Use of Bronchoalveolar Lavage to Detect Lung Damage

by Rogene F. Henderson*

The assay of bronchoalveolar washings from acutely exposed animals has proven useful as a rapid screen for lung injury from inhaled airborne toxins. The screen is useful for choosing appropriate compounds and exposure levels for subsequent in-depth studies in which complete histopathologic evaluations will be made. An inflammatory response can be detected by the appearance of polymorphonuclear leukocytes and an increase in protein content of lung washings. The release of the cytoplasmic enzyme, lactate dehydrogenase, into the acellular portion of the lavage fluid serves as an indication of cell death or membrane damage. A large increase in some lysosomal enzymes has been found in the bronchoalveolar lavage fluids from animals chronically exposed to insoluble particles. Angiotensin-converting enzyme has been found to be elevated in bronchoalveolar washings from animals with endothelial cell damage in the pulmonary capillaries. The correlation of these cellular and biochemical alterations in the bronchoalveolar lavage fluid with morphological indications of damage has served to validate this method of detecting acute lung injury. Further study is needed to validate indicators of developing chronic disease.

Introduction

As the need to evaluate the inhalation toxicity of environmentally available substances grows, there has been an increasing demand for short-term tests that can be used as reliable predictors of the effects an agent will have on the lung during long-term exposures to low levels of a pollutant. There is a need to monitor both the ability of an agent to cause cell killing and induce an acute inflammatory response in the lung and the ability of the agent to alter normal growth and repair processes so as to induce development of chronic lung pathology such as fibrosis, emphysema or neoplasia. The cytotoxicity of a compound has been estimated in cell culture tests such as that of Waters et al. (1), in which pulmonary alveolar macrophages are used as the test cell, and the endpoint observed is cell death as measured by dye exclusion or cell injury as measured by release of the cytoplasmic enzyme, lactate dehydrogenase (LDH). Many short-term tests have been developed to assay for the mutagenic activity of an agent in bacteria (2,3), in mammalian cells in culture (4), in insects (5) and in fungi (6). The works of Heppleston and Styles (7) and of Bitterman et al. (8) indicate possible screening tests that can be performed with pulmonary macrophages *in vitro* to assay for the fibrogenic poten-

tial of a material. Such *in vitro* tests, while useful from the point of view of rapidity and relative low cost, lack the ability to monitor the integrated response of the whole body to the potentially inhalable agent.

One approach to a rapid *in vivo* screen to evaluate the potential pulmonary toxicity of a respirable substance is the use of bronchoalveolar washings to detect lung damage in acutely exposed animals. In the past, analyses of body fluids such as blood and urine have proved useful for the detection of pathology in specific organs, especially the heart and the liver; however, these tests have not been sensitive to lung injury. Analysis of bronchoalveolar fluids, sampled by segmental or total lung washings, has the potential of being a useful tool as a rapid screen for lung injury.

Lavage Fluid Parameters Useful as Indicators of Lung Injury

What parameters should be altered in bronchoalveolar fluids in the injured lung? Some possible indicators of pulmonary damage are listed in Table 1. If damage to the alveolar–capillary barrier occurs and edema is present, one would expect lung washings to contain increased levels of serum proteins and enzymatic activities associated with serum proteins. If upper airway damage or irritancy is produced, airway washings should contain higher levels of mucous secretions, which can be monitored by analysis of free and hydrolyzable sialic acid in lung washings. However, serum also

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contains glycoprotein so that increased levels of sialic acid in bronchoalveolar washings may result from transudation of serum proteins, as well as from increased mucous secretions. The alveoli contain surface-active lipids necessary for maintaining their structural integrity (9). Transudation of serum protein is known to deplete the surfactant lining of the alveoli (10), and thus measurements of change in amount or type of lipid in lung washings could be used to detect some types of lung damage.

Changes in levels of enzymatic activities in the washings may also indicate levels of injury. Lactate dehydrogenase, a cytoplasmic enzyme, should be extracellular in bronchoalveolar lavage fluid only if cell lysis or cell membrane damage has occurred. Despite being highly oxygenated, lung tissues contain high levels of LDH (11). An elevation in total lung LDH activity has been reported in response to lung damage caused by O_2 (12–14), cadmium (15) or NO_2 (16), but the studies did not distinguish between LDH of lung tissue and of infiltrating cells. Other cytosol enzymes which might be expected to be elevated in lung tissue following some types of injury (17) and which might leak from injured cells are glucose-6-phosphate dehydrogenase (G6PDH) and glutathione reductase and peroxidase. The G6PDH is a marker enzyme for the hexose monophosphate shunt and has been found to be elevated in response to oxidant injury (18,19) and in activated macrophages (20). This enzyme has been demonstrated histochemically in Type I cells (21), the alveolar cells which appear to be most vulnerable to injury (22). Glutathione reductase and peroxidase are cytoplasmic enzymes involved in the lung's protective mechanisms against damage from peroxides formed by high oxygen pressures or exposure to oxidant gases.

Lysosomal enzymes, such as acid phosphatase, β-glucuronidase and β-N-acetylglucosaminidase are released by polymorphonuclear leukocytes during phagocytosis and to some extent by macrophages (23,24). In addition, Hook (25) has reported extracellular hydrolases washed from the airway of rabbits. The level and type of hydrolases (high levels of β-N-acetylglucosaminidase and α-mannosidase and low levels of β-glucuronidase and arylsulfatase) point to a Type II lamellar body origin for the enzymes. Dependent on the type of hydrolase, increased hydrolytic activity washed from the lung could be used as an indicator of increased phagocytic activity or Type II cell injury. The enzyme, alkaline phosphatase, is associated with plasma membranes of mammalian cells (26) and has been observed histochemically in Type II cells (27). A soluble form of this enzyme with a lung-specific isoenzyme pattern has been reported in pulmonary washings from healthy rabbits (28). The authors suggest that the lung specific enzyme, which may be secreted by Type II cells along with the lamellar inclusion bodies with which the enzyme has been shown to be associated (27,29), may prove to be a good indicator of pulmonary disease. Increased alkaline phosphatase activity in bronchial aspirates has been used to detect pulmonary carcinoma (27).

Airway enzymes that may play a significant role in the development of chronic injury are the proteolytic enzymes and their counterpart, the antiproteolytic proteins. The balance between these two types of activities is crucial to the maintenance of the structural integrity of lung tissue (30,31). The presence of α_1-antitrypsin has been demonstrated in lung washings from dogs (32) and patients with alveolar proteinosis (33). The α_2-macroglobulin was also found in lavage fluid

Table 1. Indicators of acute injury in bronchoalveolar lavage fluids.

Parameter	Location	Possible indication if elevated
Lactate dehydrogenase	Cytosol (glycolysis)	Cell damage (increased membrane permeability to frank cell lysis)
Glucose-6-phosphate-dehydrogenase	Cytosol (hexose monophosphate shunt)	Cell damage; leakage from cells undergoing repair
Lysosomal acid hydrolases	Lysosomes	Release during phagocytosis; PMN and/or macrophage damage
Alkaline phosphatase	Plasma membranes, Type II cell lamellar bodies, serum	Type II cell damage or increased secretions; transudation of serum proteins
Glutathione peroxidase Glutathione reductase	Cytosol	Protection mechanism activated against lipoperoxidation
Angiotension converting enzyme	Endothelial cells	Endothelial cell damage
Total protein	Extracellular	Transudation of proteins across alveolar–capillary barrier
Sialic acid	Mucus Glycoproteins	Increased mucus secretion Transudation of serum glycoproteins
Phagocytic cells PMNs Macrophages		Inflammation

from the patients and, at lower levels, in lavage fluid from normal volunteers. Plasminogen activator, a secretory product of the alveolar macrophages (34,35), has been detected in lavage fluid (33). A group in France has reported measurements of chymotrypsinlike activity, collagenase and elastase activity in macrophages and in supernatant fluids of bronchoalveolar lavage from humans (36). The same group also detected α_2-macroglobulin, α_1-antichymotrypsin and α_1-antitrypsin in the lavage supernatants.

In addition to these enzymatic indicators of injury, the cellular content of lung washings should be a good indication of lung pathology. An influx of polymorphonuclear leukocytes would be expected early in an inflammatory response while an increase in alveolar pulmonary macrophages has been shown to be a long-term component of persistent inflammation (37). The use of sputum cytology has long been used as a diagnostic tool to detect neoplasia of the respiratory tract in man (38). Saccomanno and co-workers (39,40) have used sputum cytologies to perform prospective studies on the development of lung cancer in uranium miners. Investigators at NIH have used the cellular content of bronchoalveolar lavage fluid to diagnose the type and stage of activity of various types of interstitial lung disorders (41–43).

Method of Lavage

The exposed lungs of test animals can be lavaged either *in vivo* or after excision. For the purpose of screening for pulmonary injury in acutely exposed small laboratory animals, the lavage is best done after excision of the lungs. Techniques for bronchopulmonary lavage of small animals *in vivo* have been developed by Mauderly (44) (Fig. 1), so that subjects can be lavaged serially if such a technique is consistent with the experimental objectives of the study involved. The technique performed *in vivo* requires more time and skill than lavage of excised lungs and not all species tolerate the procedure equally well (44). The rabbit, Syrian hamster and mouse have been reported to be well suited to pulmonary lavage *in vivo* with the hamster being especially tolerant of the procedure and quite easily intubated as well. In most cases, serial lavage in the same animal is better reserved for larger animals in which segmental washings can be done (45,46). Lavages are performed *in vivo* in dogs (46–49) (Fig. 2), primates (47,50,51) and man (52–55). The lavage procedure in dogs (46,47), primates (47) or in man (53) produces a mild inflammatory response in the lung. As reported in the dog (46), it is characterized by the classic signs of the inflammatory response with some interstitial swelling, an influx of polymorphonuclear leukocytes followed by increased numbers of macrophages. The response is over quickly and by 48 hr few or no changes can be seen by light microscopy. Mild but detectable transitory changes in lung function occur following lung lavage in dogs (46). These changes are associated with mechanical properties of the lung as

measured by compliance and with gas distribution as measured by respiratory pattern and gas washout techniques.

The wash volume used in pulmonary lavages has been calculated on the basis of 80% of the lung volume change at 20 cm H_2O pressure (44), or 80% of the total lung capacity as calculated from body weight by the equation of Stahl (56) or as five times the tissue volume of the lung (57). As judged by light microscopy, these volumes do not appear to damage the lung (44,57).

The appropriate number of washes can vary but two to four washes have proven adequate in reported studies (58–60). Henderson et al. (45) report that the quantity of protein (and lipid) (unpublished results) removed from unexposed (control) dog lungs by bronchopulmonary lavage decreased with each successive wash; two washes yielded ~ 75% of what could be recovered in six washes, while four washes yielded > 90% of the material collected in six washes. Hook (25) reported similar data for removal of hydrolases from the airways of rabbits (Fig. 3). In screening for lung damage in exposed animals, care must be taken not to wash excessively, particularly if the washing is done *in vivo*,
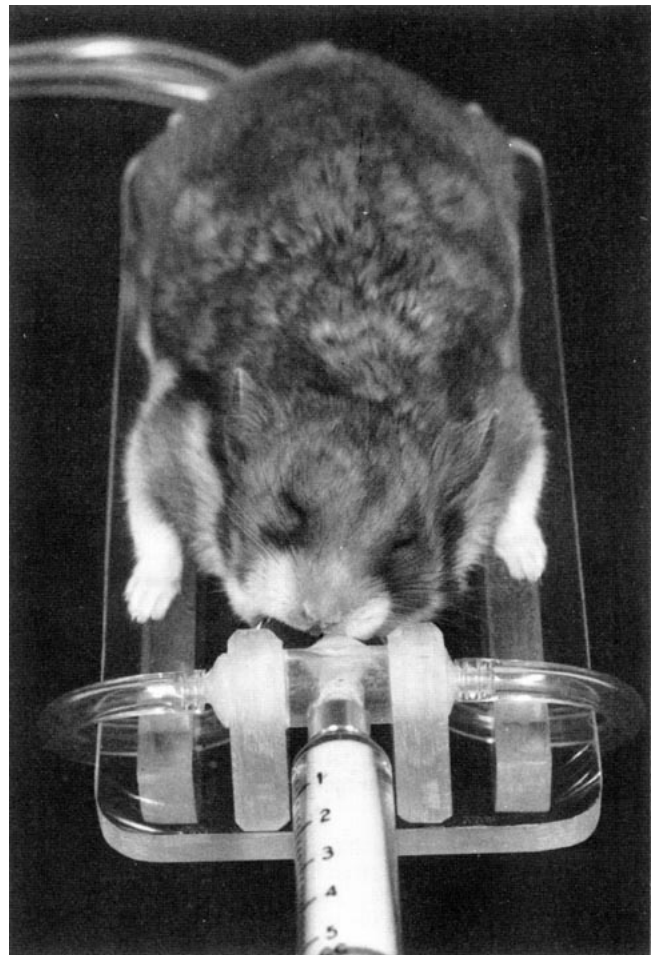

FIGURE 1. Lavage of Syrian hamster by method of Mauderly (44). The hamster is under light anesthesia (1.5% halothane in O_2).
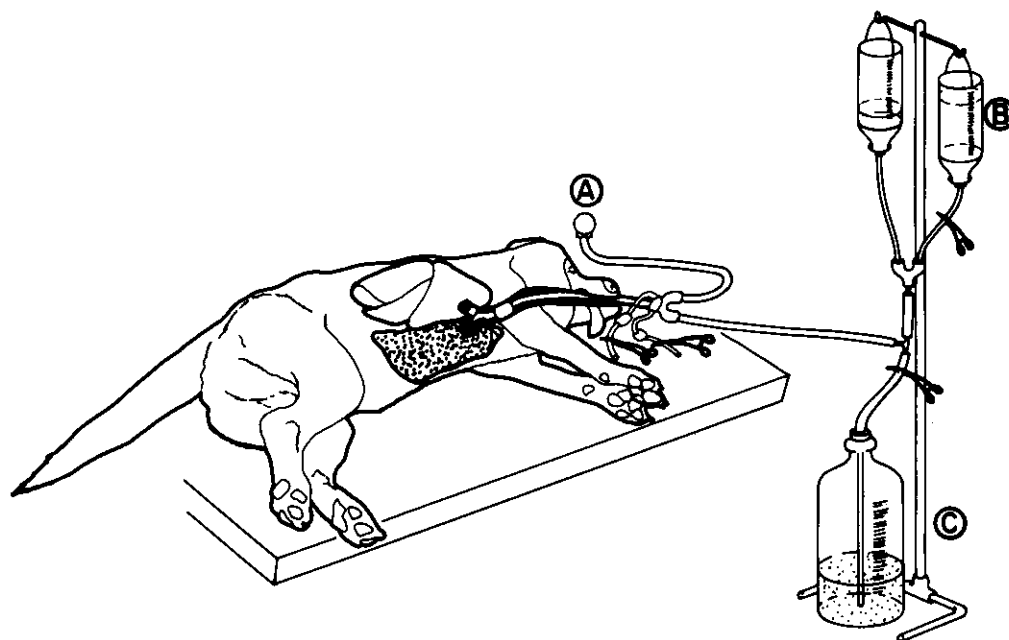
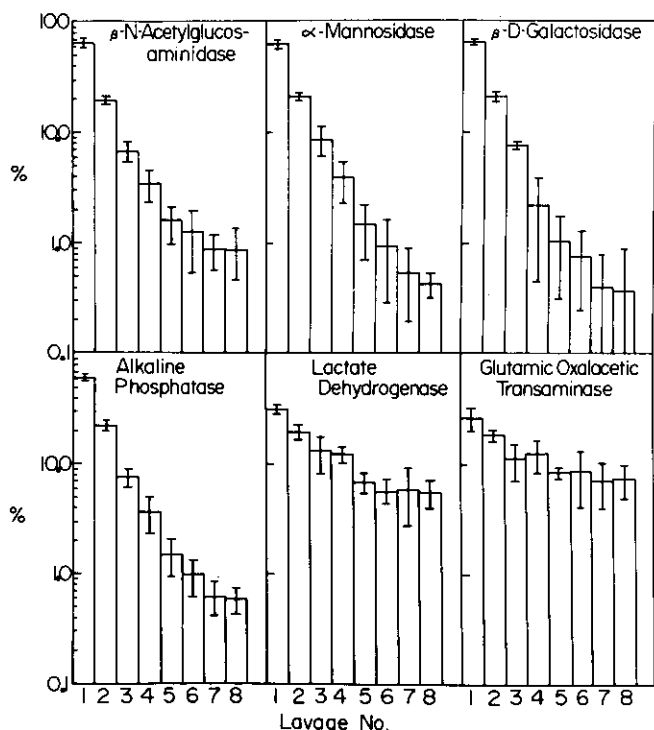FIGURE 2. Lavage of beagle dog by method of Muggenburg et al. (*48*).

FIGURE 3. Removal of extracellular enzymes from rabbit lungs by consecutive lavages. For a given lung, the volumes of consecutive lavage effluents were similar. Results are from four rabbits with vertical bars representing the mean quantity of enzyme removed by each lavage expressed as a percentage of the total enzyme removed by all eight consecutive lavages. Standard deviations are indicated. Reprinted by permission from Hook (*25*).

since the damaged lung may be less resistant to further damage from the lavage technique than is the healthy lung.

The wash fluid can be a balanced salt solution (*25,61,62*) or physiological saline (*44,45,57–60,63*). Supplementation of the physiological saline with 1.9 mM Ca^{2+} may be advisable to maintain the tight junctions between epithelial cells (*64,65*). Some workers report greater cell recovery from washing rodent lungs with physiological saline rather than a balanced salt solution (*66*).

If the lung washings are to be performed in excised lungs, care must be taken to sacrifice the animals in a manner that does not result in changes in airway fluid content due to the sacrifice method. In rats and Syrian hamsters sacrificed by cervical dislocation using a blunt guillotine, a study was made of the effect of anesthetic agents on the lavaged fluid parameters used as indicators of pulmonary injury (*67*). Anesthesia with 5% halothane produced the least changes in the parameters as compared to unanesthetized controls. Asphyxiation with CO_2 caused the greatest change and sodium pentobarbital ($\sim$ 80 mg/kg) administered intraperitoneally was intermediate in causing elevation of the lavage fluid damage indicators.

Quantitation of cellular and noncellular constituents of lavage fluid is more easily achieved in small experimental animals than in larger animals or humans. In small animals, either the total lung or a known fraction of the lung can be lavaged and the total amount of a constituent removed from a lung by a standard lavage technique can be used. For comparisons between spe-

cies the total amount removed by lavage per kilogram body weight is useful as a reference. Standardizations based on body weight avoid the problems involved in standardizing to the weight of the lung, which may be edematous in some injuries.

There is a much greater problem in quantitation of constituents in segmental washings of larger animals or of humans. A review of these problems has been given by Keogh and Crystal (42). If a standard wash volume is used, one can assume that a standard area of the lung is being washed, but this is not altogether certain. Even so, fluid recovery may vary in individuals. One approach is to assume that the recovered fluid, whatever its amount, represents an aliquot of the total fluid presented to the washed area. Therefore concentrations in the recovered fluid (amount of constituent/mL) can be used as a means of quantitating what was in the fluid lining the washed area. The other approach is to use ratios of constituents in the washings as an indication of injury states. This is commonly used in differential cell counts in the lavage fluid; noncellular constituents are often referenced to albumin (42). The obvious drawback to albumin as a reference material is that the concentration of this relatively small serum protein may be increased in lavage fluid from subjects with minimally damaged capillaries and at least one study reports a decrease in albumin concentrations in patients with cystic fibrosis (68). In segmental washings, investigators must clearly state the method of quantitation used and the rationale used in interpretation of the results.

Screening for Acute Inflammatory Response in the Lung

Validation of the Method

Several studies have been performed to determine the validity of analysis of lavage fluid as a probe to detect acute lung injury in animals exposed to toxic agents. The most useful studies for validation of the method have been those in which some correlation was made between the morphological manifestation of the induced injury and the biochemical and cytological content of the lavage fluid (69). Lung washings from animals with specific types of lung injury have been examined.

In one study (58), Syrian hamsters exposed to the nonionic surfactant, Triton X-100, were found to have a dose-dependent increase in LDH in airway washings. The major portion of the LDH appeared to originate from lung cells rather than from lysed red cells, since the iron content of the lavage fluid did not increase. The LDH levels observed in the washings from the injured animals were too high to be accounted for by transudation of serum levels of LDH.

The lipid and protein content of lung washings of beagle dogs with β-radiation-induced lung injury indi-

cated that increasing levels of protein in the lung washings was a good indication of the progression of the radiation pneumonitis (45). Lipid content of the lavage fluid at first increased and then decreased as the protein content continued to rise. The loss of lipid did not appear to be a sensitive indication of damage since it was not until severe injury was apparent that a detectable loss of lipid was observed. Radiation is known to result in damage to the endothelial cells (70), and the transudation of serum protein accompanied by loss of surfactant lipid in the airways (10) is an expected sequel of radiation injury. Since the lungs of the dogs in this experiment were severely injured, the sensitivity of protein transudation as an indicator of lung injury was not determined by this study. The work of Alpert et al. (71), in which the transudation of radiolabeled serum protein into the alveolar space was used as an indicator of injury induced by O_3 in rats, showed that protein transudation can be a very sensitive indicator of the endothelial injury caused by high O_2 exposure, if a highly sensitive measure of the serum protein can be used.

In studies of the toxicity to lung of metal salts (59,60), cytologic as well as biochemical parameters were measured in lung washings. In Syrian hamsters exposed by inhalation to a toxic metal salt ($CdCl_2$) and to a relatively nontoxic metal salt ($CrCl_3$) (59), the parameters in lavage fluid which proved to be most useful in distinguishing the response to the injurious salt from that of the essentially innocuous salt, was the LDH, G6PDH (not shown), acid and alkaline phosphatase activities, the sialic acid and protein content and the cellular content (Fig. 4). The injury caused by the cadmium was diffuse and characterized by a severe interstitial pneumonia. There was good correlation between the extent of the injury and the extent of the response in the lavage fluid. The lavage fluid assay was more sensitive at detecting the injury in the early stages after the exposures (1 day) when the airways were still open than at later stages (1 week) when the injury was severe enough that some airways were blocked and were probably not accessible to the wash fluid. In the animals exposed to $CrCl_3$, no morphological indication of lung injury was observed, as judged by light microscopy. The lavage fluid showed only an elevation in acid phosphatase activity. In Syrian hamsters exposed by intratracheal instillation to various metal salts, the lavage fluid parameters which were the best indicators of injury were LDH and β-glucuronidase activities, and sialic acid, protein and cellular content.

The lavage fluid profile was quite different in Syrian hamsters with a multifocal type injury resulting from a 48-hr exposure to NO_2 (72,73). The peak response, as measured morphologically or by the lavage fluid parameters, was at the end of the two-day exposure when the major pathological feature was a graded, dose-dependent terminal bronchiolitis (Figs. 5 and 6). The
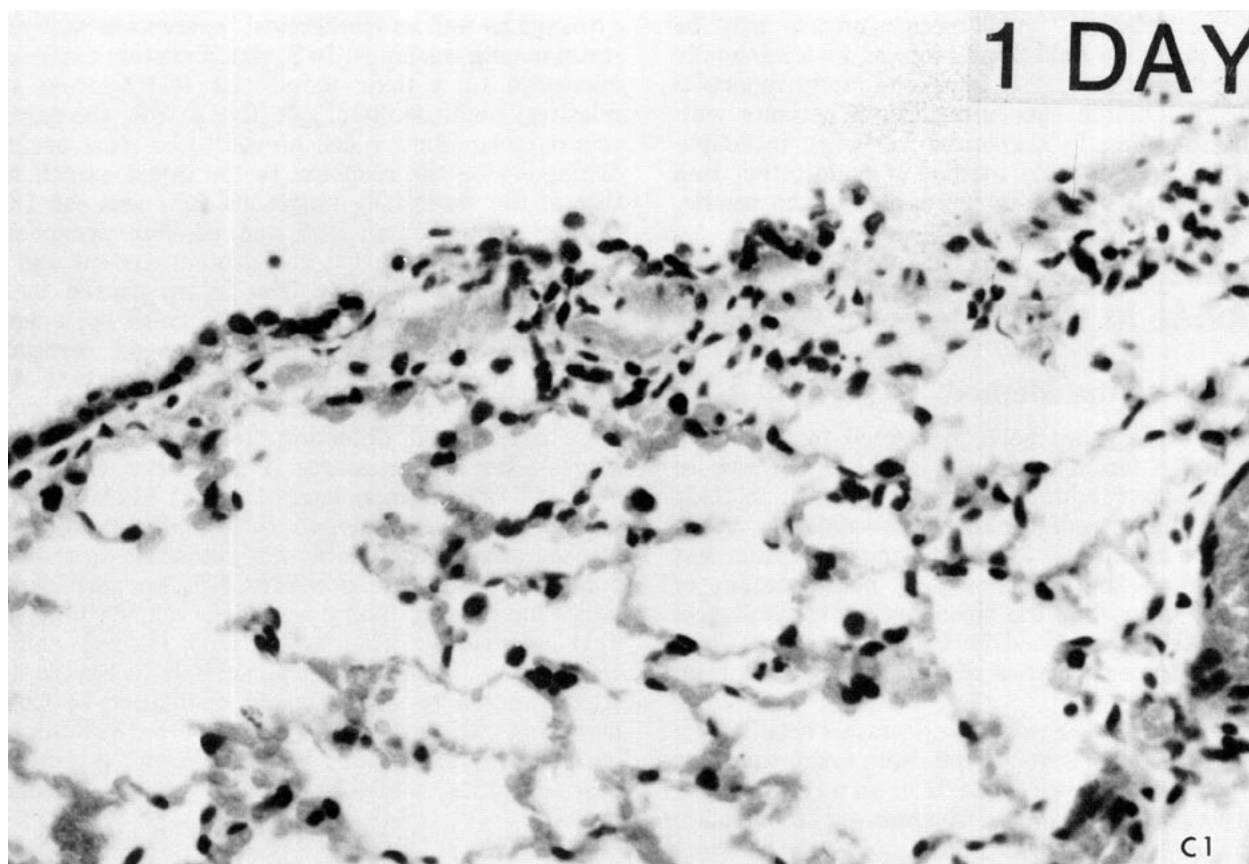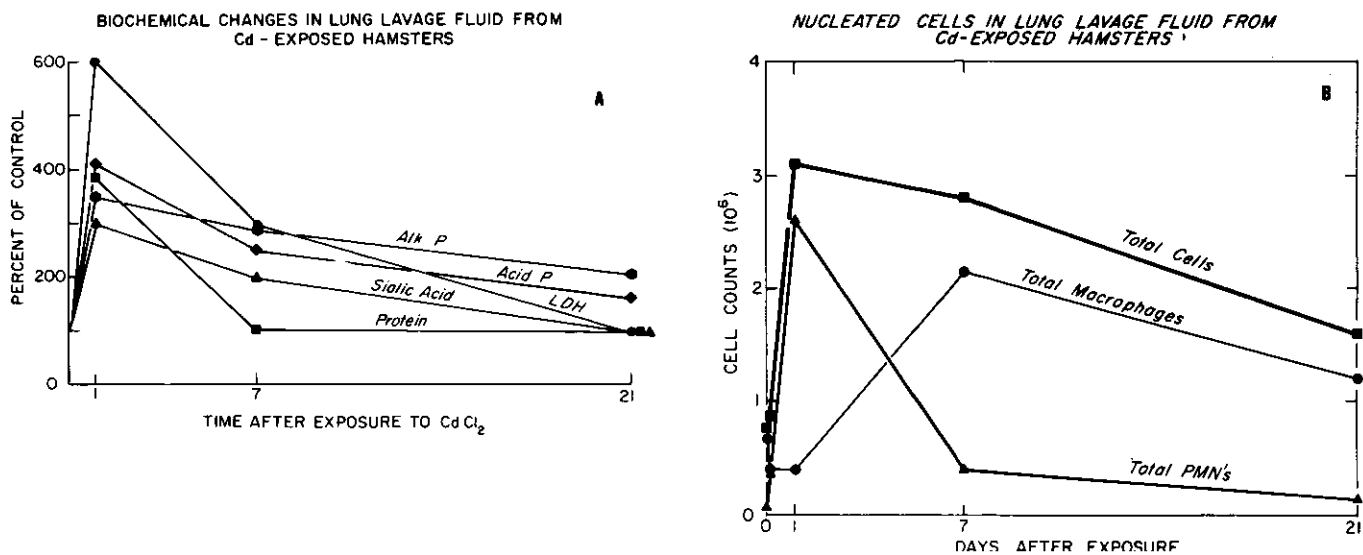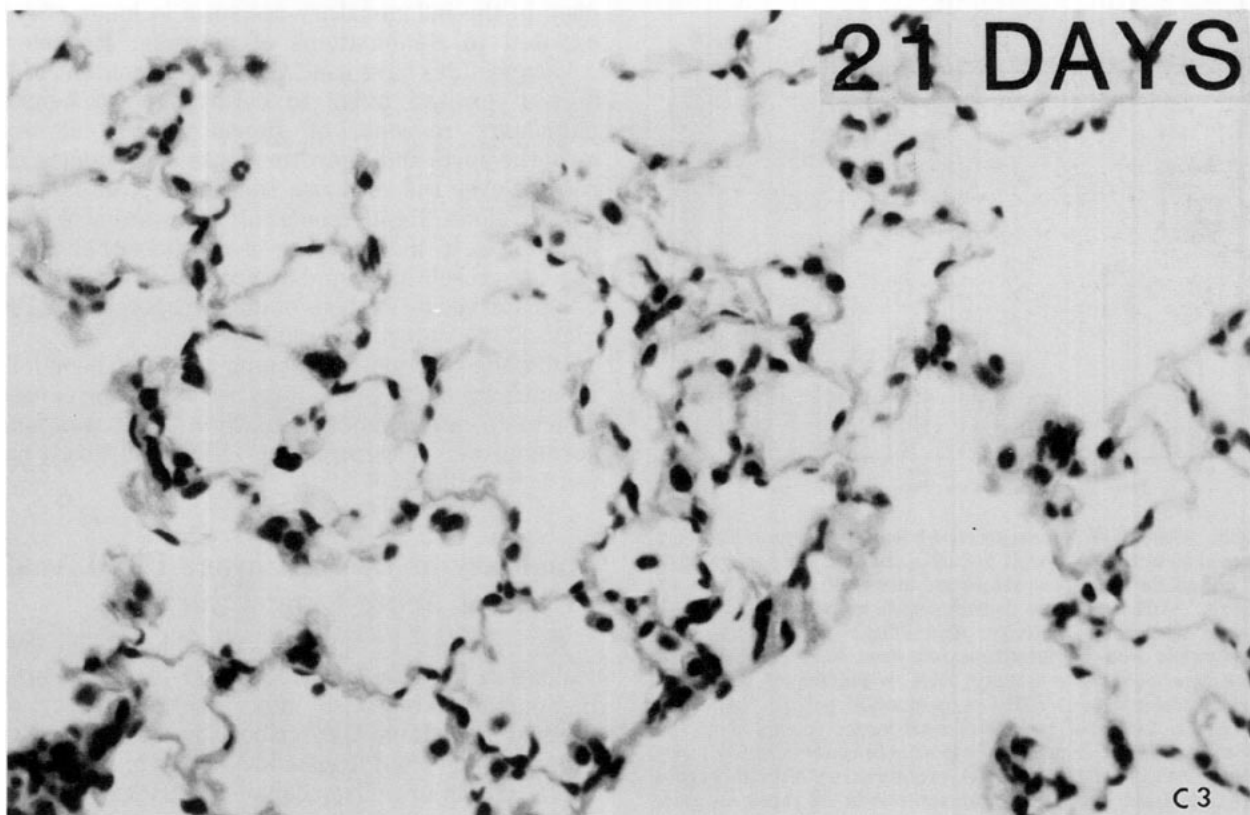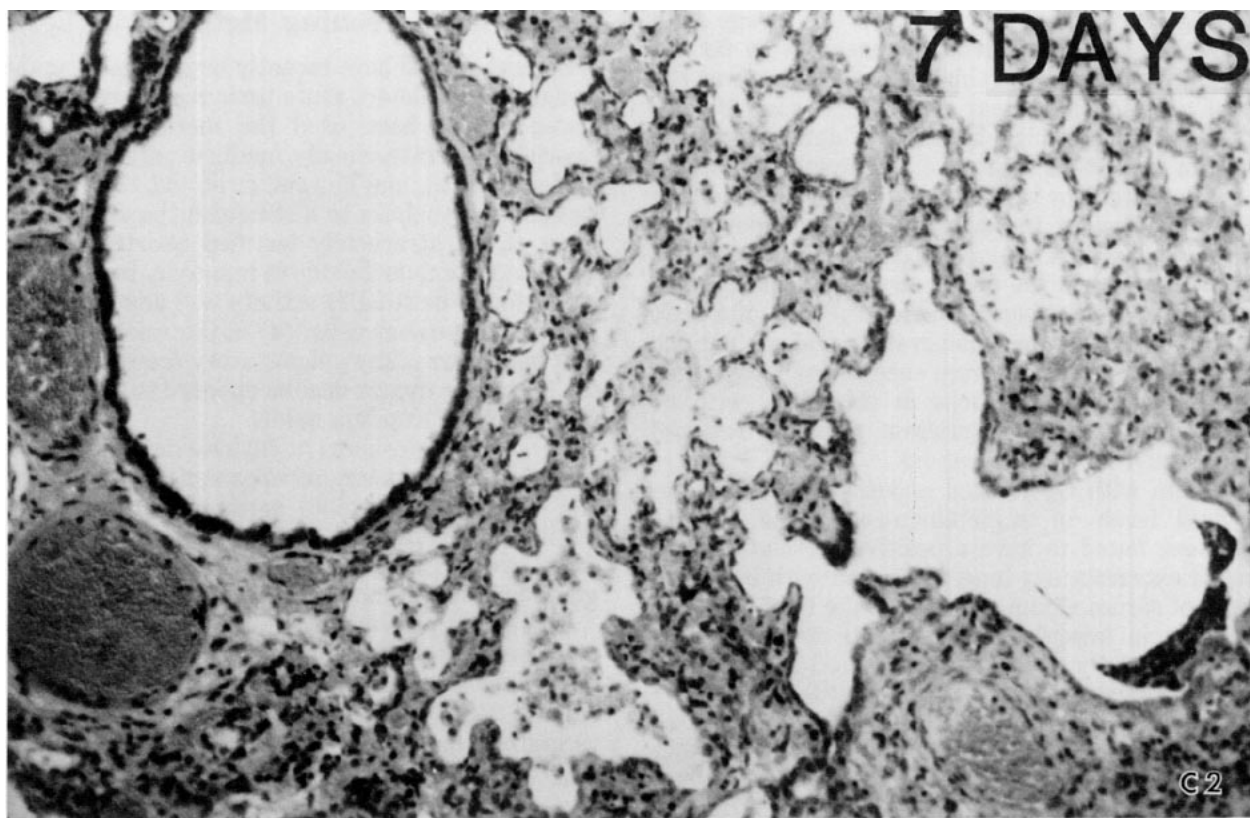
FIGURE 4.   Response to pulmonary injury as detected in lung washings of Syrian hamsters exposed by inhalation to CdCl$_2$ (4.4 ± 1.2 μg CdCl$_2$/lung): (A) biochemical response; (B) cytological response; (C) morphological changes. Reprinted by permission from Henderson et al. (85).

7 DAYS

C2

21 DAYS

C3

biochemical parameters measured in the lavage fluid also showed a dose-dependent response but by far the most sensitive indicator of this type of damage was the influx of polymorphonuclear leukocytes (Fig. 5). The most sensitive biochemical indicator of damage was the increase in sialic acid content of the washings, which was accompanied by a parallel increase in soluble protein (not shown) indicating that transudation of serum proteins was a major feature of the injury.

In rats exposed to the upper airway irritant, H_2SO_4 mist, pulmonary washings indicated the lack of a deep lung injury as only an increase in sialic acid content and acid phosphatase activity were seen in the lavage fluid (85). The sialic acid response in this case, with no accompanying increase in protein, probably resulted from increased mucous secretions.

In rabbits with O_2-induced endothelial damage, lavage fluid levels of angiotensin-converting enzyme (ACE) were found to have a positive correlation with increased extravascular lung water and with increased amounts of serum albumin in the lavage fluid (74). The ACE levels in lavage fluid from rats with thiourea-induced pulmonary edema also correlated well with the degree of capillary damage observed (75).

Roth (76) has made use of comparisons between serum and lavage fluid LDH levels to distinguish between liver and lung damage induced by intraperitoneally injected toxins known to be either liver or lung toxicants or to be toxic for both organs.
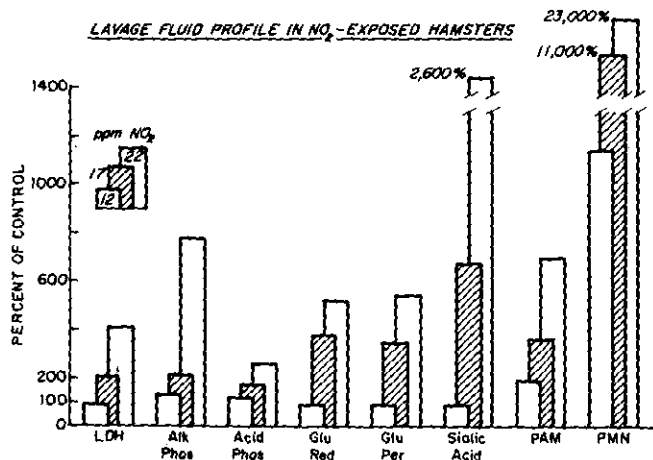
Use of the Screening Method

Several groups have recently begun to use analysis of lavage fluid to detect acute toxic responses in the lung. Three groups have used the method to assess the toxicity of intratracheally instilled particles.

Moores et al. and Morgan et al. (66,77,78) have used lavage fluid analysis to distinguish the response of rat lungs to intratracheally instilled quartz (a fibrogenic dust) and titanium dioxide (a reputedly inert dust). This group found both LDH activity (77) and the number of polymorphonuclear cells (66) in the washings were the best indicators of the inflammatory response induced by the fibrogenic quartz dust as opposed to the response to nonfibrogenic titanium oxide.

Beck and co-workers (79,80) have done similar studies comparing the toxicity of volcano ash, α-quartz, Al_2O_3 and Fe_2O_3. Lavage fluid parameters found useful in distinguishing between the toxic quartz and the inert dusts were LDH, albumin, peroxidase, β-N-acetyl-glucosaminidase and differential cell counts.

Mühle (81, personal communication) has used lavage fluid analysis to determine the relative toxicity of intratracheally instilled coal combustion fly ash and various types of titanium dioxide. Parameters useful in rating the lung toxicity were differential cell counts, LDH, alkaline and acid phosphatase, glucose-6-phosphate-dehydrogenase, and acid proteolytic activity as well as antiproteolytic activity.

Lavage fluid analysis has been used to detect synergism in the inflammatory response in lungs of animals exposed to combinations of aerosols. Rylander and colleagues (82) have used the cellular content of washings of excised lungs to determine the heightened pulmonary response of guinea pigs to aerosols of airborne dusts and cigarette smoke in sequential combinations over the response seen in exposures to either aerosol alone. Henderson et al. (83) were able to detect differences in the response of rats to either fly ash or H_2SO_4 aerosols alone and to a combined exposure to the two materials by analysis of lung washings for LDH and glutathione reductase activities.

Immune reactions in the lung have also been followed by analysis of lung washings (84). In rats exposed to endotoxin, an increased leukocyte count was found to correlate well with increasing IgG/IgA antibody ratio in the lavage fluid.

FIGURE 5. Content of pulmonary lavage fluid from Syrian hamsters exposed 48 hr to 12, 17 or 22 ppm NO_2. Lavage was performed at the end of the exposure. Values are means of samples from six animals. LDH = lactate dehydrogenase activity; Alk Phos = alkaline phosphatase activity; Acid Phos = acid phosphatase activity; Glu Red = glutathione reductase activity; Glu Per = glutathione peroxidase activity; PAM = number of pulmonary alveolar macrophages; PMN = number of polymorphonuclear leukocytes. Levels of the biochemical measurements were significantly different from controls ($p < 0.05$) by the Mann-Whitney U test for the 17 and 22 ppm NO_2 exposures. Cytological changes were significantly different from controls at all three exposure levels. Reprinted by permission from Henderson et al. (85).

Summary of Use of Lavage Fluid Analysis to Detect Acute Lung Injury

An overview of the work that has been done validation and use of lavage fluid to detect an acute inflammatory response indicates the efficacy of method (85). Table 2 describes the different respon seen in lavage fluid from animals with different types acute lung injury. Extracellular LDH levels are a indication of general cell injury. Polymorphonucl

Table 2. Lavage fluid profiles observed in different types of acute lung injury in rodents.

Location, type of injury	Causative agent	Lavage fluid profile
Diffuse, interstitial pneumonia (59)	$CdCl_2$	Increased levels of both biochemical and cytologic indicators of injury
Multifocal terminal bronchiolitis (73)	NO_2	Increased number of leukocytes (especially PMNs) and lesser increases in biochemical indicators of injury
Upper airway irritant (83)	H_2SO_4	Difficult to detect by lung washings but some increase in sialic acid and acid phosphatase activity
Subclinical, not detectable by light microscopy (59)	$CrCl_3$	Increased acid phosphatase activity
Inflammation (66,77,79,80)[a]	Quartz	Increased LDH, lysomal enzyme activity; increased leukocytes (especially PMNs)
Inflammation (76)[a]	Paraquat, bromobenzene, monocrotaline, or oxygen	Increased LDH activity; increased protein
Immunological alterations (84)	Endotoxin	Increased leukocytes; increased IgG/IgA ratio
Inflammation (82)[a]	Smoke and MnO_2	Increased leukocytes

[a] No histopathologic evaluation reported.

leukocytes are sensitive indicators of an inflammatory response. Alkaline phosphatase activity has been a consistently good indicator of damage (59,60,69,72, 73,86), and if the isoenzyme pattern were determined by isoelectric focusing (28), some indication of the source of the enzyme (Type II cells or serum) and therefore the site of the injury, might be obtained. Acid hydrolases can be used to measure release of lysosomal contents, although elevation of these lysosomal enzymes may not always indicate a significant lung injury. For example, if acid phosphatase is the only parameter elevated in lung washings (59,83), little injury is indicated. In acute injury, acid phosphatase activity has been elevated more consistently than has β-glucuronidase, an enzyme which is sometimes sporadically elevated in both control and exposed animals (59). This has not been true in chronically injured animals (see following section) in which the lavage fluid level of β-glucuronidase appears to be one of the more sensitive indicators of chronic inflammation induced by inhalation of particles. If a more detailed profile of different hydrolases is measured in the lavage fluid, one might potentially be able to detect increases in those hydrolases associated with Type II cell lamellar bodies (25,29) as an indication of Type II cell injury. Sialic acid and protein determinations in lung washings are useful because if both are elevated, damage to the alveolar capillary barrier is indicated while if only sialic acid levels are elevated, an upper airway effect on mucous secretions is indicated. Angiotensin-converting enzyme activity in lavage fluid is useful in detecting damage to the endothelial cells of the capillaries (74,75).

Screening for Chronic Pathology in the Lung

Studies in Animals

Very few studies have used bronchoalveolar lavage to follow the development of chronic pulmonary disease in animals. Fahey and co-workers (87) have found broncho-alveolar lavage to be a useful tool in detecting bleomycin-induced pulmonary fibrosis in dogs prior to changes in chest radiographs. The endpoint used to detect onset of the injury was an increase in neutrophils in the lavage fluid. Another group (88) looked at lavage fluid from guinea pigs with chronic silicosis and found increased numbers of neutrophils and giant cells and impaired functioning of the alveolar macrophages.

In animal studies, lung tissue neutral proteases remained elevated as long as 10 months after a 60-day exposure of Fischer-344 rats to levels of NO_2 which eventually caused emphysematouslike lesions in the lungs (86). If tissue levels of neutral protease are elevated in developing emphysema, the airways may also have elevated levels of proteolytic activity which could be detected in lavage samples. In comparisons of lavage fluid from old versus young rats and hamsters (63), there was a trend toward increased levels of proteolytic activity and decreased levels of antiproteolytic activity in the older animals.

In preliminary reports on the analysis of lavage fluid from rats and mice exposed chronically to diluted diesel exhaust (89), β-glucuronidase was elevated at least 10-fold after 6 months of exposure to 7.0 mg/m^3 of diesel exhaust particles. Also elevated in the lavage fluid of the chronically exposed rodents were LDH and glutathione reductase activity and soluble protein, sialic acid, macrophage and neutrophil cell counts. In rats exposed to 35 mg/m^3 coal combustion fly ash for one month, LDH and β-glucuronidase levels and neutrophil counts in lavage fluid were elevated, and the β-glucuronidase and neutrophil levels remained elevated up to 5 months after the exposure (90). From these two studies it appears that neutrophils may remain elevated in the lung on continued exposure to particles. The lysosomal enzyme β-glucuronidase was a sensitive indicator for the exposures in contrast to what has been reported in some acute toxicity studies on metal salts (59,60) in which this enzyme was found to be only sporadically elevated. Acid phosphatase, on the other hand, was not elevated in lavage fluid from animals exposed to either type of particle.
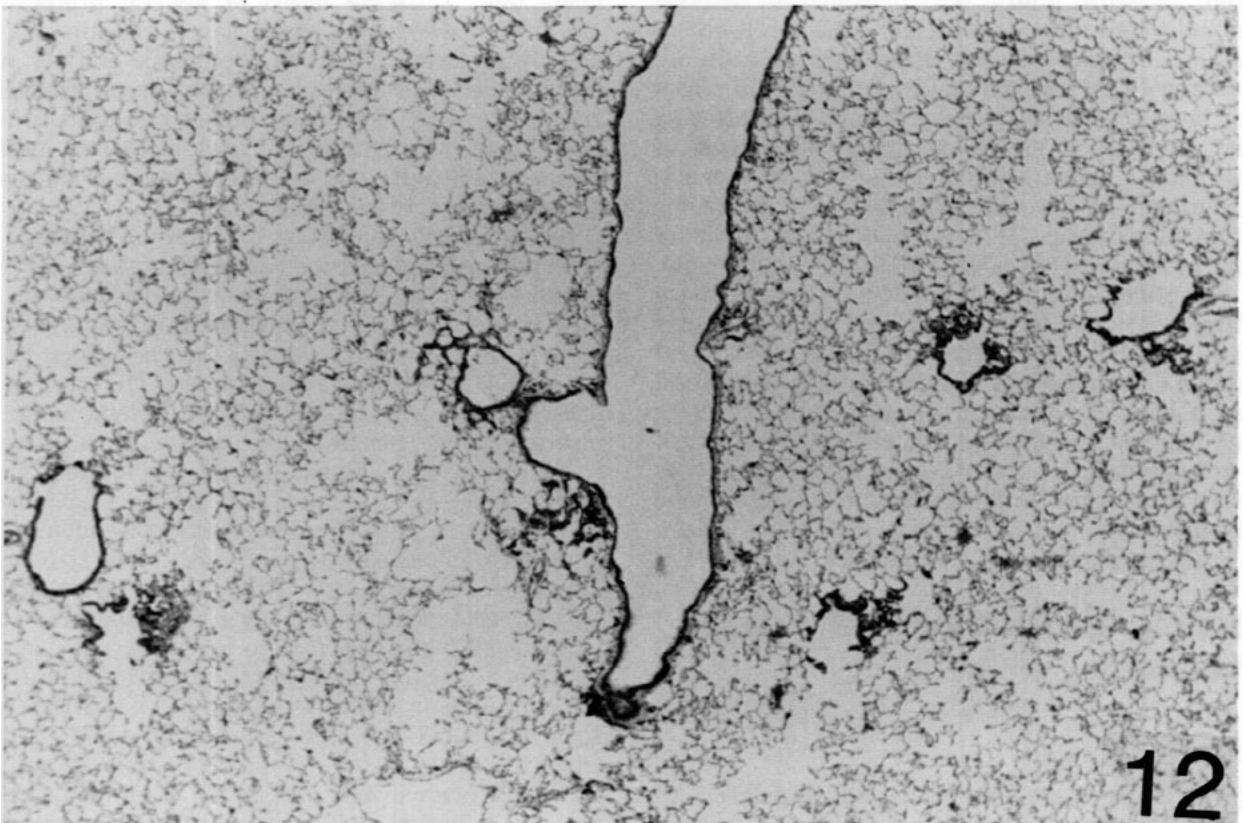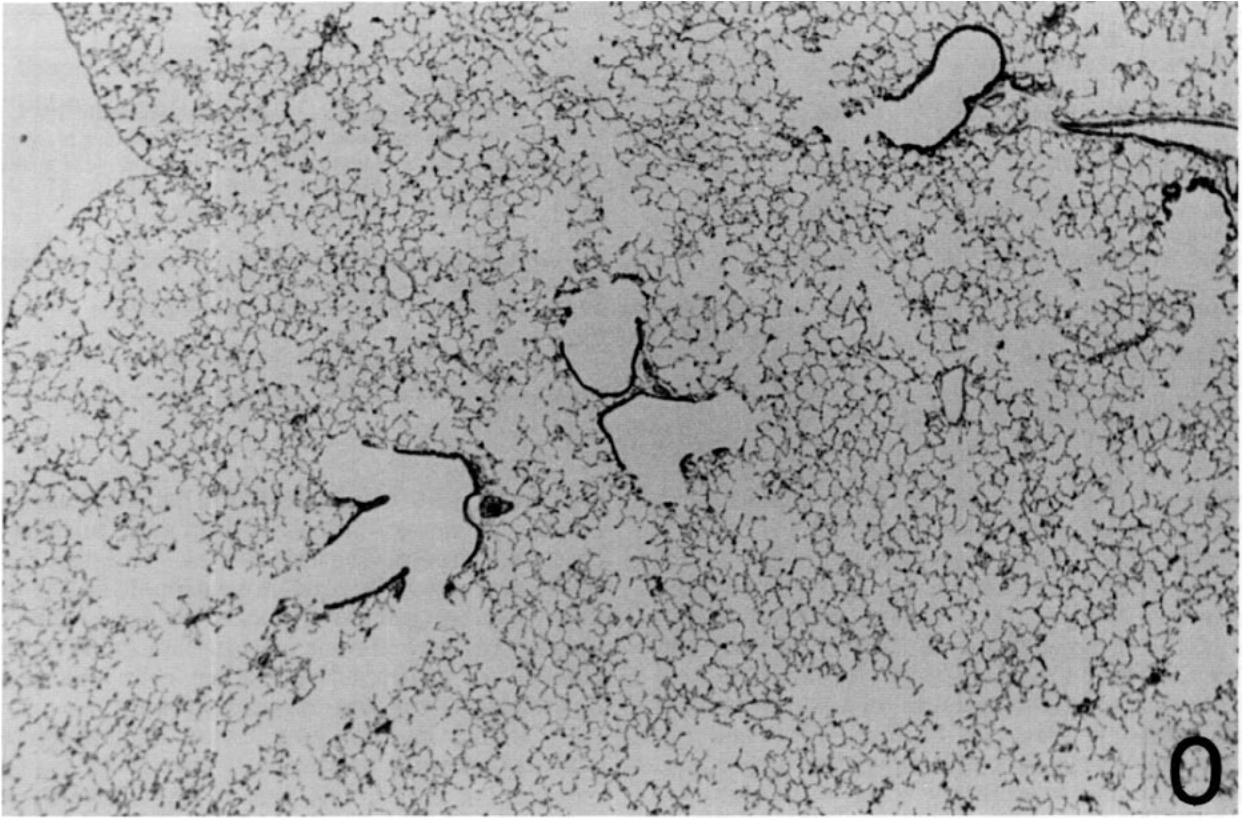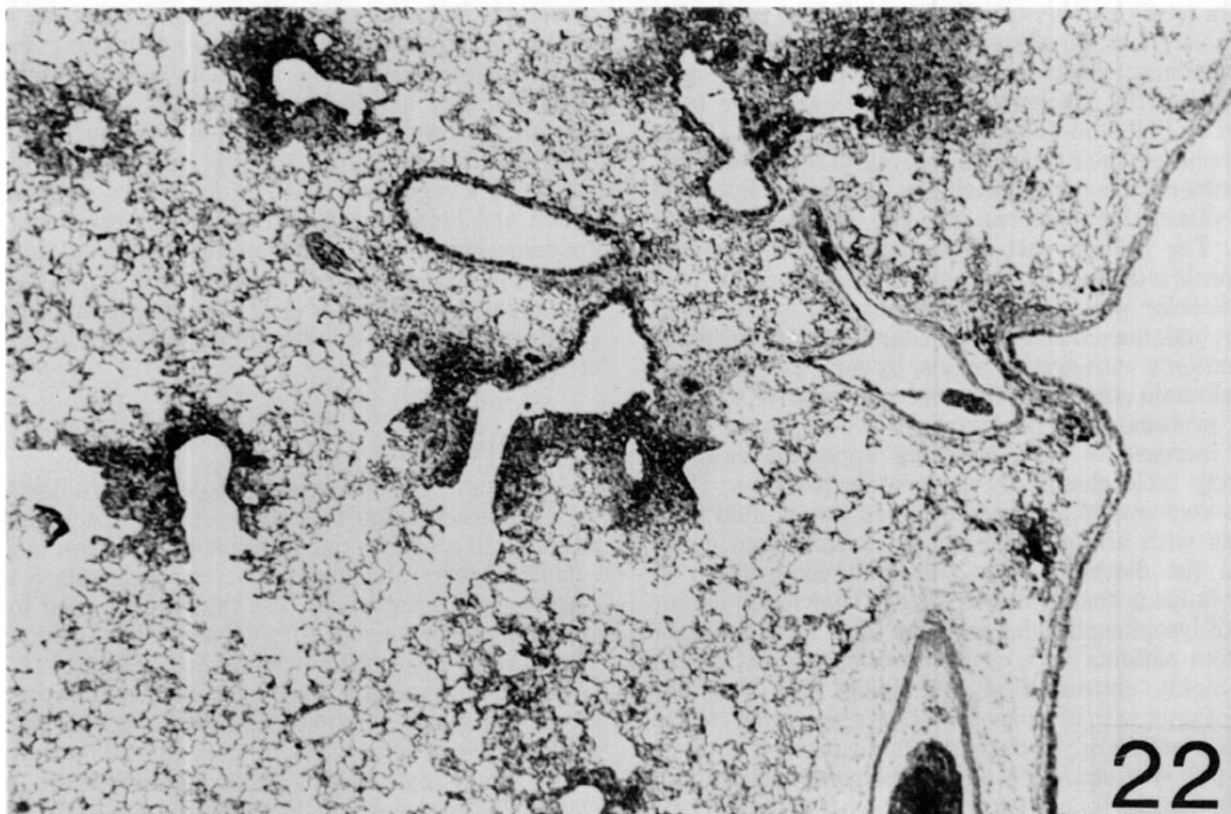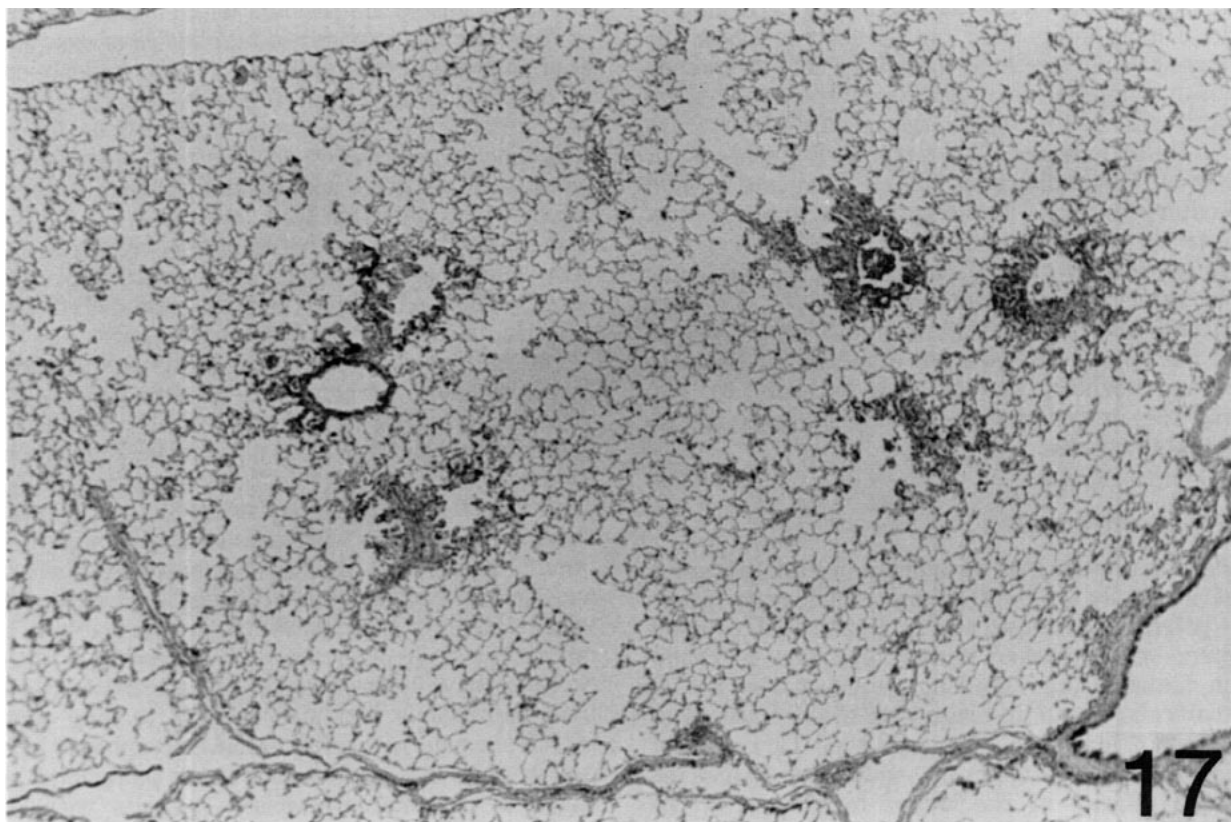
FIGURE 6.   Syrian hamster lung tissue at end of 48 hr exposure to 0, 12, 17, or 22 ppm NO₂. H & E stain (73).

More studies in animals are needed to determine if analysis of lung lavage fluid can be used to follow the development of chronic lung pathology. The frequency with which lavage can be performed in the same animal without drastically altering the course of the injury also needs to be determined. The work of Reynolds et al. (53) and of Muggenburg et al. (48) indicate a mild inflammatory response to the lavage itself. Long-term studies (4 yr) of dogs that received 10 serial lavages in 49 days showed no chronic morphologic or functional changes associated with lung lavage (46). As more specific and sensitive indicators of different types of chronic pulmonary diseases are determined, the segmental lung washings may prove to be useful tools in following the progression of developing pathology in experimental animals.

Studies in Humans

In addition to detecting developing disease processes in experimentally exposed animals, the lavage probe has the potential to be used as a diagnostic tool to detect developing or established chronic pathological conditions in humans. An excellent review of the use of bronchoalveolar lavage in diagnosing disease processes in the human lung has been published (41). Recent editorials (42,91) give opposing views on the reported safety and efficacy of the lavage techniques in the diagnosis of interstitial lung disorders (43).

Many parameters have been measured in fluid from patients receiving therapeutic lavage by the method of Kylstra (52). Lavage effluents from the lungs of patients with pulmonary alveolar proteinosis, with cystic fibrosis or from healthy smokers and nonsmokers have been studied by Bell et al. (33,68,92,93) and Hook (94). These workers have done a thorough analysis of the electrophoretic pattern of soluble proteins in the lavage fluids and found distinctive patterns for the different disease states. The protein pattern in patients with alveolar proteinosis indicated the presence of small to intermediate molecular weight serum proteins and is consistent with an undamaged alveolar capillary barrier. Washings from patients with cystic fibrosis, by contrast, indicated a low albumin content in the washings relative to other serum proteins with no elevation in IgG levels but a 3–fold increase in IgA. Smoking appeared to cause relatively little change in the protein patterns. Lynn and co-workers (95,96) have analyzed lavage fluid from patients with alveolar proteinosis, asthma and cystic fibrosis for distinctions in the lipid composition of airway fluids in these disease states. They found higher levels of lysophosphatides and free fatty acids in washings from patients with cystic fibrosis; asthmatics had more highly saturated phospholipids; and washings from patients with alveolar proteinosis were reported to contain a unique glycoprotein (97). In parallel studies, Spock (98) was unable to detect chemical mediators of smooth muscle tone (sertonin, catecholamine, bradykinin, SRS-A and prostaglandin F_{2a} and E_1) in lavage

fluid from either the patients with chronic lung disease or from animals exposed to high levels of oxygen. Low et al. (99) found that protein and carbohydrate content of cell-free lavage fluid from bronchofiberoptic subsegmental lavage of patients with diffuse interstitial lung disease was increased over that of control volunteers, and patients with granulomatous disease had increased lavage fluid lipid. Martin et al. (100) found that segmental lavage was an aid in diagnosing alveolar proteinosis because of the large amount of acellular eosinophilic bodies and periodic acid–Schiff staining material in lavage fluid from such patients.

The cellular content of lavage fluid has proven to be the most useful parameter to classify interstitial lung disorders (41,54,55). Increased lymphocytes have been noted in lung washings from patients with hypersensitivity pneumonitis, tuberculosis, pulmonary lymphomas, and sarcoidosis while high neutrophil counts have been observed in patients with idiopathic pulmonary fibrosis, familial pulmonary fibrosis or asbestosis. The number and type of lymphocytes in lavage fluid from patients with pulmonary sarcoidosis has proven useful in determining the stage of activity of the disease (41,101–103). Smokers have been shown to have increased levels of both neutrophils and macrophages in lung washings (41). Hemosiderin-laden alveolar macrophages in lavage fluid have been used to detect occult pulmonary hemorrhage in immunocompromised patients with radiographically demonstrable pulmonary infiltrates (104). Lawrence and co-workers (105) reported marked increases in IgG secreting cells in bronchoalveolar lavage fluid from patients with active interstitial lung disease.

Proteolytic activity and how it is balanced by inhibitors may play a role in development of chronic lung disease. Orlowski et al. (106) have recently reported finding cathepsin-B-like activity and prolyl endopeptidase in lavage fluid from human patients, most of whom had lung cancer, and have suggested that such enzymes may effect collagen turnover and lung remodeling. Kucich and co-workers (107) are developing immunological assays for elastin fragments to be used in the detection of developing emphysema by the analysis of lung washings.

Summary

At present, the analysis of lavage fluid is a useful tool as a rapid screening test to determine the pulmonary response of acutely exposed animals to environmentally available, respirable materials. It represents a short-term *in vivo* cytotoxicity test that can be used to rank the pulmonary toxicity of a series of test compounds. The screen is useful for choosing appropriate compounds and exposure levels for subsequent in-depth studies in which complete histopathologic evaluations will be made. The most useful parameters for detecting acute injury by analysis of lavage fluid are LDH, lysosomal enzymes, ACE, protein content and cell counts. Results to date indicate that bronchoalveolar

washings may also prove to be useful in the detection of developing chronic lung pathology in animals exposed to toxicants. However, specific and sensitive assays for parameters that can be related to stages in the developing disease process must be defined.

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