral blood lymphocytes which are readily available, and should therefore be amenable to development into population-based markers of environmental exposure to oxidants. Ex vivo peripheral blood cells can also be used for follow-up of exposed individuals.

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ABBREVIATION LIST

EMSA - Electrophoretic mobility shift assay

IL-2 - Interleukin 2

PAO - Polyamine oxidase

PHA - phytohemagglutinin

TPA - tetradecanoyl phorbol acetate

FIGURE LEGENDS

1. Suppression of NF κ B DNA-binding by oxidative stress. T cells were pre-treated with polyamine oxidase (Stim.+PAO, for 2 days at 5×10^{-4} U/ml + spermidine at 5 μ M, and then washed and incubated for 2 hours in fresh medium), or irradiation (Stim.+Irr., for 5 minutes at 6 Gy and then incubated for 2 hours in fresh medium), or hydrogen peroxide (Stim.+H₂O₂, for 2 hours at the indicated concentration and then washed and incubated for 2 hours in fresh medium). Cells were then stimulated with PHA (1 μ g/ml) + TPA (5ng/ml) for 6 hours. In addition, control cultures of untreated cells were either not stimulated (Unst.) or stimulated with PHA + TPA for 6 hours (Stim.). Nuclear extracts were prepared and 10 μ g of protein was incubated with ³²P-labeled NF κ B sequence and electrophoresed. The lanes were loaded with DNA without nuclear extract (Free), DNA with extract from untreated and unstimulated cells (Unst.), DNA with extract from untreated and stimulated cells (Stim.), same as Stim. + 50x excess of unlabeled probe (Stim.+Comp.), and DNA with extracts from pre-treated cells that were also stimulated (Stim.+PAO, Stim.+Irr., Stim.+H₂O₂). The gel was dried and autoradiographed. The arrow marks the specific DNA-protein complex.

2. Suppression of AP-1 DNA-binding by oxidative stress. Same as Figure 1 except that ³²P-labeled AP-1 sequence was used.

3. Suppression of NFAT DNA-binding by oxidative stress. Same as Figure 1 except that ³²P-labeled NFAT sequence was used.

4. Enhancement of catalase activity by oxidative stress. T cells were exposed to polyamine oxidase (PAO, for 2 days at 5×10^{-4} U/ml + spermidine at 5 μ M, and then washed and incubated for 2 hours in fresh medium), or irradiation (Irr., for 5

minutes at 6 Gy and then incubated for 2 hours in fresh medium), or hydrogen peroxide (H_2O_2 at the indicated concentration, μM , for 2 hours and then washed and incubated for 2 hours in fresh medium). Cells were lysed and cytosolic catalase activity was measured by following kinetically the decomposition of hydrogen peroxide in a spectrophotometer, and calculated per protein concentration. Catalase activity in untreated cells was 1.2 ± 0.09 ($\text{sec}^{-1} \cdot \text{mg protein}^{-1}$). Results shown are the increase in catalase activity in stressed cells over untreated cells \pm SD. The increase in catalase activity was significant at $p < 0.01^*$, 0.005^{**} , 0.0005^{***} .

Figure 1.

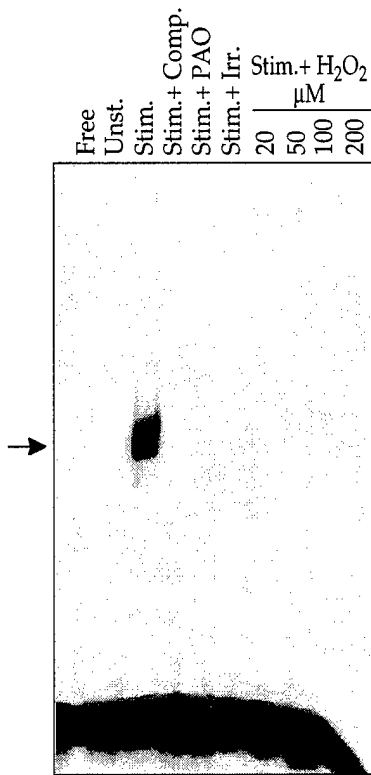


Figure 2.

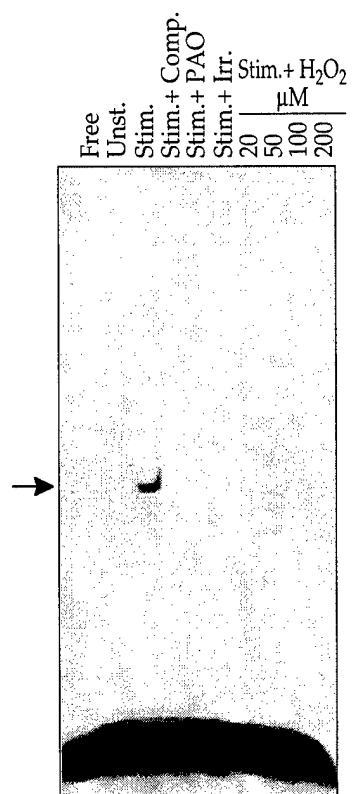


Figure 3.

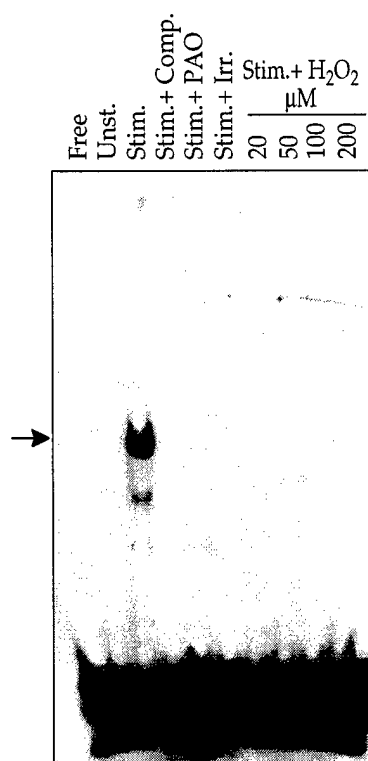
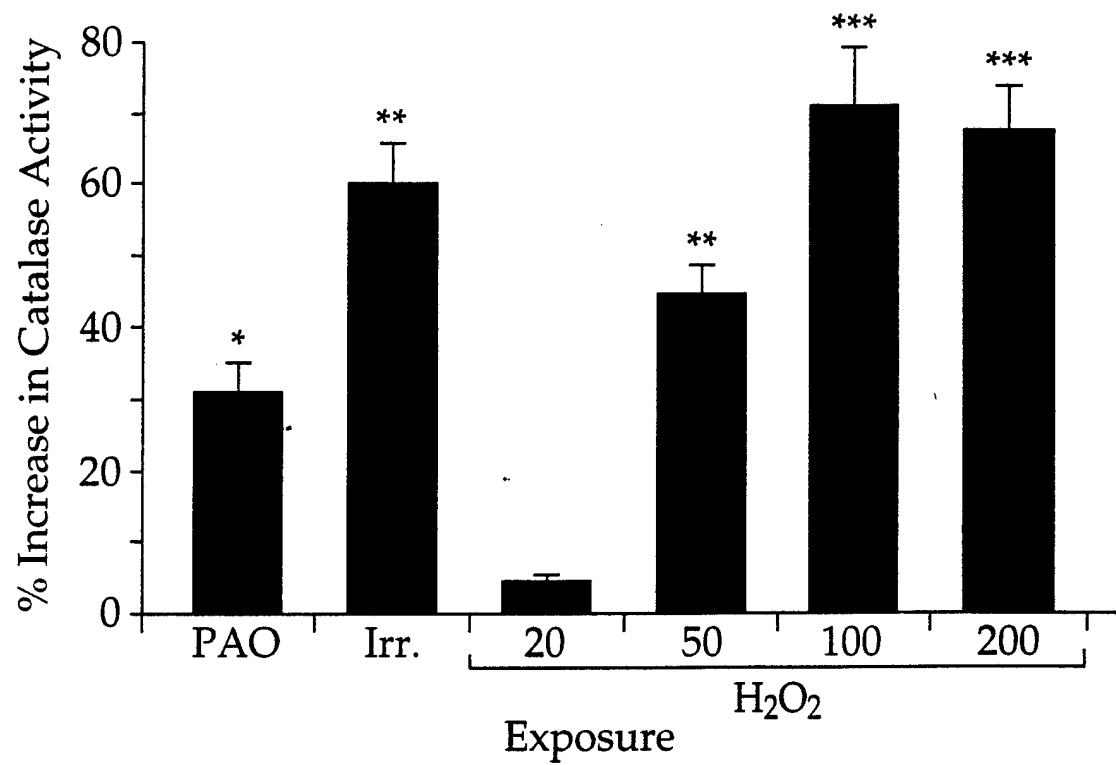


Figure 4.



Ozone-induced IL-8 expression and transcription factor binding in respiratory epithelial cells

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Jaspers, I., E. Flescher, and L. C. Chen. Ozone-induced IL-8 expression and transcription factor binding in respiratory epithelial cells. *Am. J. Physiol.* 272 (*Lung Cell. Mol. Physiol.* 16): L504-L511, 1997.—Ozone, one of the most reactive oxidant gases to which humans are routinely exposed, induces inflammation in the lower airways. The airway epithelium is one of the first targets that inhaled ozone will encounter, but its role in airway inflammation is not well understood. Expression of inducible genes involved in the inflammatory response, such as interleukin (IL)-8, is controlled by transcription factors. Expression of the IL-8 gene is regulated by the transcription factors nuclear factor (NF)- κ B, NF-IL-6, and possibly activator protein-1 (AP-1). Type II-like epithelial cells (A549) were grown on a collagen-coated membrane and exposed in vitro to 0.1 ppm ozone or air. Exposure to ozone induced DNA-binding activity of NF- κ B, NF-IL-6, and AP-1. IL-8 mRNA and IL-8 protein levels were also increased after ozone exposure. These results link ozone-induced DNA-binding activity of transcription factors and the production of IL-8 by epithelial cells thus demonstrating a potential cellular cascade resulting in the recruitment of inflammatory cells into the airway lumen.

nuclear factor- κ B; nuclear factor-interleukin-6; activator protein-1; A549 cells

OZONE IS a common urban air pollutant to which humans are routinely exposed. The National Ambient Air Quality Standard for ozone, 0.12 ppm for a daily 1-h average, is exceeded in more than 60 cities of the United States (38). Laboratory animal and human clinical studies have demonstrated that ozone causes reversible decrements in pulmonary function, increased permeability of the epithelium, influx of inflammatory cells, impaired pulmonary defense capacity, and tissue damage (21). The morphological and biochemical changes measured after ozone exposure result from the direct effects of the interaction of ozone (or its derived products) with cells and the inflammatory response against that initial damage (38).

An important cellular mechanism by which the lung reacts to inhaled noxious gases or particles is the recruitment of inflammatory cells, especially neutrophils, from the vasculature into the airway lumen. These neutrophils release soluble factors, including proteolytic enzymes and reactive oxygen intermediates (ROI), which can initiate changes in lung function and morphology (17). Increased numbers of infiltrated neutrophils in the bronchoalveolar lavage fluid (BALF) of humans exposed to ambient concentrations of ozone (0.1 ppm for 6.6 h) were observed 18 h postexposure (12), whereas markers of airway inflammation in the BALF of exercising individuals exposed to 0.4 ppm for 2 h could be observed as early as 3 h postexposure (30).

It is not yet known which cell type initiates the lower airway inflammatory response after ozone exposure. The pulmonary epithelium, comprised of several different cell types, is one of the first targets that inhaled ozone will encounter and could therefore be a plausible initial mediator of pulmonary inflammation. Specifically, epithelial cells in the proximal alveolar region are susceptible to damage by inhaled ozone (6). Alveolar type II cells play an important role in defense mechanisms of the respiratory tract by acting as a physical barrier to inhaled agents and as the main source of pulmonary surfactant (35). More recently, the airway epithelium has been implicated as an "effector" tissue responding to exogenous stimuli by releasing a variety of cytokines (1), such as interleukins (IL)-1, -3, -6, and -8, granulocyte macrophage colony-stimulating factor, granulocyte colony-stimulating factor (G-CSF), and tumor necrosis factor- α (TNF- α ; see Ref. 11). IL-8, a potent neutrophil chemotactic factor (5), is a potential mediator of pulmonary inflammation.

DNA binding of transcription factors is a necessary step in the expression of inducible genes involved in inflammatory and immune responses. Oxidative stress appears to be an important regulator of IL-8 gene expression (10), which has been shown to be under the synergistic control of nuclear factor (NF)- κ B and NF-IL6 sites in its promoter region (18). An NF-IL-6-like sequence was identified between nucleotides -94 and -81, whereas NF- κ B binds to a region between nucleotides -80 and -71 (18). Activation of NF- κ B and NF-IL-6 DNA-binding activity is thought to occur through the induction of signaling cascades involving ROI and protein kinases (28, 36). An activator protein-1 (AP-1) sequence was also identified, but its activity may be dispensable (18). DNA-binding activity of AP-1 is regulated by a redox mechanism involving a conserved cysteine residue in the DNA-binding domain and/or phosphorylation of the AP-1 subunits Fos and Jun (9). The role of ozone in the activation of protein kinases and the induction of DNA-binding activity of NF- κ B, NF-IL-6, and AP-1 is currently not known.

In this study, we investigated whether in vitro exposure of A549 cells, a human alveolar type II-like cell line, to a low level of ozone induces the DNA-binding activity of the transcription factors NF- κ B, NF-IL-6, and AP-1 and whether this is associated with ozone-induced activation of IL-8 gene transcription and translation. Furthermore, we examined whether these cells display polarity in their release of IL-8. Our results demonstrated that in vitro exposure of A549 cells to 0.1 ppm ozone induced the activity of the transcription factors NF- κ B, NF-IL-6, and AP-1 and increased the expression of IL-8 mRNA and production of IL-8. The

release of IL-8 was initially and predominantly toward the apical side, supporting the proposed mechanism of neutrophil movement along a chemotactic gradient (5) toward the airspace lumen.

MATERIALS AND METHODS

Cell lines and cell culture. A human pulmonary type II epithelial-like cell line (A549 from American Type Cell Culture, Rockville, MD), derived from a patient with alveolar cell carcinoma of the lung, was used in this study. These cells retain features of type II alveolar epithelial cells, including cytoplasmic multilamellar inclusion bodies, but cannot be definitively characterized as of type II origin or function (19). A549 cells were cultured in F-12K media plus 10% fetal bovine serum (FBS; GIBCO-BRL, Gaithersburg, MD) plus 1% penicillin and streptomycin (GIBCO-BRL).

In vitro exposure. A549 cells were grown on Vitrogen-coated (Collagen, Palo Alto, CA) Costar clear transwells (25 mm diameter, 0.4 μ m pore size; Costar, Cambridge, MA) until a confluent monolayer was established ($\sim 2 \times 10^6$ cells/well). To avoid interference of serum components with possible ROI-mediated ozone effects, cells were cultured in phenol red-free F-12 nutrient mixture (Ham's; GIBCO-BRL) without FBS 1 h before and throughout the exposure. Just before exposure, the apical media was aspirated while 2 ml media remained in the basolateral compartments to supply cells with nutrients. The cell monolayers were kept at 37°C and exposed to preheated/prehumidified 0.1 ppm ozone, balanced with 5% CO₂, or exposed to incubator air (which hereafter will be called air-exposed cells) for 2.5 or 5 h. This ozone concentration and exposure duration are frequently achieved in the ambient air of southern California (21). Ozone was produced by passing 0.5% O₂ (in argon carrier) through an ozonizer (Sander), and the exposure concentration was monitored continuously using an Ozone Analyzer (model 8810; Monitor Labs, San Diego, CA). Unexposed incubator control cells remained covered by F-12 media on the apical side throughout the exposure period. To evaluate whether the effects seen after ozone exposure were unique to ozone or were caused by a nonspecific state of oxidative stress, cells received a bolus addition of 0.1, 0.5, and 1 mM H₂O₂, which remained in the apical compartment for 5 h.

Immediately after exposure, cells were removed from the membranes using 0.1% trypsin-EDTA (GIBCO-BRL), and viability was assessed using the trypan blue exclusion method.

Electrophoretic mobility shift assay. Nuclear factors for the mobility shift assay were prepared from A549 cells immediately after exposure as described by Flescher et al. (14). Briefly, cells were washed with phosphate-buffered saline. Extraction buffer made of 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 10 mM KCl, 0.1 mM EDTA, 0.1 mM ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), and seven protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 μ g/ml aprotinin, 5 μ g/ml antipain, 100 μ M benzamidin, 5 μ g/ml leupeptin, 5 μ g/ml pepstatin, and 5 μ g/ml soybean trypsin-chymotrypsin inhibitor, pH 7.9) was added to the monolayers, and the cells were removed by manual scraping with a rubber policeman and transferred to a microcentrifuge tube. The cells were allowed to swell on ice for 15 min, and Nonidet P-40 at 0.625% was added. The tube was vortexed and centrifuged for 30 s in a microcentrifuge. The nuclear pellet was resuspended in buffer containing 20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and the seven previously listed protease inhibitors. The tube was vigorously rocked on ice for 15 min on an orbital

shaker platform, and the nuclear extract was centrifuged for 5 min in a microcentrifuge. The supernatant contained the nuclear extracts. Protein concentrations of the nuclear extracts were determined using the Bradford method (Bio-Rad protein assay kit). Oligonucleotides containing the NF- κ B and AP-1 sequences (Promega, Madison, WI) were labeled using [γ -³²P]ATP and T4 polynucleotide kinase (New England Biolabs, Beverly, MA). The oligonucleotide containing the NF-IL-6 consensus sequence (5'-TTCAACCTGTTTCGAGT-TTCTCGAGGAATCA-3'; see Ref. 8) was labeled using [α -³²P]CTP and Klenow enzyme (Boehringer Mannheim, Indianapolis, IN). Four micrograms of nuclear extract, 20,000 counts/min labeled probe, and 2 μ g poly dI/dC were mixed in a total volume of 20 μ l. The mixture was incubated for 60 min on ice, loaded on a 4% polyacrylamide gel, and subjected to electrophoresis at 20 mA for 1.5–2 h. The gel was dried and autoradiographed on Kodak XAR-5 film at -70°C for 1–3 days.

RNA analysis. RNA was extracted at 0, 4, and 16 h postexposure using RNazol (Biotech, Houston, TX). The amount of RNA present was determined by spectrophotometric absorbance at 260 nm. RNA (15 μ g) was loaded onto a 1.5% agarose gel and subjected to electrophoresis at 15–20 volts overnight. The electrophoresed RNA was transferred onto membrane filters (Nytran; Schleicher & Schuell, Keene, NH) and fixed by ultraviolet cross-linking. Membrane-bound RNA was prehybridized and then hybridized to [³²P]cDNA probe (IL-8 cDNA; R & D Systems, Minneapolis, MN) at 52°C in 1 \times sodium chloride-sodium phosphate-EDTA (SSPE), 2 \times Denhardt's, 0.1% sodium dodecyl sulfate (SDS), and 0.1 mg/ml denatured tRNA and salmon sperm DNA. As control, 28S cDNA (Clontech Laboratories, Palo Alto, CA) was radiolabeled and hybridized as described above. After hybridization, the filters were washed at a final stringency of 5 \times SSPE-0.1% SDS at 52°C and exposed to Kodak X-OMAT S film at -70°C for 1–3 days. Autoradiogram signal strengths of hybridized mRNA were quantified using a laser densitometer (BioImage; Millipore, Ann Arbor, MI). All IL-8 mRNA levels were normalized to 28S expression and expressed as degree of induction over control mRNA isolated from incubator control cells at time 0.

Measurement of IL-8 levels. Immediately after the exposure period, all media were aspirated, and 1 ml fresh phenol red-free F-12 nutrient mixture was added to the apical and basolateral sides. Cells were cultured for an additional 4, 16, and 24 h, and at each time point the conditioned culture media was collected for measurement of IL-8 levels. Release of IL-8 by epithelial cells into the apical and basolateral compartments was assayed with a human IL-8 enzyme-linked immunosorbent assay kit (R & D Systems), according to the instructions given by the manufacturer. Briefly, the IL-8 present in the samples reacts with an immobilized murine monoclonal antibody against IL-8. An enzyme-linked polyclonal antibody specific for IL-8 reacts with the IL-8 bound to the monoclonal antibody. Addition of the enzyme substrate develops color in proportion to the bound IL-8. Any unbound proteins are washed away with a buffered surfactant solution. To assess total IL-8 released by A549 cells, the amounts of IL-8 secreted into the apical and basolateral compartments were combined and expressed as picograms IL-8 per well.

Statistical analysis. All data were analyzed relative to control. This was done using a two-way analysis of variance to test for exposure and postexposure time effects. The Fisher's post hoc test was used to determine if the exposed groups were different from one another. Three Transwells per exposure group were used in this study.

RESULTS

DNA-binding activity of NF- κ B, NF-IL-6, and AP-1. Figure 1 shows that neither exposure to air or 0.1 ppm ozone nor treatment with 1 mM H₂O₂ for 5 h caused a significant decrease in viability of A549 cells compared with unexposed control cells. The effects of ozone exposure on the DNA-binding activity of NF- κ B, NF-IL-6, and AP-1 in A549 cells are shown in Fig. 2. Analysis of nuclear factors isolated from A549 cells exposed to 0.1 ppm ozone for 5 h using the mobility shift assay showed the induction of the DNA-binding activity of all three transcription factors. Exposure to air had no effect on the activity of NF- κ B and NF-IL-6 but slightly induced the DNA-binding activity of AP-1. Nuclear factors isolated from unexposed control cells also showed a weak band of DNA-binding activity of AP-1 (data not shown), similar to the activity of nuclear extracts from air-exposed cells. These results suggest that AP-1 may be constitutively present in A549 cells. In addition, treatment with H₂O₂ induced the DNA-binding activity of NF- κ B (Fig. 2A, lane 2) but did not induce NF-IL-6 DNA binding (Fig. 2B, lane 2). Specificity of the transcription factor DNA-binding activity was examined by adding an excess of the specific unlabeled oligonucleotide to the reaction mixture. The addition of 50 \times nonradiolabeled oligonucleotide successfully competed for the DNA binding of the respective transcription factor.

Induction of IL-8 transcription after ozone exposure. The induction of the DNA-binding activity of transcription factors that control IL-8 gene expression (22) suggested that ozone exposure may affect IL-8 mRNA levels in A549 cells. To examine the effect of ozone and air exposure on IL-8 mRNA levels, RNA was isolated at

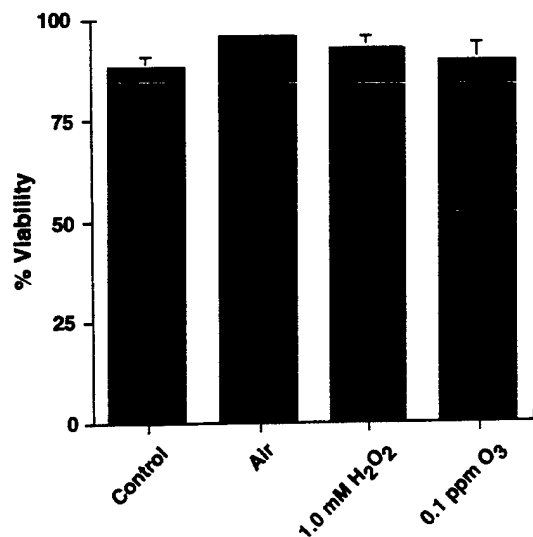


Fig. 1. Effects of air and ozone exposure or treatment with 1 mM H₂O₂ on cell viability. Exposure to ozone or air or treatment with H₂O₂ did not decrease cell viability compared with control cells. After exposure to 0.1 ppm ozone or air or treatment with 1 mM H₂O₂ for 5 h, cell viability was assessed by trypan blue exclusion. Values shown represent means \pm SE of 3 separate experiments. Error bar for air-exposed cells is too small to be shown.

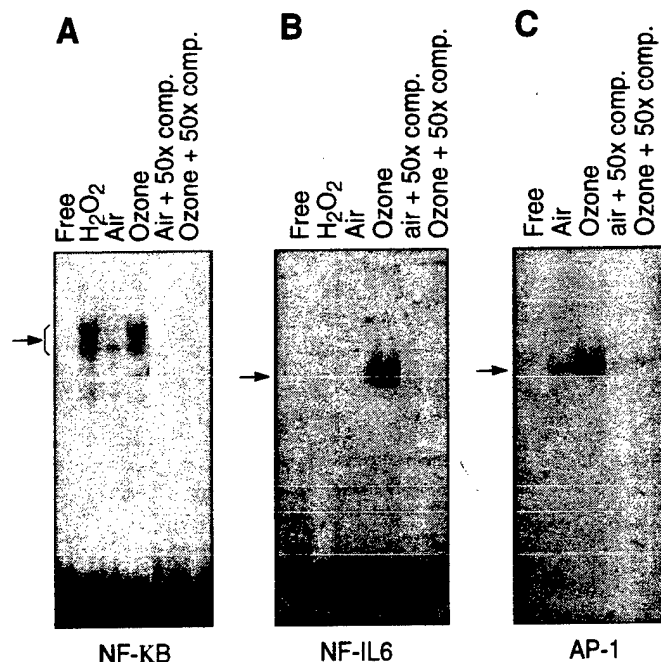


Fig. 2. Exposure to ozone induces nuclear factor (NF)- κ B, NF-IL-6, and activator protein-1 (AP-1) DNA-binding activity. A549 cells were exposed to air or 0.1 ppm ozone or treated with 1 mM H₂O₂ for 5 h, nuclear extracts were prepared, and 4 μ g protein were incubated with ³²P-labeled NF- κ B (A), NF-IL-6 (B), and AP-1 (C) sequences and then electrophoresed. Lanes were free (DNA without nuclear extract) or loaded with air (nuclear extract from air-exposed cells), ozone (nuclear extracts from ozone-exposed cells), air + 50 \times comp (same as air plus 50 \times excess of unlabeled probe), or ozone + 50 \times comp (same as ozone plus 50 \times excess unlabeled probe). In A and B, lane 2 is H₂O₂ (nuclear extracts isolated from H₂O₂-treated cells). Gels were dried and autoradiographed. Arrows mark specific DNA-protein complexes.

0, 4, and 16 h postexposure. As illustrated in Fig. 3, exposure to 0.1 ppm ozone for 5 h induced a significant increase in IL-8 mRNA levels within 4 h after exposure compared with air-exposed cells. At 16 h postexposure, the mRNA levels in the ozone-exposed cells decreased. Exposure to air also elevated expression of the IL-8 gene in A549 cells compared with unexposed control cells but was significantly lower than in ozone-exposed cells.

Comparison of ozone- and H₂O₂-induced expression of IL-8. To investigate whether the changes seen after ozone exposure are caused by general oxidative stress or some other more ozone-specific phenomena, we treated cells with various concentrations of H₂O₂ for 5 h. The autoradiograph in Fig. 4A compares IL-8 mRNA isolated from H₂O₂-treated and ozone-exposed A549 cells 4 h postexposure. Treatment with various concentrations of H₂O₂ did not increase IL-8 mRNA levels, in contrast to what is seen after exposure to ozone. Similarly, Fig. 4B shows that IL-8 production 4 h after exposure was significantly higher in ozone-exposed cells compared with unexposed control cells, air-exposed cells, or H₂O₂-treated cells. Neither air exposure nor treatment with various concentrations of H₂O₂ significantly increased IL-8 production compared with

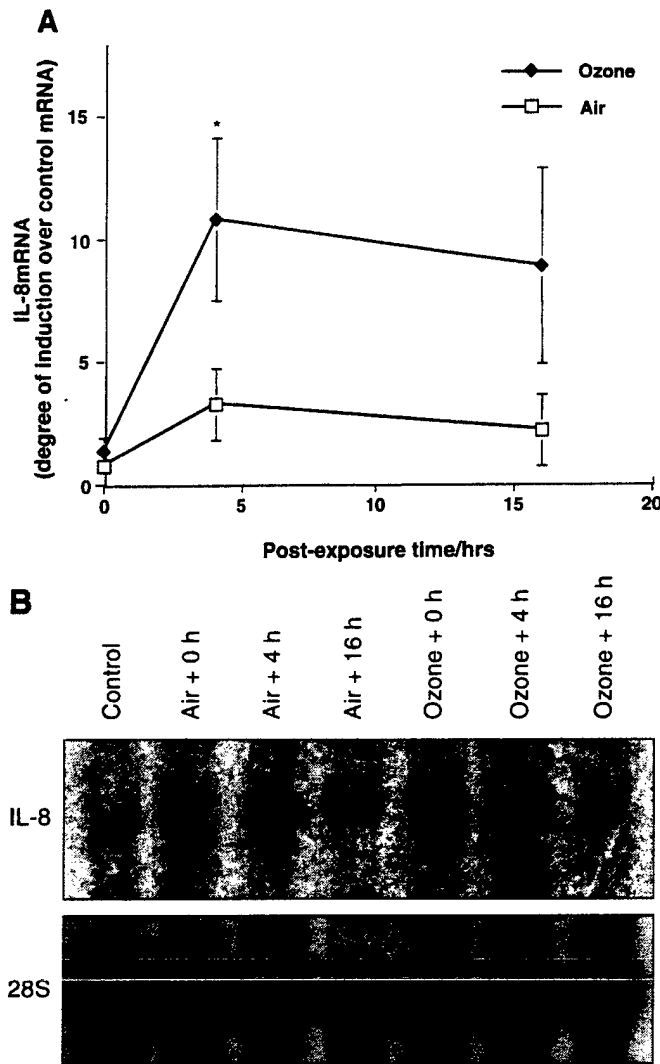


Fig. 3. Exposure to ozone increases interleukin (IL)-8 mRNA levels. RNA was isolated from A549 cells at 0, 4, and 16 h after exposure to 0.1 ppm ozone or air for 5 h. A: IL-8 mRNA levels were normalized to 28S rRNA and expressed as percent of control cells. Values shown represent means \pm SE of 3 individual experiments. *Significantly different from air-exposed cells; $P < 0.05$. B: representative autoradiograph showing hybridization to IL-8 (top) and 28S (bottom) cDNA. Northern blot analysis shows that exposure to ozone induces expression of IL-8 gene within 4 h after exposure.

unexposed control cells. This suggests that exposure to air or treatment with H_2O_2 had no significant effect on the regulation of IL-8 synthesis beyond the documented spontaneous production of IL-8 by A549 cells (3), as detected by us in control cells.

Effects of exposure duration on IL-8 expression. To examine whether the effect of ozone on the expression of IL-8 was dose dependent, we analyzed IL-8 mRNA and protein levels after 2.5- and 5-h exposures to 0.1 ppm ozone. Figure 5, A and B, compares expression of IL-8 after 2.5- and 5-h exposures. Figure 5 shows that exposure of A549 cells to 0.1 ppm ozone for 5 h induced a significantly higher expression of IL-8 than exposure for 2.5 h. At both time points, IL-8 production was

significantly higher in ozone-exposed cells than in air-exposed cells.

Comparison of IL-8 levels in the apical and basolateral compartments. Because airway epithelial cells in vivo are thought to possess polarity in their morphology and release of mediators, we examined next whether the ozone-induced accumulation of IL-8 was different in the apical versus basolateral compartments. Figure 6

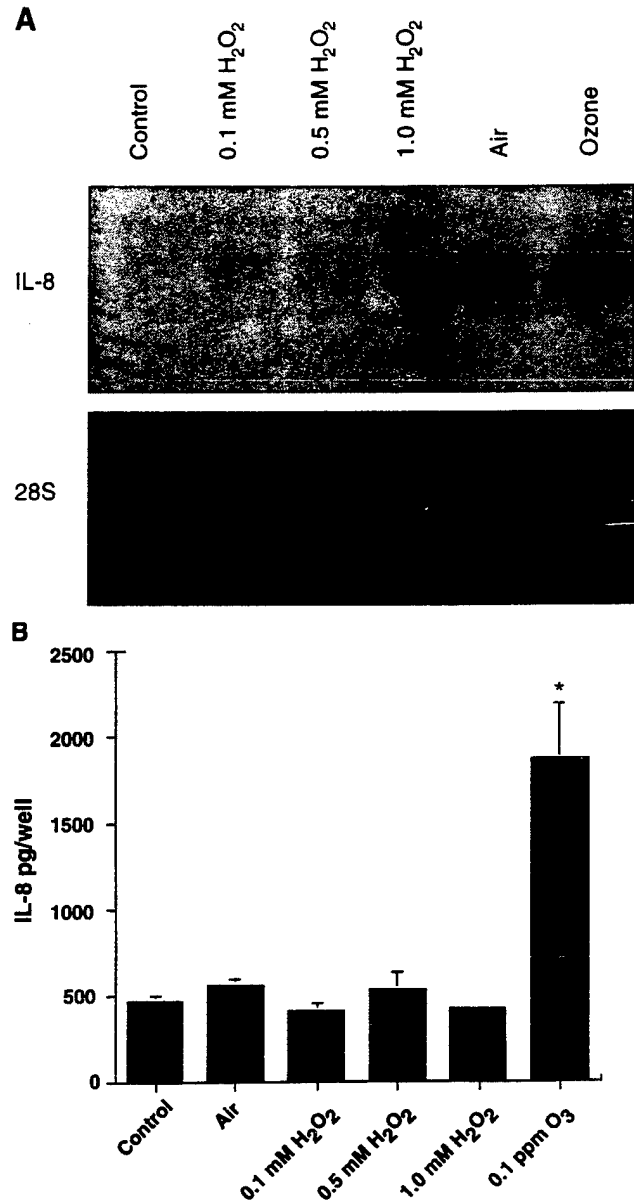


Fig. 4. Comparison of ozone- and H_2O_2 -induced effects on IL-8 expression. A: representative autoradiograph showing hybridization of RNA isolated from A549 cells 4 h postexposure or posttreatment to IL-8 (top) and 28S (bottom) cDNA. Northern blot analysis shows that 5 h of exposure to ozone induces greater expression of IL-8 gene than either exposure to air or treatment with 0.1, 0.5, or 1.0 mM H_2O_2 for 5 h. B: analysis of IL-8 content in conditioned media 4 h after exposure shows that ozone induces significantly higher release of IL-8 than exposure to air or treatment with H_2O_2 . *Significantly different from control, air, or treatment with 0.1, 0.5, or 1.0 mM H_2O_2 ; $P < 0.05$. Values are means \pm SE.

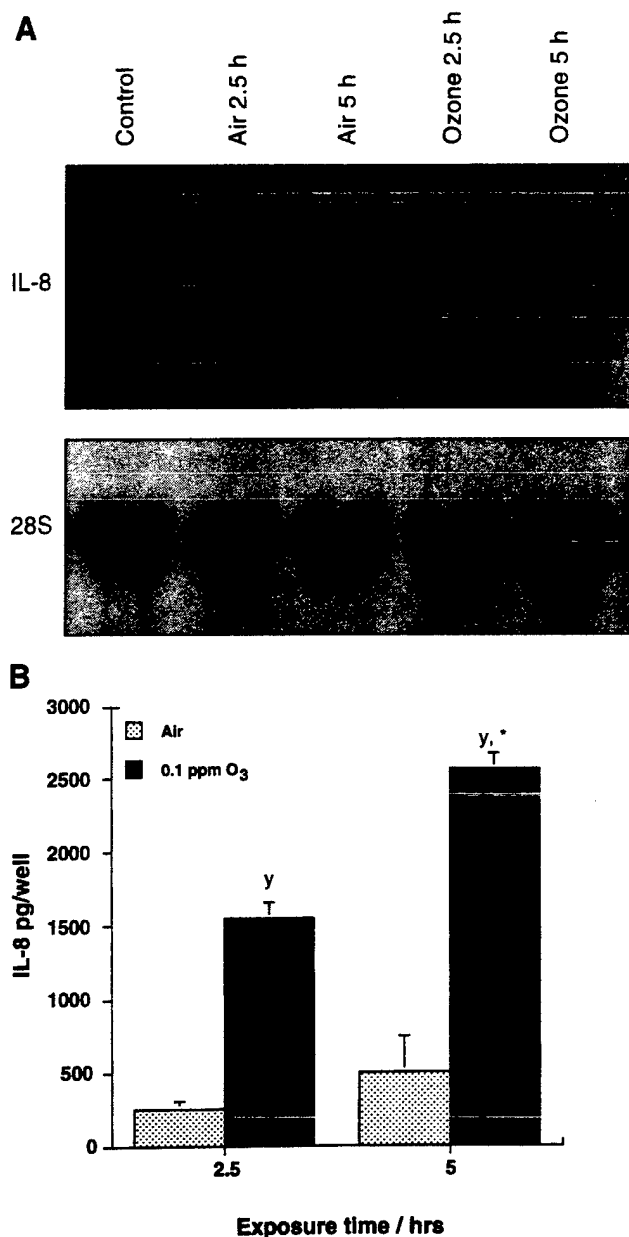


Fig. 5. Effects of exposure duration on IL-8 expression. A: representative autoradiograph showing hybridization of RNA isolated from A549 cells 4 h postexposure to IL-8 (top) and 28S (bottom) cDNA. Northern blot analysis shows that 5 h of exposure to ozone induces greater expression of IL-8 gene than exposure for 2.5 h. B: analysis of IL-8 content in conditioned media 4 h after exposure shows that exposure to 0.1 ppm ozone for 5 h induces significantly higher release of IL-8 than exposure for 2.5 h and that ozone induces greater expression of IL-8 than air exposure independent of exposure duration. ^ySignificantly different from air-exposed cells; ^{*}significantly different from 2.5-h exposure; $P < 0.05$. Values are means \pm SE.

shows that a significant rise in IL-8 in the apical compartments could be observed as early as 4 h after exposure, whereas this did not occur until 16 h postexposure in the basolateral compartments. At 24 h postexposure, IL-8 content in both apical and basolateral compartments decreased in ozone-exposed cells. The greatest difference between apical and basolateral IL-8 content was seen at 4 h postexposure. This difference

was still significant at 16 h postexposure. Air exposure did not significantly increase IL-8 release into the apical or basolateral compartments compared with unexposed control cells.

DISCUSSION

The cellular mechanisms that induce cytokine production in lung cells after pollutant exposures are still unclear. Previous studies have suggested that IL-8 synthesis, which is under the synergistic control of the transcription factors NF- κ B and NF-IL-6, and possibly AP-1 (18), is initiated by oxidative stress (10). We therefore investigated whether exposure of respiratory epithelial cells to the oxidant pollutant ozone induces changes in the DNA-binding activities of NF- κ B, NF-IL-6, and AP-1 and whether these changes are reflected in increased IL-8 mRNA and IL-8 protein levels. Our results demonstrated that in vitro exposure of A549 cells to an environmentally relevant concentration of ozone (21) induced the DNA-binding activity of transcription factors responsible for the control of IL-8 gene expression. The activation of NF- κ B and NF-IL-6,

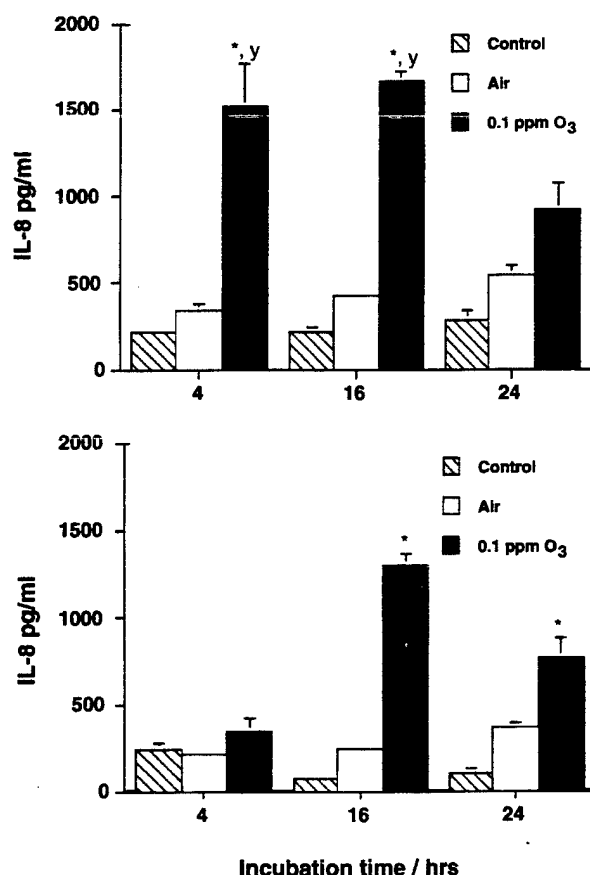


Fig. 6. Comparison of IL-8 levels in apical (top) and basolateral (bottom) media. Conditioned media was collected at 4, 16, and 24 h after exposure to air or 0.1 ppm ozone for 5 h. Enzyme-linked immunosorbent assay of IL-8 production in apical and basolateral media shows that release of IL-8 is initially only toward the apical side. ^{*}Significantly different from air-exposed cells; ^ysignificantly different from basolateral media; $P < 0.05$. Values shown represent means \pm SE of 3 individual experiments. Some error bars of IL-8 levels in air-exposed cells are too small to be shown.

which are essential in the expression of other inflammatory cytokines, such as IL-6, G-CSF, and TNF- α (2, 24), could comprise a proximal step in the ozone-induced inflammatory response at a concentration that is not overtly cytotoxic.

Other studies investigating pollutant-induced activities of transcription factors and expression of inflammatory cytokines concur with our findings regarding the ozone-induced activation of the transcription factors NF- κ B and NF-IL-6 and the production of IL-8 by human pulmonary epithelial cells. For example, the DNA-binding activities of NF- κ B and NF-IL-6 as well as the production of IL-8 were induced in asbestos-exposed pulmonary epithelial cells, and the response was thought to be mediated through asbestos-induced oxidative stress (31). In another study, the activation of NF- κ B in lung tissue and the expression of the chemokine cytokine-induced neutrophil chemoattractant were observed in rats exposed to a high level of ozone (16). It is possible that the induction of DNA-binding activity of transcription factors, specifically NF- κ B and NF-IL-6, may result in the generation of other inflammatory cytokines, such as G-CSF, TNF- α , and IL-6, by pulmonary cells. Therefore, activities of these transcription factors may constitute a common and early step in pollutant-induced inflammatory processes.

In our study, we observed dose-dependent increases in the transcription of the IL-8 gene and production of IL-8 in ozone-exposed respiratory epithelial cells. Although these changes correlated with increased transcription factor activities, further experiments, such as using the recently developed inhibitor for nuclear translocation of NF- κ B (20) or NF-IL-6 knockout mice (33), are necessary to establish the dependency of ozone-induced IL-8 release and transcription factor binding. Nevertheless, the rise in NF- κ B and NF-IL-6 DNA-binding activities and levels of IL-8 mRNA after the same exposure conditions, as well as the dependency of IL-8 transcription on the activities of NF- κ B and NF-

IL-6 shown by others (18, 22), suggest such a relationship.

Ozone is thought to exert its toxic effects through the formation of ROI at or near the epithelial membrane (26). It is likely that pulmonary cells respond specifically to the membrane-associated ROI production rather than a general state of oxidative stress, such as that produced by H₂O₂. Indeed, as shown in our study, while inducing the DNA-binding activity of NF- κ B, similar to that shown by others (29), apical stimulation of A549 cells with 1 mM H₂O₂ failed to induce the DNA-binding activity of NF-IL-6, a transcription factor essential for the expression of IL-8 (18, 22). Furthermore, IL-8 mRNA and IL-8 protein levels were significantly lower in H₂O₂-treated cells than in ozone-exposed cells. These results indicate that the ozone-induced signaling cascade is initiated, at least partially, by an ozone-specific mechanism rather than by a general state of oxidative stress. Evidence for the existence of an ozone-specific mechanism is supported by recent findings that lipid ozonization products (LOP), formed through the reaction of ozone with membrane lipids, may be responsible for the effects seen after ozone exposure (27).

The airway epithelium is known to possess polarity in its morphology. Primary human tracheal epithelial cells grown on membranes with a gas-liquid interface retain that polarity in vitro, displaying ciliogenesis and mucus production toward the apical side (15). Interestingly, the results of our study indicate that A549 cells retained polarity in their release of IL-8 after exposure to ozone. Comparison of the apical and basolateral conditioned media of ozone-exposed A549 cells showed that the IL-8 release was mainly toward the apical side, hence creating a concentration gradient. Our results are in contrast to a study published by Devlin et al. (13), which showed polarity in the release of IL-6, but not IL-8, by a human bronchial epithelial cell line exposed to ozone. The discrepancy in the results could stem from differences in the cell line and exposure duration.

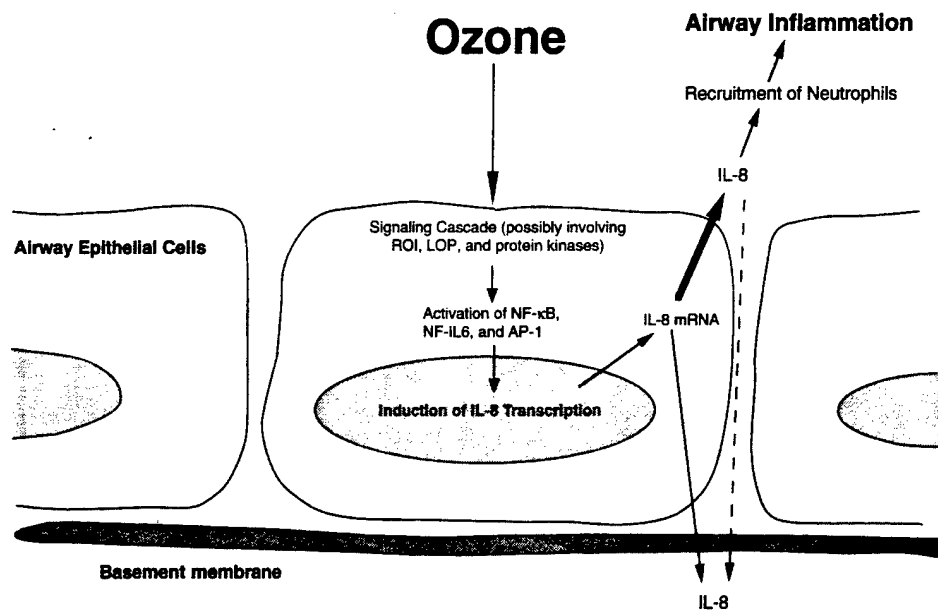


Fig. 7. Model of proposed biochemical events induced in A549 cells upon exposure to ozone, leading to airway inflammation. ROI, reactive oxygen intermediates; LOP, lipid ozonization products.

The cell line used in our study has type II-like cell characteristics, whereas an SV40-transformed bronchial epithelial cell line was used by Devlin et al. (13).

Although in vitro exposures to ozone in a cell culture system do not directly mimic in vivo exposure conditions, it is a useful model to study intracellular mechanisms of pollutant-induced injury in a homogenous cell population (3, 7, 23). Culturing respiratory epithelial cells on collagen-coated membranes, with nutrients supplied from the basolateral side, facilitates the direct exposure of these cells to gaseous pollutants on the apical side, without the influence of other cellular and biochemical reactions. Although these reactions are important parameters in the initiation of lung injury, they also make the investigation of the exact role that respiratory epithelial cells play in pollutant-induced inflammatory responses very difficult. As in other in vitro studies, one has to be careful in extrapolating the results obtained in this study to the in vivo situation, especially since the cell line used throughout this study cannot be definitively characterized as of alveolar type II cell origin or function.

In conclusion, our study showed that exposure to ozone induced the DNA-binding activities of NF- κ B, NF-IL-6, and AP-1, as well as the expression of IL-8, in a dose-dependent manner and that this response was specific to ozone and could not be induced by treatment with H₂O₂. Our results suggest that interaction of ozone with epithelial plasma membranes induced an intracellular signaling cascade, resulting in the generation of an IL-8 concentration gradient. The proposed sequence of cellular events, as illustrated in Fig. 7, is as follows. Formation of LOP or ozone-derived ROI could activate protein kinases, such as protein kinase C and tyrosine kinases (4, 27, 28, 32, 36). Upon phosphorylation, NF- κ B, NF-IL-6, and AP-1 would be able to translocate into the nucleus (9, 25, 34, 37), bind to the DNA sequence in the IL-8 promoter region, and facilitate transcription. The ozone-induced IL-8 release would be initially predominantly toward the apical side, thus creating a chemotactic gradient along which neutrophils could migrate into the airway lumen. IL-8 detected in the basolateral side may be derived from site-directed secretion or diffusion of apically released IL-8. The absence of obvious cell injury indicates that ozone could physiologically stimulate cytokine production in respiratory epithelial cells. The expression of IL-8 shown in this study lies within the time frame of neutrophil infiltration observed in humans (17). Therefore, our findings may help explain the influx of inflammatory cells into the lung lumen of humans exposed to ambient levels of ozone (12).

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TYROSINE PHOSPHORYLATION: A REQUIRED STEP IN THE OZONE-INDUCED PRODUCTION OF IL-8 BY RESPIRATORY EPITHELIAL CELLS. I. Jaspers, L.C. Chen, E. Flescher. Nelson Institute of Environmental Medicine, New York University Medical Center, Tuxedo, NY 10987

Studies conducted in our laboratories have shown that ozone-induced NF-kB and NF-IL6 DNA-binding activity and production of IL-8 in respiratory epithelial cells. Phosphorylation of NF-kB and NF-IL6 appears to be essential in the nuclear-translocation and binding of these transcription factors to the IL-8 promoter region. We, therefore, investigated whether activation of protein tyrosine kinases is required in the signaling cascade leading to IL-8 production in ozone-exposed respiratory epithelial cells. In this study, we analyzed whether the PTK inhibitors Herbimycin A and Genistein can prevent the ozone-induced activation of NF-kB and NF-IL6 as well as the expression of IL-8 in respiratory epithelial cells. A human alveolar type-II-like cell line (A549) was exposed to 0.1 ppm ozone or air in the presence or absence of Herbimycin A and Genistein. Treatment with Herbimycin A inhibited the ozone-induced DNA-binding activity of NF-kB and NF-IL6. Furthermore, both Genistein and Herbimycin A significantly decreased the ozone-induced production of IL-8 in A549 cells. We conclude that tyrosine phosphorylation is an essential step in the ozone-induced signaling cascade leading to the expression of IL-8 in respiratory epithelial cells. Sponsored by EPA R819342 and DAMD17-95-1-5058

This abstract is funded by:

TYROSINE KINASES, BUT NOT PROTEIN KINASE C, ARE REQUIRED FOR SURFACTANT PROTEIN A STIMULATION OF ALVEOLAR MACROPHAGES. Tania Schagat, Michael James Tino, and Jo Rae Wright. Department of Cell Biology, Duke University Medical Center, Durham, NC, USA.

Surfactant Protein A (SP-A) is an immunomodulatory protein specific to the lung. It stimulates a variety of events in alveolar macrophages including phagocytosis, chemotaxis, and production of reactive oxygen species. However, the signal transduction pathways involved in the SP-A stimulation of these events are unclear. We hypothesize that SP-A stimulates the phosphorylation of specific proteins within alveolar macrophages and that this phosphorylation activity is necessary for the SP-A activation of macrophage function, specifically phagocytosis and chemotaxis. Protein phosphorylation was examined by Western blot, using monoclonal antibodies against phosphoproteins; phagocytosis was examined by fluorescence microscopy after alveolar macrophage exposure to fluorescently-labeled *Streptococcus pneumoniae*; chemotaxis was measured using a modified Boyden chamber. We found that SP-A stimulates the rapid tyrosine, but not serine/threonine phosphorylation of specific proteins in alveolar macrophages, and that this phosphorylation activity is inhibitable by genistein, a specific inhibitor of tyrosine phosphorylation. The role of phosphorylation in phagocytosis and chemotaxis was then examined using genistein and calphostin, a protein kinase C (PKC) inhibitor. Genistein inhibited both SP-A-stimulated and nonopsonic phagocytosis, whereas calphostin had no effect. Genistein also inhibited SP-A stimulation of chemotaxis, but it had no effect on nondirected cell movement; again, calphostin had no effect. These data suggest that tyrosine phosphorylation, but not PKC activity, is necessary for both SP-A and non-SP-A-stimulated phagocytosis and SP-A directed chemotaxis in alveolar macrophages. It remains to be determined what proteins are being tyrosine phosphorylated and whether this response is specific to SP-A stimulation of alveolar macrophages.

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THE G_i AND G₁₂ PROTEINS ARE EXPRESSED AT CRITICAL TIME POINTS DURING GROWTH AND AT SPECIFIC CELLULAR LOCALIZATION IN DISTAL BRONCHIOLAR EPITHELIAL CELLS. M.D. Aleixo, S.H. Chin, L. Escobari, and T.B. Kinane. Pediatric Pulmonary and Renal Units, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114.

Heterotrimeric guanine nucleotide binding (G) proteins transduce a wide variety of receptor mediated signals. Pertussis toxin, which decouples a subfamily of G proteins (G_i), attenuates mitogenic responses to thrombin and bombesin/gastrin activating, which play a key role in lung development. Recently, the G₁₂ family of G proteins have been implicated in pathways that regulate growth arrest and possibly apoptosis. To determine whether G_i and G₁₂ family of G proteins were expressed in MLE12 (a transformed distal bronchiolar and alveolar epithelium cell line) immunoblotting of extracts from cells cultured for 1, 4 and 7 days using antibodies against G_{ai2}, G_{ai3}, G_{ai12} and G_{ai13} was performed. All G proteins were easily detected in MLE12 extracts. G_{ai2} and G_{ai13} were induced. G_{ai3} was unchanged with time as G_{ai12} was expressed as the cells became more confluent. The early expression of G_{ai2} is consistent with its involvement with growth pathways, as the late expression of G_{ai12} is consistent with a role in polarization or induction of growth arrest. G_{ai2}, G_{ai3} and G_{ai12} had a bright plasma membrane staining in immunofluorescence studies. G_{ai3} in other secretory epithelial cells has golgi staining and is thought to regulate golgi transport, however such a location was not present in these cells. However, G_{ai2} in addition to plasma membrane staining, had strong golgi staining which has not been described for other cell lines. This would suggest a role for G_{ai2} in the regulation of secretion.

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MECHANISM OF OXYGEN RADICAL GENERATION IN SILICA-STIMULATED ALVEOLAR MACROPHAGE

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Silica and asbestos are well-known occupational fibrogenic agents and their primary target cell is alveolar macrophage. Particle-stimulated macrophages are believed to release various mediator which can regulate the inflammation and pulmonary fibrosis as well. Even though reactive oxygen species (ROS) play the major role among these mediators, the mechanisms about the stimulation of alveolar macrophages is not clear yet.

ROS production, ATP level and the activity of PLC (Phospholipase C), PKC (Protein kinase C) and PTK (Protein tyrosine kinase) were assessed to compare the capability of silica to generate oxygen radical with asbestos and examine the mechanism to stimulate oxygen radical generation in alveolar macrophages.

As the result, we observed silica and asbestos increased generation of oxygen radical with dose-response pattern and also silica decreased intracellular ATP level with the time-course. To know the interaction between tyrosine kinase, PLC and PKC, we measured the effect of several drugs on ROS production and PI (phosphoinositide) turnover during silica stimulation. These data suggest the activation of tyrosine kinase occurs before the activation of PLC and PKC.

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vulnerable to AM-induced toxicity, which may be an initiating event of an inflammatory response that precedes pulmonary fibrosis. (Supported by Medical Research Council of Canada Grant No. MT-13257).

378 METABOLIC FUNCTIONS OF ALVEOLAR TYPE II CELLS IN ACUTE SILICOSIS

R D Levy, A F Hubbs, B S Ducatman, G Singh, V Vallyathan, L Bowman and P R Miles. *Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, WV and Department of Pathology and Laboratory Medicine, Veterans Administration Hospital, Pittsburgh, PA. Sponsor: C. Kominami.*

We have examined some metabolic functions of alveolar type II cells during experimental silicosis in rats. Consistent with previous studies, hypertrophied and hyperplastic alveolar type II cells and alveolar lipoproteinosis were observed at two and four weeks after intratracheal administration of 20 mg silica. Both hypertrophied and normal alveolar type II cells from all exposure groups contained immunohistochemically detectable lysosomes and surfactant apoprotein. Cytochrome P450 2B1 was immunohistochemically demonstrated in the Clara and normal type II cells of control and silica exposed rat lungs. However, the hypertrophied and hyperplastic alveolar type II cells contained no immunohistochemically detectable cytochrome P450 2B1. The concentration of P450 2B1 containing alveolar cells (positive cells/mm²) was unaltered by silica. These findings support the hypothesis that during experimental silicosis, the hypertrophied type II cells are a newly-induced population of alveolar cells metabolically distinct from the pre-existing type II cells.

379 MUTATIONAL SPECTRA IN THE HPRT GENE OF RAT LUNG EPITHELIAL CELLS AFTER QUARTZ EXPOSURE

D G Hassenbein, J M Carter, B W Howard and K E Driscoll. *The Procter & Gamble Co., Cincinnati, OH.*

Previously we reported that exposure of rats to inflammatory doses of quartz and other particles results in an increased frequency of alveolar epithelial cells with mutation in the hprt gene. To better understand mechanisms of particle-induced mutation in the rat lung, the present study was undertaken to characterize the types of mutations occurring in the hprt gene of rat lung epithelial cells after *in vivo* quartz exposure. Briefly, rats were intratracheally instilled with saline or quartz (1-20 mg/kg body weight) and animals sacrificed 3-15 months after exposure. Alveolar epithelial cells were isolated and mutation in the hprt gene selected for by culture in 6 thioguanine (6TG) containing media. 6TG resistant colonies were expanded and RNA extracted. Mutations were characterized by direct cloning and sequencing of PCR amplified hprt cDNA. As reported previously the number of alveolar epithelial cells with hprt mutations was increased for cells from rats exposed to quartz. Comparison of the types of mutations generated revealed differences between saline and quartz exposed rats. Almost all hprt mutations in cells from saline exposed rats were point mutations, with only 1 frameshift and no deletions detected. In contrast, 47% of mutations found in rats after quartz exposure were point mutations with 40% frameshifts and 13% deletions and gene rearrangements. These data suggest that differences exist in the mechanisms underlying mutations occurring in the saline control and quartz exposed rats. Studies are continuing to examine mutational spectra associated with exposure to particles other than quartz.

380 IN VITRO TOXICITY IN RAT TYPE II PNEUMOCYTES AND ALVEOLAR MACROPHAGES OF TEXTILE PAINT COMPONENTS LINKED TO THE "ARDISTYL SYNDROME"

P H M Hoet, M Leyva, F L Clottens, M Demelits, B Nemery. *Laboratory of Pneumology, K.U. Leuven, Leuven, Belgium. Sponsor: R. Lauwerys.*

In an attempt to elucidate the mechanisms for the severe pulmonary interstitial disease (Ardistyl syndrome) which affected textile printing sprayers in Spain (Moya, C. *et al.* Lancet 1994; 344: 498-502) and in Algeria (Ould Kadi, F. *et al.* Lancet 1994; 344: 962-963), the *in vitro* toxicity of components of the incriminated paint was tested in primary cultures of rat type II pneumocytes and alveolar macrophages. Acramin FWR (a polyurea), Acramin FWN (a polyamideamine), Acraflx FHN (a polyamine salt) or Acramoll W (a co-polymer of butylacrylate) were added, for 24 h, to the culture medium (Waymouth's medium with 10% FCS) in concentrations ranging from 0.000128 % to 2 % (v/v) of the commercial products. Acramoll W showed no toxicity. The viability of the type II pneumocytes, assessed as LDH release,

diminished with increasing compound concentration, with TD₅₀ values (95 % confidence intervals) of 18-52 ppm for Acramin FWR, 3-19 ppm for Acramin-FWN and 16-65 ppm for Acraflx FHN. The toxicity to alveolar macrophages was in the same order of magnitude. Phorbol uptake, a specific function of type II pneumocytes, was significantly decreased during the first hour of exposure to 6.4 ppm of Acramin FWR, Acramin FWN or Acraflx FHN. The present study indicates that these polymers have a surprisingly high pulmonary toxicity *in vitro*, thus confirming previous *in vivo* observations.

381 OZONE-INDUCED SIGNAL TRANSDUCTION IN RESPIRATORY EPITHELIAL CELLS: ROLE OF PROTEIN KINASE C

I Jaspers, E Fleischer, L C Chen. *Nelson Institute of Environmental Medicine, New York University Medical Center, Tuxedo, NY.*

We have previously shown that ozone-exposure induces the DNA-binding activity of the transcription factors NF- κ B and NF-IL6 as well as the expression of IL-8 in respiratory epithelial cells. Phosphorylation of NF- κ B and NF-IL6 allows their translocation into the nucleus and binding to the IL-8 promoter region. We, therefore, investigated whether protein kinase C (PKC) may be an essential step in the signal transduction leading to IL-8 production in ozone-exposed respiratory epithelial cells. In this study, we examined whether PKC is activated in ozone-exposed epithelial cells and whether the PKC inhibitors Calphostin C and Chelerythrine inhibit the ozone-induced expression of IL-8. Activity of PKC was measured in A549 cells, a human alveolar type-II-like cell line, exposed to 0.1 ppm ozone or air. Both air- and ozone-exposed cells displayed increased PKC activity. The effects of Calphostin C and Chelerythrine on the expression of IL-8 were measured at the transcriptional and translational levels. A549 cells exposed to 0.1 ppm ozone for 5 hours showed significantly increased expression of IL-8 as compared to air-exposed cells, which was not inhibited by Calphostin C or Chelerythrine. From these results we conclude that the ozone-induced signaling cascade leading to expression of IL-8 in respiratory epithelial cells is PKC-independent. (Sponsored by EPA R819342 and DAMD17-95-1-5058).

382 ALPHA PARTICLES LIKE THOSE EMITTED BY RADON INCREASE INTRACELLULAR SUPEROXIDE AND HYDROGEN PEROXIDE PRODUCTION IN HUMAN LUNG FIBROBLASTS

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The mechanism(s) by which high-LET alpha(α)-particles like those emitted by inhaled radon and radon progeny cause lung cancer has not been elucidated. Conceivable, DNA damage induced by α -particles may be mediated by the generation of reactive oxygen species in addition to direct α -particle-DNA interactions. In this study, we set out to examine this possibility by assessing the intracellular generation of superoxide ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) in normal human lung fibroblasts following exposure to α -particles. Ethidium bromide (EB) and 2',7'-dichlorofluorescein (DCF), fluorescent products of the membrane-permeable dyes hydroethidine (HE) and 2',7'-dichlorofluorescein diacetate (DCFH-DA), were used to flow cytometrically monitor the intracellular production of $O_2^{\cdot -}$ and H_2O_2 , respectively. Irradiation of fibroblasts with α -particles (0.04 - 0.19 Gy) caused significant increases in intracellular $O_2^{\cdot -}$ and H_2O_2 production when compared to sham irradiated fibroblasts. Our results to date are consistent with the possibility that α -particles may mediate their DNA-damaging effects at least in part via an ROS-related mechanism. (Supported by and conducted under the auspices of the U.S. Department of Energy).

383 THE CHANGE OF SURFACTANT-ASSOCIATE PROTEIN SP-B, C RNA EXPRESSION AND K-RAS MUTATION IN RAT TYPE II PNEUMOCYTE TREATED WITH EXTRACT OF AIRBORNE PARTICLES

Z Xian-si. *Dept. Preventive Medicine, School of Basic Medicine, Shanghai Tiedao University, Shanghai, P.R.China. Sponsor: J R Landolph.*

In this study, type II pneumocytes isolated from rat lung incubated in DMEM medium containing some doses of organic extract of urban airborne particles for certain time, and then refresh the medium with that containing TPA every three days. The cells were collected to extract DNA for detecting DNA adducts and K-ras mutation. The DNA adducts was detected with 32P-postlabeling assay, and K-ras gene was detected with Southern hybridization after the DNA was amplified with PCR. Meanwhile, some of the cells used

PHARMACOLOGY/THERAPEUTICS (PRECLINICAL AND CLINICAL) 8

#627 Synthetic glycoamine analogs synergize with taxol and cisplatin in inducing programmed cell death in ovarian cancer cells. Frankel, A., Glinisky, G.V., Miosane, V.V., Buckman, R., and Kerbel, R.S. *Sunnybrook Health Science Centre, University of Toronto, Toronto, Ontario, Canada, M4N 3M5, Metastatic Inc., Cancer Research Center, University of Missouri, Columbia, Missouri 65201*

Resistance to chemotherapeutic agents remains one of the most significant problems in treating ovarian cancer patients. Results with a panel of ovarian cancer cell lines exposed to taxol suggest a possible mechanism of acquired drug resistance in tumors based on the response of a cell population (multicellular resistance) as opposed to classic unicellular resistance mechanisms. Upon exposure to taxol for a period of 48 hours, a panel of ovarian cancer cell lines demonstrated a drug-resistant phenotype when grown as three-dimensional cultures (multicellular tumor spheroids), but did not exhibit this phenotype when grown in monolayer cultures. After treatment with taxol, the cells grown as three-dimensional cultures had a greater survival, an increased proliferative potential as well as colony forming ability compared to the taxol-exposed cells grown in monolayer culture. The monolayer cultures exposed to taxol had a greatly altered cell cycle profile that was not apparent in the spheroid model. Taxol-induced apoptosis in the monolayer culture as measured by flow cytometry for concomitant detection of apoptosis and cell cycle analysis was not observed in the spheroids. The levels of the anti-apoptotic protein, bcl-x_L, decreased significantly upon taxol treatment in the monolayer culture, whereas the bcl-x_L levels in the spheroids treated with taxol remained elevated, closely resembling the levels of the control spheroids. We have studied the ability of synthetic glycoamine analogs, anti-adhesive and anti-metastatic agents, to cause a reversal of multicellular resistance to taxol-induced apoptosis or to synergize with cisplatin in inducing apoptosis in ovarian cancer cells. These glycoamine analogs act by competing for specific carbohydrate-lectin interactions, particularly those involving β -galactoside-specific lectins expressed on tumor cells. Three synthetic glycoamines (Fru-Gly, Fru-D-Lau, and Lac-L-Lau) induced programmed cell death in the ovarian cancer cell line A2780. These compounds synergized in vitro with taxol as well as cisplatin to increase the apoptotic index of ovarian cancer cells. Our results support the idea that anti-adhesives and anti-metastatic agents, synthetic glycoamine analogs, may have a translational potential in combination with chemotherapeutic drugs to treat ovarian cancer patients.

#628 Taxol induces apoptosis by a p53/p21 independent mechanism in cisplatin resistant ovarian carcinoma cells. Menendez, A.T., Ladlaw, J., Raventos-Suarez, C., Gilbert, L., Granas, A., Li, M.-X., and Kramer, R. *Bristol-Myers-Squibb, Pharmaceutical Research Institute, Oncology Drug Discovery, Princeton, NJ 08543-4000*

Cisplatin and taxol are widely used in the treatment of cancer. While loss of p53 function has been correlated with resistance to DNA damaging agents, such as cisplatin, its role in the sensitivity of human tumors to taxol (an inducer of p53 and p21) has not been clearly defined. In this report we describe studies using cisplatin resistant A2780DDP human ovarian carcinoma cells that were selected by intermittent cisplatin treatment. We demonstrate that A2780DDP cells have a G to T transverse mutation in exon 5 of the p53 gene (V to F) and parental A2780S have wild-type p53. Whereas A2780DDP cells were resistant to cisplatin as compared to parental A2780S cells, taxol was equally cytotoxic in both cell lines. Although p53 and p21 levels were induced in A2780S cells after a 24 hour exposure to either cisplatin or taxol, neither p53 nor p21 were induced in A2780DDP cells under the same conditions. Cisplatin treated A2780S cells show a slow S traverse, maintain G1 arrest and undergo apoptosis after 24 hours. Cisplatin treated A2780DDP cells also showed a slow S traverse time, and undergo apoptosis but G1 was decreased; these effects required higher drug concentrations and longer drug exposure times. Taxol treated A2780S and A2780DDP cells undergo pronounced G2/M arrest and apoptosis at equal concentrations. We show in this report that taxol sensitivity (but not concomitant cisplatin resistance) in a human ovarian carcinoma cell line is independent from a clinically relevant p53 mutation.

#629 Non-steroidal anti-inflammatory drugs (NSAID) protect human T lymphoma cells against apoptosis induced by anti-cancer drugs. Azare, J., Cohen, D., and Fleischer, E. *New York University Med. Ctr., Tuxedo, NY 10987, Oncology Preclinical Res., Sandoz Res. Inst., East Hanover, NJ 07936 U.S.A.*

Anti-cancer drugs can induce apoptosis in cancer cells and their removal from the cells is mediated by the efflux pump P-glycoprotein (P-gp). We have previously shown that NSAID enhance the multidrug resistance gene (MDR1) expression and function of P-gp in transformed T lymphocytes. The purpose of the present study was to assess the ability of NSAID to protect Molt-4 cells against induction of apoptotic death. Aspirin and sodium salicylate at 2 mM (levels attainable in the plasma and enhancing P-gp function *in vitro*) reduced adriamycin (5 μ M)- and taxol (100 nM)-induced apoptosis. For instance, aspirin reduced taxol-induced apoptosis by 82%, $P < 0.0005$. These findings can be explained by enhanced removal of anti-cancer drugs from NSAID-treated cells, and suggest a potential contra-indication for the use of NSAID during lymphoma chemotherapy. (These studies have been supported by Department of the Army Grant #DAMD17-95-1-5058 and by NIOSH Grant #OH07125.)

#630 Expression of a protective TR1 gene during cell death by tumor necrosis factor and staurosporine. Chang, N.-S., Mattison, J., Cao, H., Joki, N., Zhao, Y., Grissio, M., and Lee, C. *Guthrie Research Institute, 1 Guthrie Square, Sayre, PA 18840*

Transforming growth factor- β 1 (TGF- β 1) is known to prevent L929 fibrosarcoma cell death by tumor necrosis factor (TNF). This protection involves a rapid activation of cellular protein tyrosine kinases which apparently interrupts the TNF killing pathway. Furthermore, a TGF- β 1-induced extracellular matrix protein of 46 kD appears to provide an additional signal for activation of tyrosine and serine/threonine kinases that restrict TNF killing. By expression cloning we have isolated a novel cDNA (1.4 kb), designated TR1, which encodes a putative 12.4 kD protein. Stable expression of TR1 cDNA in L929 cells protects the cells from TNF killing. Prosite analysis shows that the 12.4-kD TR1 protein contains two conservative phosphorylation sites and a motif of TGF- β family. TGF- β 1 rapidly induces TR1 gene expression (a minor 1.4 kb and a major 3.0 kb mRNA transcripts) in L929 cells within 1-hr stimulation, which correlates with the induced TNF resistance in these cells. TR1 gene is also expressed in 4-6 hr by stimulation of L929 cells with TNF or staurosporine. That is, TR1 gene is expressed when L929 cells are undergoing apoptosis. These results suggest that TR1 is a protective protein against TNF- and staurosporine-mediated cell death. Western blotting analysis using antibodies against a synthetic peptide of TR1 revealed a 40-kD protein recognized by the antibodies. The 40-kD TR1 could be rapidly induced by TGF- β 1 within 1 hr and by TNF in 4-6 hr, which correlates with the extent of gene expression as determined by Northern hybridization. The 40-kD TR1 protein is most likely derived from the 3.0 kb mRNA transcript. TR1 could be a tumor suppressor since L929 cells stably expressed TR1 had a reduced proliferation rate. The antiproliferative nature of TR1 renders the cells less susceptible to TNF killing, since in most cases TNF targets proliferating cells (Supported in part by NIH CA61879 and CA64423).

#631 p53 overexpression converts p21-mediated G1 arrest into apoptosis in human lung cancer cells: Clinical implication for p53 gene therapy. Kagawa, S., Fujiwara, T., Nishizaki, M., Ogawa, M., Inoue, F., Kizuta, A., Roth, J.A., and Tanaka, N. *First Department of Surgery, Okayama University Medical School, Okayama 700, Japan, M. D. Anderson Cancer Center, Houston, TX 77030*

p21, a cyclin-dependent kinase inhibitor, may be critical for p53-mediated growth suppression. To directly examine the role of p21, we introduced human p21 gene into a p53-deficient human lung cancer cell line H1299 using a p21-expressing adenoviral vector (AdCMVp21). Infection with AdCMVp21 resulted in high levels of p21 protein expression, and significantly suppressed the growth of H1299 cells. Flowcytometric analysis of DNA contents showed G0/G1 arrest of cell cycle and no apoptosis. To examine the effect of p21-mediated G1 arrest on the induction of apoptosis by p53 gene transfer, H1299 cells were sequentially infected with AdCMVp21 and AdCMVp53. Overexpression of p53 on cells arresting at the G1 phase resulted in a rapid cell death, indicative of apoptosis. Time-course-flowcytometric analysis showed that cells in the G1 phase directly underwent apoptosis without the entry into the S phase. Thus, p53-mediated apoptosis is not affected by p21-mediated G1 arrest. These results suggested that p53 expression could overcome p21-mediated G1 arrest by inducing apoptosis and that p53 gene transfer may be an effective inducer of cell death even on the resting tumor cells.

PHARMACOLOGY/THERAPEUTICS (PRECLINICAL AND CLINICAL) 9: Polyamines, Ether Lipids, Signal Transduction Inhibitors, and Antifolates

#632 A synergistic antiproliferative effect of a polyamine analogue and a triplex forming oligonucleotide (TFO) on MCF-7 breast cancer cells. Balabhadrapathruni, S., Thomas, T., Shirahata, A., and Thomas, T.J. *UMDNJ-Robert Wood Johnson Medical School, New Brunswick, NJ 08903, Josai University, Saitama 350-02, Japan*

Targeting of oligonucleotide oligopyrimidine sequences in the promoter regions of specific genes by TFOs is a novel strategy to inhibit the transcription of disease-related genes. Recent investigations have revealed that synthetic analogues of the natural polyamines are excellent promoters of triplex DNA. We studied the effects of a series of spermine analogues (E₁H₂NCH₂CH₂NHCH₂CH₂NHE₁; where x, y and z are 3 or 4; for spermine x=3, y=4, and z=3; E₁=ethyl and a 37-mer TFO (targeted to the promoter region of ornithine decarboxylase) as triplex dual agents and in combination on the proliferation of MCF-7 cells by [³H]-thymidine uptake. This TFO exerted a 25% inhibitory effect at the 48 h time point. Bisiethylspermine and norspermine (x=3, y=3, z=3) had no significant effect on [³H]-thymidine uptake, whereas bisethylhomospermine (x=4, y=4, z=4) exerted a 20% inhibitory effect at 5 μ M concentration. Combinations of TFO with bisethylspermine and norspermine exerted no significant effect on DNA synthesis. In contrast, a combination of TFO and bisethylhomospermine inhibited [³H]-thymidine uptake by 90%, suggesting a synergistic action of this combination. These data indicate that selective use of polyamine analogs is a viable strategy to develop an anti-gene therapeutic approach for breast cancer.