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The Role of Toxicological Interactions in Lung Injury

by H. P. Witschi* and P. J. Hakkinen*

Interactions between two or more toxic agents can produce lung damage by chemical–chemical interactions, chemical–receptor interactions or by modification, by a first agent, of the cell and tissue response to a second agent. Interactions may occur by simultaneous exposure and if exposure to the two agents is separated in time. Chemical–chemical interactions have been mostly studied in the toxicology of air pollutants, where it was shown that the untoward effect of certain oxidants may be enhanced in the presence of other aerosols. Interactions at the receptor site have been found in isolated perfused lung experiments. Oxygen tolerance may be an example, when pre-exposure to one concentration of oxygen mitigates later exposure to 100% oxygen by modifying cellular and enzymatic composition of the lung. Damage of the alveolar zone by the antioxidant butylated hydroxytoluene (BHT) can be greatly enhanced by subsequent exposure to oxygen concentration which, otherwise, would have little if any demonstrable effect. The synergistic interaction between BHT and oxygen results in a resulting interstitial pulmonary fibrosis. Acute or chronic lung disease may then be caused not only by one agent, but very likely in many instances by the interaction of several agents.

Introduction

Toxicological interactions have been broadly defined as "a circumstance in which exposure to two or more chemicals results in a qualitatively or quantitatively altered biological response relative to that predicted from the action of a single chemical. The multiple chemical exposures may be simultaneous or sequential in time and the altered response may be greater or smaller in magnitude" (*1*).

If exposure to two or more chemicals occurs simultaneously, we may expect a modified biological response if the two agents have the potential to react directly with one another. The action of one agent may be cancelled by the other (neutralization reactions, chelation of heavy metals), or two agents may react with each other to form a more toxic species (nitrosamines from secondary amines and nitrites). Another mechanism involves competition between chemicals at their target sites which may be the molecular site of absorption, activation, detoxification, injurious action or excretion.

It is important to recognize that a modified biological response may also occur if exposure to two agents is separated in time. Induction of mixed function oxidases is one of the best studied examples. Pretreatment with or inadvertent exposure to a variety of agents will in many tissues profoundly modify the response to a second chemical (*2*). Interactions of this type are most often investigated and interpreted in terms of altered metabolic pathways. Examples include potentiation of haloalkane-induced liver necrosis by ketogenic agents (*3*), the shift of 4-ipomeanol toxicity from lung to liver (*4*) or protection against chemical carcinogenesis by various dietary regimens (*5*). Chemicals may also alter cell proliferation or produce shifts in cell-age distribution and alter the biological response for example to ionizing radiation (*6*). Others induce irreversible changes in target cells which then become enhanced or expressed following exposure to a second agent. This is the case in two-stage carcinogenesis (*7*). We may thus label this type of interaction, which does not necessarily require simultaneous exposure to two or more chemicals, as interactions involving altered cell and tissue responsiveness.

All three types of interaction mentioned above —chemical–chemical, chemical–receptor and altered cell and tissue responsiveness—may play a role in the pathogenesis of acute and chronic lung disease. This will be illustrated with selected examples from the literature.

Interactions as a Result of Simultaneous Exposure

Chemical–Chemical Interactions

Air Pollutants. The irritating effects of SO_2, the second most abundant air pollutant, are greatly en-

*Biology Division, Oak Ridge National Laboratory and The University of Tennessee–Oak Ridge Graduate School of Biomedical Sciences, Oak Ridge, TN 37831.

hanced if guinea pigs are exposed simultaneously to aqueous aerosols of various salts soluble in water (*8,9*). Both the solubility of SO_2 in a given aerosol droplet and the ability of the droplet to catalyze the oxidation of SO_2 to H_2SO_4 play a role in increasing airway resistance. An aerosol in which SO_2 is insoluble (e.g., iron oxide fume, activated carbon) does not potentiate the response to SO_2 while potentiation is readily demonstrable by exposure to aerosols in which H_2SO_4 is soluble (e.g., sodium or potassium chloride, ammonium thiocyanate, soluble salts of manganese, iron and vanadium).

The particle size of an aerosol also plays a role. For example, a synergistic increase in guinea pig airway resistance was observed when sodium chloride particles with 0.2 μm diameter were combined with SO_2, but not when particles had a diameter of 2.5 μm (*10*). The same study also showed a greater than additive increase in guinea pig airway resistance when particles of sulfuric acid mist of 0.8 μm, but not of 2.5 μm, diameter were combined with SO_2.

Aerosol–gas interactions were also examined by Last et al. (*11,12*). Biochemical changes in the lungs of rats (DNA, RNA and protein content and activities of various lysosomal enzymes) exposed to O_3 and a H_2SO_4 aerosol exceeded the sum of the response induced by either agent alone. Rats exposed to an O_3 and H_2SO_4 mixture showed a synergistic increase in the rate of mucus glycoprotein secretion from tracheal explants (*13*). LeMesurier and co-workers (*14*) found that rats exposed to a combination of trichloroethylene vapor and cigarette smoke for 15 days had reductions in surfactant secretion to a level far below that found with either agent alone. Potentially important chemical–chemical interactions in the lung may also occur between an inhalant and a bloodborne agent. It was recently shown that this may not only produce acute lung damage, but may also lead to the development of chronic pathologic conditions (*15*).

Direct interactions between two chemicals may on occasion mitigate rather than enhance toxicity. Ammonia is known to reduce H_2SO_4 mist toxicity, as was first demonstrated in guinea pigs (*16*). Anecdotal evidence of ammonia protecting against SO_2 is given in an account of the London fog disaster of 1952 (*17*). Animals housed in pens that had not been regularly cleaned suffered far less pulmonary toxicity than those kept in clean surroundings. Presumably, the ammonia fumes emanating from the animal waste in the dirty pens combined with the SO_2 in the immediate atmosphere to form ammonium sulfate which is much less toxic than either SO_2 or H_2SO_4. Attention must be paid to this neutralizing effect of NH_3 in long-term inhalation studies (*18*).

Phalen and co-workers (*19*) examined the effects of several aerosols (ammonium sulfate, ferric sulfate and H_2SO_4) in combination with O_3 on the rates of tracer particle clearance in rats. It was found that the effects of the aerosols combined with O_3 were qualitatively similar to those observed with O_3 alone. However, the combination often produced effects quantitatively less than those observed with O_3 alone. A reduction in the lethality of nitric acid fumes and an increase in the lethality of formaldehyde vapors were observed during simultaneous exposure of mice to certain aerosols (*20*). In another gas–aerosol interaction, the toxicity of NaOH aerosols was lessened by reaction with ambient CO_2 and water vapor resulting in the formation of the much less alkaline and toxic sodium carbonate. The damage to the deep lung was further lessened by the changes in aerodynamic behavior brought about by the new size and density of the particles which helped to limit deposition to the upper respiratory tract and nasal passages (*21*).

In attempts to document unequivocally interactions between inhaled gases in humans somewhat ambiguous results were obtained. Linn and co-workers (*22*) did not find significant alterations in pulmonary function in humans exposed to a mixture of NO_2 and SO_2 compared to exposure to each gas alone, although several nonspecific symptoms (e.g., headache, cough, substernal irritation) were reported more frequently with the mixture. When humans were exposed to a combination of SO_2 and O_3, a much greater decline in maximum expiratory flow was recorded than found with exposure to either gas alone (*23*). Similar changes in pulmonary function were not found in later studies with this combination of pollutants (*24–26*). These conflicting results may be at least partially explained by interexperimental variations in levels of particulates within the exposure chamber, and differences in temperature and humidity. In fact, a less severe decline in ventilatory function was observed when some of the same subjects used in the Hazucha and Bates study (*23*) were exposed under stricter conditions of particulate, temperature, and humidity control (*26*).

Direct reaction between two chemicals may thus modify the response following inhalation of agents or following simultaneous exposure to an inhalant and a bloodborne agent. The biological response can be increased or mitigated. However, it must be pointed out that studies on interactions between air pollutants in the laboratory setting may differ from the actual interactions which may occur in man in the outside world. This is particularly true when, in experiments, natural "filtering" processes are circumvented. The role of the nasal–pharyngeal region in absorbing water-soluble compounds such as SO_2 and ammonia must be remembered because this may play a role when particulates are present to combine with these agents (*9*). It must also be kept in mind that with a small number of human subjects in a given study, one may fail to include individuals such as asthmatics or healthy humans who breathe oronasally at rest (*27–29*). These people are or may be unusually sensitive to air pollutants. Any results obtained in a limited study may underestimate the risk to sensitive individuals. For example, asthmatics develop

broncho-constriction at considerably lower concentrations of inhaled SO_2 than healthy people without asthma (*29*). This makes it somewhat difficult to extrapolate from laboratory studies and to estimate fully and precisely the risk of multichemical exposure to all segments of the population.

Oxygen Free Radicals. There is evidence to show that pulmonary oxygen toxicity in experimental animals is greatly enhanced by the concomitant presence of several agents in the lung. Examples are the dipyridylium herbicides paraquat and diquat (*30–32*), the antibacterial drug nitrofurantoin (*33*), and disulfiram (*34*). A characteristic feature of the interaction between O_2 and these chemicals is the greatly accelerated and increased mortality which is preceded and accompanied by the development of massive intraalveolar and perivascular edema.

It is thought that both the dipyridylium herbicides and nitrofurantoin in the presence of oxygen undergo cyclical reduction and oxidation, with concomitant consumption of NADPH and generation of large amounts of superoxide anion. Evidence for NADPH depletion and superoxide anion formation has been found both in *in vitro* and *in vivo* conditions (*33–38*). The superoxide anion would then undergo further transformation to such reactive species as singlet oxygen, hydrogen peroxide and hydroxyl radicals.

Paraquat is actively taken up by the lung, whereas diquat is not (*39*). Given at equimolar doses, about 5 to 10 times as much paraquat as diquat is found in rat lung a few hours following intravenous injection (*38*). However, the lethal interaction between oxygen and paraquat or diquat is not necessarily proportional to the tissue concentrations of the herbicides. With low doses (5 mg/kg IV) the LT_{50} (time for 50% of the animals to die) in 100% O_2 is considerably shorter for paraquat compared to diquat. However, following injection of 80 mg/kg, diquat-treated animals die sooner. If paraquat and diquat are given at equitoxic doses (20 mg/kg with an LT_{50} in 100% O_2 of 11–12 hr for both compounds), rats treated with diquat are much more susceptible to 40% O_2 than are rats treated with paraquat (*32*). Diquat has a lower redox potential than has paraquat (*40*), and diquat radicals have been shown to cycle more rapidly in tissue homogenates than paraquat radicals (*41*). Exacerbation of pulmonary injury caused by simultaneous exposure to paraquat or diquat and to 100% oxygen depends thus not only on amount of the toxic agent present, but also on rates of formation of the ultimately toxic molecular species.

A similar mechanism was postulated for the interaction between O_2 and nitrofurantoin (*33*). Increased destruction of oxygen free radicals by enzymatic scavengers should therefore abolish or at least mitigate oxygen toxicity. There is strong evidence to suggest that endogenous superoxide dismutase (SOD) (mitochondrial and cytoplasmic) plays a key role in protecting the lung against oxygen toxicity by dismutating singlet oxygen to hydrogen peroxide (*42*). Administration of exogenous SOD might be a rational procedure to counteract oxygen toxicity. While several *in vitro* studies have indicated SOD to be an effective antidote, results of *in vivo* experiments are somewhat conflicting. McLennan and Autor (*43*) reported that continuous delivery of SOD at a rate of 140 units/hr protected rats exposed to 95% O_2 against pulmonary damage. Continuous delivery of the SOD was achieved with osmotic minipumps implanted into the peritoneal cavity. On the other hand, Crapo et al. (*44*) failed to protect rats against the lethal effects of 100% O_2 when SOD was injected or administered as an aerosol. It was also not possible to protect mice against potentiation of butylated hydroxytoluene (BHT)-induced lung damage by 70% O_2 with a continuous, 7-day-long infusion of SOD, given IP or SC by use of an osmotic minipump at a rate of 62 units/hr (Hakkinen, unpublished observations). SOD reportedly was used with some success to protect animals under other experimental conditions where oxygen free radicals are thought to be ultimately responsible for injury, such as whole-body or localized skin X-irradiation or in acute paraquat poisoning (*45–49*). However, administration of 10 mg/kg of SOD IP twice a day to rats deficient in vitamin E for 3 days before and 3 days after paraquat failed to prevent paraquat toxicity (*50*). While there appears to be no question that endogenous SOD is a key enzyme in defending lung tissue against oxygen toxicity (*42,51*), it appears to be more difficult to achieve effective levels of exogeneous SOD at the target sites of oxygen toxicity. This is, of course, a common problem in all attempts to provide antidotal therapy based on direct interactions between a toxic molecular species and compounds designed to neutralize or to deactivate them. More research in this important area is needed, particularly since it has been found that other free radical scavengers such as cysteamine and cysteine may give some protection against free radical-mediated lung injury. Continuous infusion of cysteamine hydrochloride to rats exposed to 100% O_2 reduced pulmonary edema, protected against oxidation of the lung sufhydryl pool, prolonged survival and decreased mortality (*52*).

Competition between Agents at Cellular Targets, Storage and Uptake Sites

A great deal of information is now available on the pulmonary uptake, metabolism and disposition of endogenous and exogenous chemicals. The role of the lung as a metabolic organ is now well recognized (*53*). Many of those studies have been done with isolated perfused lung preparations. It is now evident that competition between chemicals at sites of uptake and metabolism plays an important role in drug metabolism and disposition and in interaction between exogenous and endogenous agents (*54*).

The naturally occurring amines 5-hydroxytryptamine (5-HT, serotonin) and norepinephrine accumulate in the

lung against a concentration gradient (*55*). It has been shown that uptake of 5-HT occurs by an energy-requiring Na^+-dependent mechanism; the site of uptake is the capillary pulmonary endothelium. Ouabain and imipramine inhibit 5-HT uptake (*55, 56*). The anorexigenic agent chlorphentermine, which itself accumulates in lung tissue, may decrease both the uptake of 5-HT by the pulmonary endothelial cells and also its subsequent metabolism by monamine oxidase (*57*). Since both chlorphentermine and 5-HT are taken up at least partially by Na-dependent transport mechanisms, it is likely that chlorphentermine inhibits 5-HT uptake by occupying sites on a carrier molecule. It has also been suggested that chlorphentermine liberates 5-HT from storage sites (*58*). Since anorexigenic agents such as aminorex and chlorphentermine have been shown to produce pulmonary hypertension, it is conceivable that a combination of uptake inhibition, decreased metabolism and release of stored 5-HT from lung storage sites plays a role in the etiology of drug-induced pulmonary hypertension (*57*).

Several exogenous basic amines, such as *d*-propranolol, methadone and phenoxybenzamine, are concentrated in the lung (*54*). Compounds with similar physicochemical characteristics may inhibit their uptake and binding or accelerate the release of agents already taken up (*59*). An attempt was made to exploit the possible competition between different compounds at recognition sites of the cell membrane for a rational approach to the treatment of paraquat poisoning. It was found that several drugs, among them propranolol and endogenous amines such as 5-HT and histamine, inhibit uptake of paraquat by lung slices *in vitro* (*60*). However, propranolol injection 1 hr following paraquat did not protect rats (*49*). In human cases of paraquat poisoning the benefits of propranolol treatment remain ambiguous (*61, 62*).

There are, however, other examples which show that competition between molecules at sites of uptake or metabolism in the lung prevents or at least alleviates toxicity. Treatment of animals with SKF-525A or with piperonyl butoxide protects mice and rats against Clara cell necrosis produced by 4-ipomeanol, 3-methylfuran and CCl_4 (*4*) or against alveolar Type I cell damage by BHT (*63*). All these compounds presumably require metabolic conversion to a reactive intermediate by mixed function oxidases prior to causing cell death. Since both SKF-525A and piperonyl butoxide or a metabolite thereof are competitive inhibitors for these agents at cytochrome P-450, toxicity is prevented.

An interesting observation is that mice kept on red cedar shavings can be completely protected against BHT-induced lung damage (*64*). It has been known for some time that certain wood beddings induce drug-metabolizing enzymes in the liver and may decrease the toxicity of certain compounds by enhancing pathways of inactivation or detoxification (*65*). Protection against BHT damage in lung could thus be caused by enhanced detoxification of BHT by pulmonary mixed function oxidases. Inhalation of inducing agents increases pulmonary aryl hydrocarbon hydroxylase activity to peak activity within a few hours, whereas intraperitoneal injection of inducers usually results in much slower increases in enzyme activity in lung (*66,67*). However, mice are protected against BHT toxicity not only when they are placed on red cedar shavings shortly before BHT injection, but even if placed upon it up to 3 hr after BHT (*64*). This makes it unlikely that protection against BHT damage is a result of enhanced detoxification following enzyme induction. Rather, it must be assumed that volatile compounds emanating from the bedding and inhaled by the animals act as competitors for BHT at its site of uptake or biotransformation in the lung. These compounds are in all likelihood certain cedar terpenes, such as cedrol, or sesquiterpenes (*64*). The experiments done by Malkinson (*64*) show that it is possible to prevent pulmonary toxicity caused by a bloodborne agent by inhalation of suitable antagonists or competitors even after administration of the toxic agent. Efforts should be made to determine whether such a mechanism of interaction can be exploited to protect lung against other toxic agents.

Interactions Resulting from Exposures Separated in Time

Acute diffuse lung injury caused by many toxic inhalants and bloodborne agents often produces profound changes in cellular structure and function (*68*). This in turn, may modify the biological response to a subsequent exposure to another toxic agent. As a consequence, toxicological interactions may also play a role in producing or protecting against lung injury even if the exposure to two or more agents is not simultaneous, but separated in time.

Interactions Where the Response to a Second Agent is Modified

Induction of Pulmonary Mixed Function Oxidases. Induction of hepatic microsomal metabolism greatly increases the toxicity of several compounds, such as CCl_4 or acetaminophen, whereas the untoward effects of other chemicals such as 2-acetylaminofluorene or aminoazo dyes may be mitigated or abolished (*69–72*). Similarly, enzyme induction in lung by one agent may modify the response produced by a second agent (*4,73*). However, relationships among increased biotransformation, formation of active metabolites and enhancement of pathways of detoxification are often complex. It must also be kept in mind that the organ involved in the formation of the active and presumably toxic metabolite of a compound may not always be the only site of toxicity. In the mouse, bromobenzene toxicity in lung and kidney appears to be mediated through formation of bromobenzene epoxide in the liver. Phenobarbital pretreatment did not increase mixed function oxidase

activity in mouse lung or kidney, but aggravated toxicity and also the amount of covalently bound bromobenzene metabolites in both organs. Pretreatment with piperonyl butoxide had the opposite effect (*73*). The data suggest that bromobenzene is converted in the liver to an active intermediate of sufficient stability to reach other tissues via the bloodstream and to cause damage at sites distant from its original formation. It is important to realize that toxicological interactions may result in shifts in target organs and this may affect pulmonary toxicity.

This has also been documented in studies with 4-ipomeanol and 3-methylfuran. Evidence showing that both these compounds undergo metabolic activation to highly reactive intermediates is discussed elsewhere in this volume. In both mice and rats 4-ipomeanol produces acute Clara cell necrosis. Pretreatment of animals with 3-methylcholanthrene, an inducer of mixed-function oxidases in both liver and lung, increases hepatotoxicity and a greater amount of covalently bound metabolite is found in the liver. At the same time, pulmonary toxicity is diminished or even abolished. Toxicity can only be modulated by 3-methylcholanthrene in mouse strains where aryl hydrocarbon hydroxylase is inducible, whereas no shifts in toxicity are produced in noninducible strains (*4*). A second enzyme inducer, phenobarbital, did not shift 4-ipomeanol toxicity from lung to liver, but decreased overall toxicity and lung damage in rats (*74*). An interesting observation is that pretreatment with small doses of 4-ipomeanol makes rats resistant to a subsequent otherwise lethal dose of the same compound. This is not a unique observation; mice can be made resistant to CCl_4 hepatotoxicity with small doses of CCl_4 (*75*). Destruction of cytochrome P-450 by the first small dose may prevent the liver or lung from converting the second dose of an agent to its ultimately toxic metabolite. Pretreatment with 4-ipomeanol also produces resistance to other toxic furan compounds and to the edematagenic agent α-naphthylthiourea (ANTU). However, ANTU pretreatment will not protect against 4-ipomeanol (*76*), although ANTU has been shown to inhibit pulmonary mixed function oxidases (*77*). The reasons for this are unknown but may be due to the apparently different types of lung injury produced by ANTU and 4-ipomeanol and the different mechanisms of toxicity (*4*).

Data on modulation of lung injury by agents altering the activity of pulmonary mixed function oxidases are, at present, much less complete than data on modulation of hepatotoxicity by such agents. This may be because the role of biotransformation in the pathogenesis of toxic lung damage has only recently been appreciated. Also, quantitative information on primary lung injury is less readily obtained than on toxic liver injury.

From the examples discussed it is obvious that agents which enhance or destroy the activity of pulmonary mixed function oxidases may influence toxic response. However, it cannot be anticipated a priori whether toxicity of a given compound will be enhanced or mitigated, since both detoxifying pathways and toxifying ones may be stimulated. Enzyme inducers also may shift toxicity from one organ to another. Effects seen in one species cannot necessarily be extrapolated to other species, as in the case of bromobenzene toxicity in rats and mice (*73*).

Enzyme inducers represent one example where we find that pulmonary injury is modified by two agents, even if exposure occurs separated in time. The temporal sequence of exposure is important because inducers must be given before the second challenging agent; if the exposure is reversed, no interactions occur.

Induction of Tolerance. It has been mentioned before that small doses of 4-ipomeanol, ANTU or BHT afford protection against a second, larger dose of the same agents. The phenomenon can be called tolerance and, as such, has been known for a long time in pulmonary toxicology (*78*). Some newer observations offer additional insight into this particular form of interaction.

The cells lining the alveolar and capillary walls undergo profound morphological changes when exposed to hyperoxia. Exposure of adult rats to 100% O_2 until death destroys almost half of the capillary endothelial cells. In animals exposed to 85% O_2, about 40% of the capillary endothelial cells are destroyed. The endothelial cells that survive 7 days in 85% O_2 show hypertrophy and from this time on they no longer become damaged even if 85% O_2 exposure is continued (*79*). Activities of glucose-6-phosphate dehydrogenase (G6PD), catalase (C) and the mitrochondrial and cytosolic superoxide dismutases are also increased by exposure to 85% O_2. This increased activity of pulmonary enzymes coincides with the development of "tolerance." Animals made tolerant by prior exposure to 85% O_2 for 5 to 7 days are able to survive indefinitely at 100% O_2 (*51,79–83*).

In another study of O_2 tolerance, Crapo and co-workers (*82, 84*) have shown that rats made tolerant to 100% O_2 by prior exposure to 85% O_2 exhibited partial cross-tolerance to 75 ppm NO_2. Rats made tolerant to 75 ppm NO_2 by prior exposure to 25 ppm NO_2 did not show significant cross-tolerance to 100% O_2. As mentioned above, O_2 tolerance coincided with increased SOD activity. However, the development of NO_2 "tolerance" did not result in a significant increase in pulmonary SOD activity, although catalase activity was significantly increased. This may explain why NO_2 pretreatment fails to give cross-protection against 100% O_2. Differences in diffusion of O_2 and NO_2 into the deep lung and resulting differences in distribution of lesions produced by the two agents may play a role in the development of tolerance and cross-tolerance found between these two gases.

Finally, rats exposed to O_3 (approx. 0.8 ppm for 7 days) have been shown to develop cross-tolerance to 96–98% O_2. O_3-exposed animals had significant increases in lung superoxide dismutase, glutathione peroxidase (GP) and glucose-6-phosphate dehydrogenase (G6PD) activities by day 7 of O_3 exposure and became tolerant

to O_3. Survival of these animals was markedly increased when exposed to 96–98% O_2 (*85*). Thus, the oxidant component of photochemical smog that causes a significant increase in SOD activity (O_3) produces cross-tolerance to hyperoxia, while NO_2, which does not elevate SOD activity, shows no cross-tolerance to subsequent O_2 exposure.

Tolerance to oxygen may also be produced by the bacterial lipopolysaccharide, endotoxin. This phenomenon has been well documented (*86–89*). Although the mechanism of protection remains unclear, small doses of endotoxin given to oxygen-exposed adult rats have been shown to significantly increase survival and decrease lung fluid accumulation if given up to 36 hr following the onset of exposure to 95% oxygen. Unlike the situation where protection against 100% oxygen is achieved by pretreatment with lower oxygen concentrations, endotoxin-induced protection requires no pretreatment schedule (*88*). Interestingly, endotoxin also protects against lung injury produced by the bloodborne edematogenic agent thiourea (*90*).

The biochemical response of the endotoxin-treated adult rat lung exposed to hyperoxia is very similar to that observed in untreated neonatal animals. Whereas untreated adult rats upon exposure to 100% O_2 show no increase in antioxidant enzyme levels (SOD, glutathione peroxidase and catalase), a rapid and significant increase occurs in the lungs of neonatal animals and endotoxin-treated rats (*89, 91–93*). However, in endotoxin-treated adult mice, exposure to oxygen produced neither an increase in lung antioxidant enzyme nor protection from oxygen toxicity (*87*).

A potentially important form of tolerance induction, which might directly relate to human toxicology, was found with the anticancer drug cyclophosphamide. Mice given a small "priming" dose of cyclophosphamide show increased survival following a subsequent larger dose. Lung damage, as measured by increased ventilation rate, was significantly reduced if the priming dose (50 mg/kg) was given either 7 or 14 days before 250 mg/kg of cyclophosphamide. However, if the animals were "primed" on the day prior to the large dose of the drug, lung damage seemed to be enhanced (*94*). The mechanism(s) by which cyclophosphamide pretreatment enhances or mitigates lung toxicity remains to be elucidated. It is tempting to speculate that the cell types proliferating in the lung at the given time following cyclophosphamide-induced injury determine the reaction to the second insult. Collis and co-workers (*94*) have observed acute interstitial pneumonitis and alveolar Type II cell hyperplasia one week after 300 mg/kg cyclophosphamide. Further Type II cell hyperplasia was observed 2 weeks following cyclophosphamide. We found that treatment of mice with 200 mg/kg cyclophosphamide resulted in an increase in ^{14}C-thymidine incorporation into pulmonary DNA between days 4 and 21 after treatment with one peak occurring at day 7 (*95*). The correlations of these increases in thymidine incorporation with proliferation of specific cell types and with induction of tolerance remain to be established. However, the observation by Collis et al. (*94*) suggests that following treatment with a small dose of pulmonary toxin a second large dose may produce increased or decreased lesions, depending upon the time interval between administration. This emphasizes the importance of temporal relationships.

Finally, it must be mentioned that in numerous studies an attempt was made to protect animals against oxidant-induced lung injury by treatment with dietary antioxidants such as vitamin E, vitamin K, selenium and other agents. Such treatments may produce biochemical changes in the lung which, in turn, may alter the response to oxidant exposure. Much of the data have recently been reviewed (*83*).

Interactions Where the Development of Lesions Produced by a First Agent Is Enhanced

Administration of inducers of mixed function oxidases or induction of tolerance alters lung metabolism and possibly lung structure in such a way that the response to a second, challenging toxic agent is enhanced or mitigated. However, it is also possible to amplify the lesions produced by a first agent with exposure to an otherwise nontoxic dose of a second agent.

A single dose of the antioxidant BHT causes acute lung damage in mice. The original observation was made in 1972 (*96*) and has since been confirmed in several laboratories (*64,97–101*). Diffuse lung lesions develop after oral or intraperitoneal administration of BHT and are dose-dependent (*102,103*). All mouse strains examined so far are susceptible to BHT (*64,98,102*). Other laboratory rodents, however, particularly rats, hamsters and rabbits, do not develop lung damage after BHT (*63,98,104*).

The morphologic and biochemical changes in mouse lung produced by BHT are very similar to the general pattern of primary pulmonary injury caused in many species by toxic agents (*68*). They can be summarized as follows: within the first 24 hr, many Type I alveolar epithelial cells show acute cytoplasmic edema undergo necrosis and eventually disintegrate, leaving behind a denuded basement membrane. Type II alveolar epithelial cells are not visibly damaged by BHT. They begin to proliferate; peak mitotic activity is seen on days 2 to 4 after BHT at which time as many as 80% of all dividing cells in the alveolar zone are Type II epithelial cells. Around days 4 and 5 after BHT, secondary damage to the capillary endothelial cells becomes apparent (*105*).

Interstitial cells appear not to become damaged by BHT, but rather proliferate and invade the empty areas left by damaged cells. Macrophages show extensive phagocytic activity and become filled with cell debris. Five to seven days after BHT, epithelial cell proliferation has largely subsided and most proliferating cells are capillary endothelial and interstitial cells (*105,106*), Two weeks after BHT, scattered alveolar wall hyper-

cellularity and occasional intraalveolar macrophages can still be seen by microscopy whereas at 3 weeks after BHT the lungs look virtually normal again (*107*).

Thus BHT initially produces a diffuse lung lesion. More important, cell proliferation following the initial insult proceeds in an orderly way; epithelial cells divide first, endothelial and interstitial cells somewhat later (*106*). Since earlier work had shown that oxygen inhibits cell division in the lung even in concentrations which do not damage a normal lung (*108*), the effects of oxygen on BHT damaged lungs were studied. Detailed analysis of time effect and dose effect suggested that exposure to 40 to 100% O_2 for as few as 16–24 hr would inhibit division of epithelial cells in lung, but not of capillary endothelial or interstitial cells (*109*). From these observations it was predicted that exposure of animals to oxygen early after BHT-induced lung injury would destroy epithelial cells while allowing fibroblasts to proliferate. The result should be fibrosis.

This prediction was found to be true. If animals were treated with BHT and placed immediately into an atmosphere of 50 to 80% O_2, extensive and diffuse pulmonary fibrosis developed within 2 weeks. In animals treated with BHT alone, total lung hydroxyproline was somewhat elevated, whereas in animals exposed to 70% O_2, no abnormal accumulation was found. However, the combined effect of BHT and O_2 were synergistic (*107*). Severity of fibrosis was found to be determined by the concentration of oxygen, duration of oxygen exposure, and extent of initial lung damage caused by BHT (*110*). Fibrosis developed in BHT damaged lungs, not only following exposure to oxygen, but also following treatment with other agents which interfere with cell profliferation in lung, such as a low dose of X-rays (50–200 rad) (*111*) and high doses of prednisolone (*112*). Prednisolone has recently been shown to interfere with epithelial cell proliferation in lung (*101*).

Exposure to 70% oxygen also produces fibrosis if animals are pretreated with lung-toxic agents other than BHT. Methylcyclopentadienyl manganese tricarbonyl (MMT) is an octane enhancer that is added to unleaded gasoline. It causes diffuse alveolar damage followed by cell proliferation in mice, rabbits, rats and hamsters following either topical, intraperitoneal or oral administration (*113–115;* Hakkinen, unpublished observations). In experiments similar to those done with BHT and oxygen, total lung collagen content in mice was significantly increased if MMT-treated mice were placed in 70% oxygen for 6 days following injection (*110*). We have also found statistically significant increases in total lung hydroxyproline content if cyclophosphamide-, bleomycin- or cadmium chloride aerosol-treated mice are placed immediately into 70% oxygen for 5 to 9 days (*95;* Hakkinen, unpublished observations).

In all these experiments, oxygen alone, given in concentrations up to 80% for a maximum of 6 days, did not produce any significant fibrosis. However, it enhanced greatly the development of fibrosis in previously damaged lungs. In this context it is interesting to note that intermittent exposure to > 95% O_2 (48 hr in oxygen, followed by 24-hr cycles of air alternating with oxygen) results in marked accumulation of hydroxyproline in rat lung (*116*). This might represent another example of fibrosis resulting from initial injury (first exposure) and subsequent interference with tissue recovery subsequent to intermittent exposures.

Acute lung damage caused by a variety of chemicals belonging to different classes and presumably having different mechanisms of initial toxicity may thus be greatly enhanced by a second toxic agent which interferes with recovery of the damaged alveolar tissue. The result is a quantitatively different lesion, abnormal accumulation of interstitial collagen. Following one acute episode of interaction between two such agents, a lesion develops which has been found to persist from 6 months up to 1 year (*117*). It must be emphasized that this only occurs if an acutely damaged lung is exposed to the second toxic agent within a few days. Exposure to oxygen or to X-rays after epithelial cell proliferation in lung will not produce fibrosis (*107, 110, 111*). The timing of exposure to two agents is thus critically important in the development of a toxicological interaction.

Aggravation of the response to a first agent in the lung by a second agent is thus an important example of toxicological interactions. It might explain two well-known human pathologic conditions: development of adult respiratory distress syndrome where it is suspected that oxygen therapy aggravates the initial lung damage (*118*), and rapid development of diffuse pulmonary fibrosis in patients treated concomitantly with chemotherapeutic agents and irradiation of the thorax (*119*). In 29 patients treated for oat cell carcinoma of the lung with combination chemotherapy consisting of bleomycin, adriamycin, cyclophosphamide and vincristine, no pulmonary complications arose whereas pulmonary fibrosis was a significant problem when the regimen was combined with thoracic irradiation (*120*). An interaction between anticancer drugs and X-ray irradiation was also found in mice. Administration of either adriamycin (14 days before), bleomycin (13 days before) or cyclophosphamide (either 3 hr before or 2 hr after) before thoracic irradiation decreased the survival times observed (*121*).

Conclusions

An attempt was made to discuss the possible role of toxicological interactions in the pathogenesis of acute and chronic lung injury. There is no question that under many circumstances exposure of the lung to two potentially toxic agents will result in a much more severe lesion than would exposure to each single agent alone. It is also possible that lung damage may be mitigated or even prevented if two agents cancel out each others action.

Interactions may occur if exposure is simultaneous or separated in time. In both cases, the nature and severity of the pulmonary lesions can often be pre-

dicted by knowing the chemical properties and mechanism of action of the two agents. However, if exposure is not simultaneous, an additional important factor needs to be considered: the temporal sequence of exposure. In all instances where the response to a second, challenging agent is modified by a first agent, the sequence of exposure cannot be reversed, and if it is, no interaction occurs. The only possible exception is induction of O_2 tolerance by endotoxin. On the other hand, whenever a second agent amplifies the response to a first agent, such as in animals treated with BHT and then exposed to oxygen or X-rays, an abnormal biological response is only seen if the exposure to the second agent occurs within a critical period following the initial injury.

Finally, it is realized that the classification of the different examples of toxicological interactions often was arbitrary and perhaps artificial. However, it is hoped that this general framework will help the further study and analysis of the role of interactions in a systematic way.

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