MORPHOLOGICAL METHODS FOR EVALUATION OF PULMONARY TOXICITY IN ANIMALS

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INTRODUCTION

The mammalian respiratory system has a variety of important functions in addition to the primary one of gaseous exchange (1, 2). The corresponding diversity of structural components of the respiratory tract, compounded by the inhomogeneity of morphologic responses of the lung to damaging agents, necessitates extremely careful selection and implementation of the several morphological methods required for its examination. Methods must be sensitive enough to reveal the presence and nature of subtle effects, and also to provide information on which useful hypotheses of pathogenesis can be based.

This review is designed to present the important considerations in the choice of methods and is a guide to references describing them in more detail. It is not intended to be a detailed critique of methods or a complete laboratory protocol. Much of the review deals with the routine necessary for the satisfactory search for and documentation of toxic effects. Emphasis is on the sine qua non for detecting subtle effects, which provide the most discriminating information relevant to pulmonary toxicity. The remainder of the review briefly addresses special methods for investigating various aspects of the pathogenesis of pulmonary lesions likely to be encountered and which are necessary for furthering the understanding of pulmonary pathobiology.

ROUTINE EVALUATION

Gross Examination

The methods to be described in this and subsequent sections are postmortem procedures, although most are applicable to surgical specimens. Radiographic studies,

therefore, are not discussed. They can provide indications of gross and subgross morphologic changes in vivo, however, and are particularly pertinent to chronic studies involving the larger experimental animals.

TRACHEOBRONCHIAL TREE AND PARENCHYMA The animal is deeply anesthetized by sodium pentobarbital and killed by exsanguination. The trachea and lungs are carefully exposed after the diaphragm is punctured, and search is made for abnormalities of the pleural cavity and its parietal and visceral surfaces (e.g. excessive fluid, adhesions). The trachea is transected 3–5 rings distal to the larynx, and the distal portion with attached lungs and other thoracic viscera removed. The surfaces of the trachea and lungs are examined for signs of abnormalities (e.g. indications of edema, hemorrhage, consolidation, emphysema, scarring, possible tumor nodules). These can be documented photographically or schematically in outline drawings. The partially collapsed state of the normal regions of the excised lung results in exaggerated appearance of the abnormalities and enables detection of small lesions that sometimes cannot be discerned in the inflated state. The extent to which the major airways and pulmonary parenchyma need be opened depends on the amount of gross damage. If there is no sign of edema or an exudative lesion, the examination of airways and parenchyma is left until after fixation. Even where major airways are opened, samples of lungs should be retained for perfusion fixation by the airways. The weight and fluid-displacement volume of the lungs can be obtained after tying off the major vessels and dissecting away the heart and mediastinum, if the degree and nature of the abnormalities observed indicate these would be useful quantitative parameters. The volume of fresh unfixed lungs is better measured from radiographs, however, as recommended by Dunnill et al (3).

NASOPHARYNX AND LARYNX These structures should be surveyed for damage and the need for more extensive examination determined. In laboratory rodents, the nasal sinuses and turbinates can be examined by removing the overlying nasal bone with forceps or by sagittal section. In larger animals such as the dog, a sagittal section is made. Except in cases of tumors or severe upper respiratory irritation by inhaled materials, microscopic methods are usually necessary for detection of changes in these regions.

Fixation

CHOICE OF FIXATIVE AND METHOD OF FIXATION Criteria for suitable fixation are production of least artifact, reproducibility, and simplicity and cost. The major aim with respect to production of least artifact is to retain as close as possible the in vivo appearance of the lung immediately preceding death. With pulmonary tissue, in addition to the usual fixation artifacts that have to be considered (e.g. shrinkage, mechanical distortion, changes in cellular organelles), there is the need to prepare pulmonary parenchyma for microscopic examination such that the correct configurations and relationships of airspaces are retained. Fixation by immersing small pieces of lung in various fluids is a common routine procedure. With the exception of severe exudative processes or where there are solid lesions such as tumors, however, immersion-fixed lungs do not provide proper definition of either

normal or abnormal components. The preferred method of distending the lungs by perfusion of fixative through the airways eliminates these disadvantages by returning the lung to a state similar to that in vivo.

The work of Heard and colleagues (4, 5) is the basis for most of the methods of perfusion via the airways used today. After the lungs have been examined grossly, they are inflated with fixative via the trachea at 30 cm of fluid pressure measured from the surface of the fixative bath in which the lungs are immersed. We have used pumps to provide the necessary height of fixative in the reservoir for large animals (e.g. horses) but have found the marriott bottle to be the most suitable device for lungs from animals the size of dogs or monkeys down to mice. We routinely use 30 cm of water pressure, since this is clearly on the plateau of the pressure-volume curve for all of these species and should not result in tearing or rupture of tissues. Fixation of dog lungs at 25 cm of water pressure has resulted in incompletely filled or distended alveoli. This is characterized by folds in the interalveolar septum, which at total lung capacity should be straight. Specimens prepared at pressures that result in incomplete distension of the alveoli and airways are not suitable for morphometric analysis using stereological procedures, and are less suitable for scanning electron microscopy because of local variations in the degree of distension and therefore interrelationships of the component parts. The airway perfusion method can be applied equally well to one lung or, as is sometimes necessary in large animals, to one lobe or bronchopulmonary segment. A more extensive discussion of general methods of fixation can be found in the report by Dunnill et al (3).

Fixation by perfusion through the airways not only maintains the dimensions and configurations of the tissues at approximately total lung capacity, but also provides a large volume of fixative in intimate contact with the various surfaces, which is essential to rapid fixation. The distance the fixative must diffuse for complete penetration is minimal. This method has for general studies the additional advantage of providing a relatively unobstructed view of cell surfaces for scanning electron microscopy by flushing off mucous coat and alveolar lining material. It has the disadvantage of causing some translocation of exudates and particles and of providing a specific artifact of increased tissue spaces around pulmonary vessels, the so-called edema artifact.

The choice of fixative is also a major consideration in view of the large numbers of fixatives that have been used for the respiratory system. The main components of these fixatives are usually one or more aldehydes, buffer, and various salts with high purity water so that the fixative has a constant pH and osomolality. Many investigators today use a mixture of glutaraldehyde and formaldehyde made from paraformaldehyde, which results in rapid penetration and thorough fixation. Cacodylic acid is generally preferred as the buffer because it results in resilient lungs; that is, blocks of lung compressed by cutting rapidly resume their original fixed volume when placed in fresh fixative. A small amount of calcium is commonly added to the fixative to preserve phospholipids associated with pulmonary surfactant as well as those that are components of the various cell membranes. Although iso-osmotic fixatives are used, we prefer a hypertonic fixative (approximately 550 milliosmoles). All of the above desirable characteristics are achieved using a modification of Karnovsky's formaldehyde/glutaraldehyde fixative with added calcium chloride

(paraformaldehyde, 40 g/liter; glutaraldehyde, 100 ml of 50% solution/liter; calcium chloride, 0.5 g/liter; cacodylic acid, 12.8 g/liter) which is diluted 1 to 4.5 before use with cacodylic acid (32 g/liter) and the pH adjusted to 7.2 with 1.0 N HCl (6). The fixative is relatively simple to prepare and can be stored in the refrigerator for several months. It has the advantage of being a good storage fluid for keeping fixed tissues at room temperature. Using this fixative at 30 cm of pressure, fixation is rapid and complete. Fixation times of 2 and 4 hr are acceptable, but we prefer to maintain the 30 cm of pressure overnight or for 18 hr. Samples cut from these lungs are placed in fresh room temperature fixative where they may be stored without damage or deterioration for more than one year.

Fixation of lungs at a standard pressure of 30 cm of the fluid provides the most reproducible appearance for general purposes. Considerations of reproducibility and least artifact become more critical relative to morphometry. Here again, for purposes of pathology we find perfusion of the excised lung to be the method of choice. The alternative approach such as used for morphometry of normal lungs is perfusion via the trachea with the lungs in situ within the thoracic cavity (7).

Perfusion of excised lungs by trachea or major bronchus is a relatively simple procedure for rodents, once a series of delivery tubes leading from marriott bottle reservoirs is provided. Larger reservoirs are needed for lungs of larger species. Although the perfusion method cannot be performed as rapidly as immersion of samples in fixative, the greater effectiveness in enabling detection and evaluation of subtle or mild lesions more than outweighs the greater cost in time taken. Where large numbers of animals per treatment group are involved, at least a significant proportion of lungs should be fixed by airway perfusion.

FIXATION OF LUNG BY PERFUSION THROUGH AIRWAYS As will be evident from the foregoing discussion, our preferred routine method of fixation is perfusion by the airways with modified Karnovsky's fixative at 30 cm of fluid pressure (6). We find that after partial collapse of the lungs has occurred on excision, no degassing is necessary to obtain complete distribution of the perfusate. Degassing is in fact contraindicated for most purposes because it increases the cumbersomeness of the technique, lessens the degree of reproducibility of reinflation, and makes redistribution of components of any lesion more likely.

FIXATION OF LUNG BY IMMERSION Massively consolidated or edematous parenchyma, or large solid lesions such as tumors, have to be fixed by immersion in fixative fluid. For subsequent study by light microscopy, formol-Zenker is preferable to formalin because it heightens the contrast of hematoxylin and eosin staining, especially the eosinophilia of proteinaceous transudates or exudates. The shrinkage caused by immersion in fixative is used to advantage in enumeration of tumor nodules in lungs of strain A mice, which is the basis of a carcinogenesis bioassay system (8). Tissue to be examined by electron microscopy is immersed in the modified Karnovsky's fixative described previously.

Immersion fixation is also used when the redistribution of intraluminal particles, cells, or exudates might interfere with the objectives of the study, as in determining the fate of inhaled particles (9, 10).

FIXATION OF NASOPHARYNX AND LARYNX After gross examination, these structures in small animals (i.e. rodents) can be fixed in toto in the modified Karnovsky's fixative after flushing surfaces with fixative to remove trapped air bubbles and mucous coat. Samples of tissues from recognized lesions and representative portions of the nasoturbinate region, pharynx, and larynx need to be dissected out in large animals.

Sampling for Microscopic Examination

The size and diversity of components of the respiratory tract pose a considerable sampling problem in the thorough search for lesions. This is compounded by the inhomogeneity of morphologic responses of the tract to irritants, as was mentioned in the introduction. These two features together require that sampling be both wide in distribution and specific in anatomic localization. The number of large blocks taken for examination by light microscopy and scanning electron microscopy will be determined by the compromise between thoroughness and the practical limit in terms of cost of preparation and examination. But there is a minimum below which the risk of spurious conclusions due to serious sampling errors becomes unacceptable. Any sampling of parenchyma must take into account vertical (gravitational) gradients affecting the distribution patterns of certain lesions and the difference between hilar and peripheral regions of lobes.

TRACHEOBRONCHIAL TREE AND PARENCHYMA The sampling procedure varies according to the size of the lung. In the case of rodents such as rats and hamsters, sampling of the trachea presents few problems other than the need to be aware of possible differences in the mucosa over the cartilaginous and intercartilaginous portions of the trachea as has been found in the rat (11). A block containing a longitudinal section of distal trachea and the bifurcation into bronchi suffices for nonparenchymal regions. The preferred planes of section for rodents' lungs are illustrated in Figure 1. These are vertical sections in the sagittal plane for the left lung and from the hilus along the axis of major airways for the cranial, middle, and caudal lobes of the right lung. All of these blocks can be sectioned whole for histologic examination. Although the sagittal section of the left lung is a common section for major attention, we prefer the sections from the right middle and caudal lobes. One reason is that unless the section of the left lung is cut very close to the midline, most of the airways are cut transversely. The longitudinal sections of airways present in blocks from the right middle and caudal lobes reveal bronchial and acinar orientations of lesions much more readily, especially by scanning electron microscopy (SEM). A second reason is that the blocks from the right lung are of a more convenient size to mount whole for light microscopy.

More care in sampling is required for lungs of larger animals such as dogs and monkeys because of the bulk of tissue to be surveyed and the increased likelihood of regional variations in response being manifested. To minimize sampling errors, standard parenchymal sampling sites covering both dorsoventral and hilarperipheral axes should be chosen. The nine sampling sites we take from parenchyma of the four lobes of the right lung of the dog are illustrated in Figure 2. Because we frequently use one lung of dogs and monkeys for biochemical studies or those

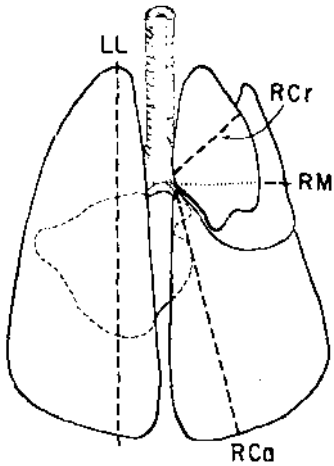

Figure 1 Schematic outline of the dorsal view of a rat's lungs illustrating the vertical planes of section for sampling tissue. The contour of the accessory lobe is indicated by the narrow broken line. LL, left lung; RCr, right cranial lobe; RM, right middle lobe; RCa, right caudal lobe.

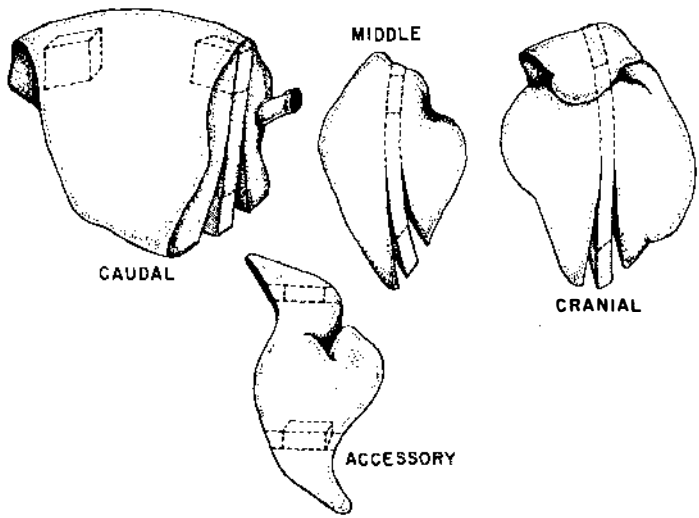

Figure 2 Schematic outline of the lateral view of the four lobes of a dog's right lung illustrating nine sampling sites.

requiring special fixation, such as freezing, we derive most morphologic information on the basis of one lung. If the two lungs are available, samples can be taken from both. Samples of major airways typically consist of proximal trachea, bifurcation of trachea, and lobar bronchus.

Evaluation of pulmonary toxicity invariably involves the comparison of lungs from two or more groups of animals. For this specific comparison, by qualitative or quantitative (morphometric) means, we use the same sampling sites for tissue blocks in all animals (12) rather than the method of stratified random sampling using a random number table together with a numbered sampling grid (13). The latter method relates more to statistical confidence with which the sample represents the lung from which the sample is taken, rather than to precise comparison among different lungs where the lesion can be affected by specific anatomic location.

When detailed comparisons are required within or among groups of animals, a useful approach is to select a specific bronchopulmonary segment of the lung for more specific study. Sections of segmental bronchus, terminal bronchiole, and more distal lobular tissue can be sliced out of the desired bronchopulmonary segment under a dissecting microscope. These sections, as well as those of trachea and lobar bronchus, can then be closely compared.

NASOPHARYNX AND LARYNX Blocks are taken representing proximal and distal regions of nasal sinuses and turbinates, and the pharynx and larynx. Again, more are required for larger animals. For some studies it is desirable to dissect mucosa from the nasal septum or turbinates and prepare it as a whole mount for morphological examination. Further details of the use of whole mounts and sections of nasal regions can be found elsewhere (14, 15).

Microscopic Examination

The need for examination of a wide sampling of pulmonary tissue has already been stressed. Requirements for cost effectiveness in the evaluation of lungs from large numbers of animals in toxicity trials means that the microscopic methods most useful are those that provide for examination of large samples, that is, light microscopy (LM) and SEM. For initial detection and analysis of lesions we use correlated LM and SEM. The best way to do this generally is to take complementary blocks of tissue from the same sampling site, embed one in plastic suitable for large 1 μ sections and process the other for SEM. The surface and sectioned views can then be compared for interpretation. The advantage of the large 1 μ section is that it not only provides the best resolution for LM, but also enables precise selection of anatomic locations for thin sections to be examined by transmission electron microscopy (TEM).

This is a discussion of routine microscopic methods and we recognize that the word can take on shades of difference in meaning according to the objectives of the investigations. Often, most microscopic screening has to be by LM of paraffin-embedded tissue because of the bulk of specimens. Equally so, it must be realized that in the search for subtle effects or in the description of damage once it is found, at least a significant number of lungs from animals in the critical experimental groups should be examined by correlated LM, SEM, and TEM.

LIGHT MICROSCOPY Survey by LM of sections carefully prepared from vacuum-embedded paraffin blocks and stained by hematoxylin and eosin provides the basis for other modes of microscopic investigation (Figure 3). More definitive study of cellular components of lesions is made on the 1 μ sections cut from large plastic-embedded blocks and these provide the essential link between LM and TEM (see segment on TEM below). The paraffin sections also provide the basis for a large variety of special staining methods (16).

SCANNING ELECTRON MICROSCOPY The large, approximately 12 X 10 X 4 mm samples of tissue selected for SEM are the complementary halves of blocks used for LM and are cut so as to include longitudinal sections of airways in the surface to be examined (Figures 4–6). The tissue blocks are dehydrated in graded ethanol and then dried by the critical point procedure using CO_2 (6, 17). The dried tissue is attached to standard SEM stubs put in a high vacuum coating device on a tilting and rotating stage and coated first with carbon then with gold-palladium (18). Such tissues can be stored in a desiccator for prolonged periods and still be useful for SEM.

Although not a routine procedure, to enable precise correlation between surface features seen by SEM and cross-sectional features of selected areas, blocks can be removed from the SEM stub after evaluation and prepared for LM and TEM. They are placed in 100% ethanol, which is next substituted by propylene oxide, and are then embedded in an Epon-Araldite mixture. The tissue is examined by LM of 1 μ sections, and specific regions can be selected for TEM (18). Information on

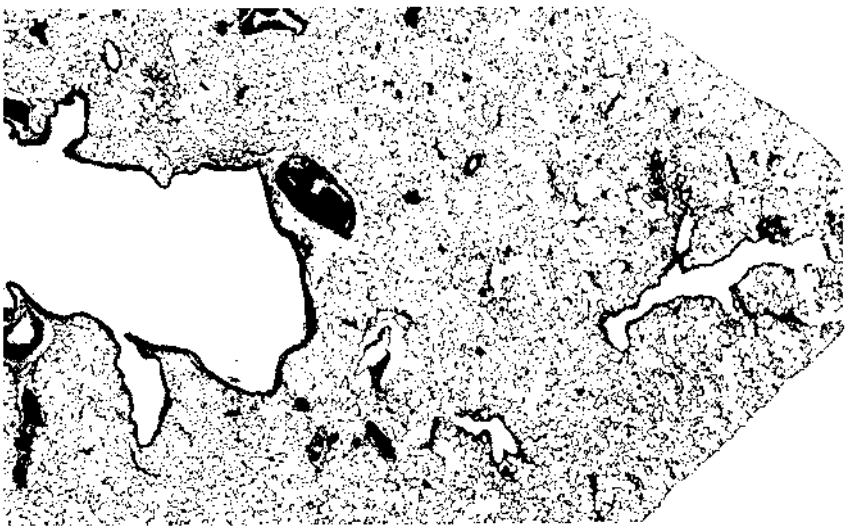

Figure 3 Light microscopy of properly prepared paraffin sections provides a relatively simple and rapid means of screening all levels of the tracheobronchial tree and parenchyma. Normal rat lung, H & E stain, 12 X.

interior aspects of tissues and cells can be obtained by SEM after the tissue has been fractured either before (19) or after (20) drying. It can also be obtained from plastic-embedded tissue after iodine and acetone surface etching (21).

TRANSMISSION ELECTRON MICROSCOPY Because lesions in the lung are frequently focal and have a specific orientation relative to the acinar structure of the pulmonary parenchyma, it is essential to know precisely the anatomic location in the small airways or acinus from which the TEM blocks are taken. This precise location can be learned by several routes. The oldest is a modification of the procedure used by Grimley (22) wherein large, 2 X 2 cm blocks of tissue are embedded as for TEM, and alternate 30 μ and 10 μ sections cut on a large microtome commonly used for metal or bone. The 10 μ sections are evaluated using light microscopy, and the precise lesion area is dissected from the adjacent 30 μ section, cemented on a block from a beem capsule, and ultrathin sections cut (23). It has the disadvantage of relatively low resolution for LM due to the thickness of the section. This can be avoided by embedding slightly smaller blocks (i.e. 12 X 10 mm) and cutting 1 μ sections on a Sorval JB-4 microtome using glass knives. These thin sections can be stained using various dyes and provide high resolution for evaluation of the tissue by LM (Figures 7*A*, *B*). The areas of interest are selected in the one-micron section, identified in the block, and the surrounding tissue removed leaving a plastic mesa containing the required region (24). This mesa is sectioned in the usual manner and examined by TEM (Figure 7*C*).

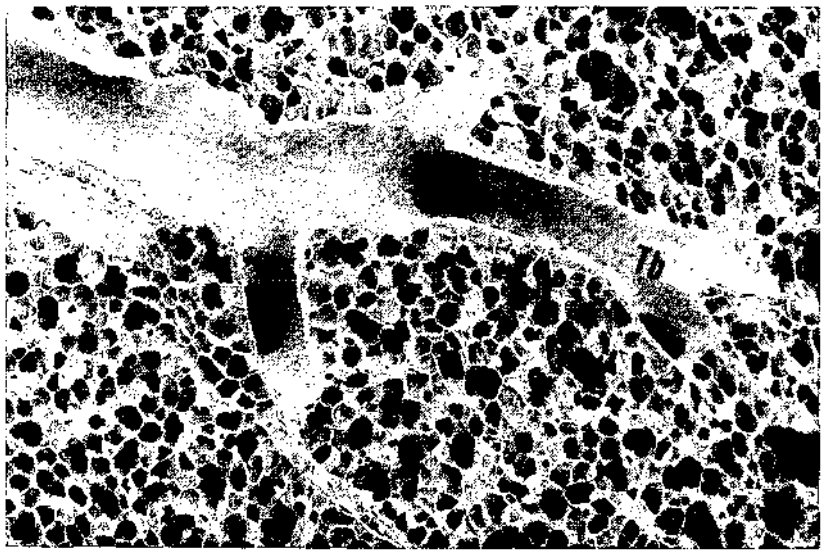

Figure 4 SEM enables evaluation of surfaces and is intermediate in resolution between LM and TEM. It allows the examination of relatively large areas and provides a great depth of field as illustrated in this micrograph of normal rat lung. Tb, terminal bronchiole; Ad, alveolar duct; 30 X.

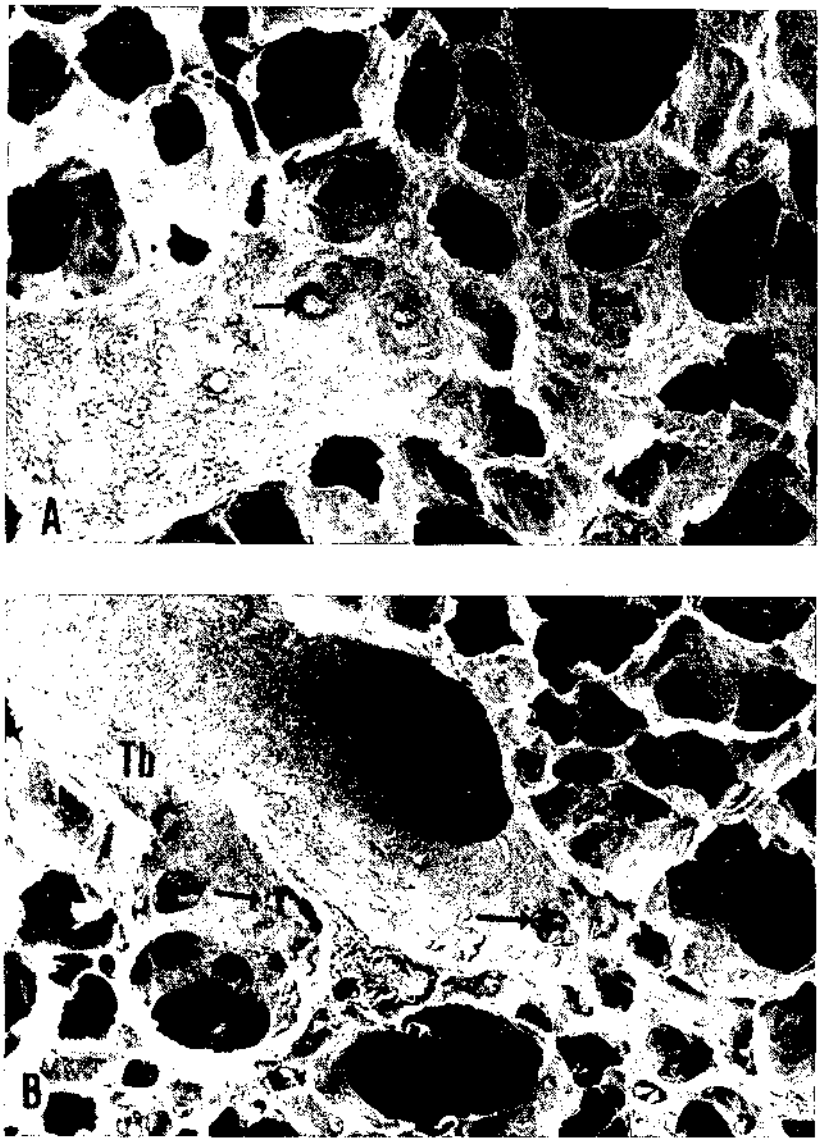

Figure 5 *A.* The transition region from terminal bronchiole to alveolar duct is an area frequently damaged by inhaled irritants. Occasional macrophages (*arrow*) can be observed within proximal alveoli of this alveolar duct from a normal rat, 170 X. *B.* Compared to the normal, in the rat following exposure to ozone (0.8 ppm for 7 days) the terminal bronchiole has a flattened surface appearance (Tb) and proximal alveoli contain clusters of infiltrating inflammatory cells and debris (*arrows*), 160 X.

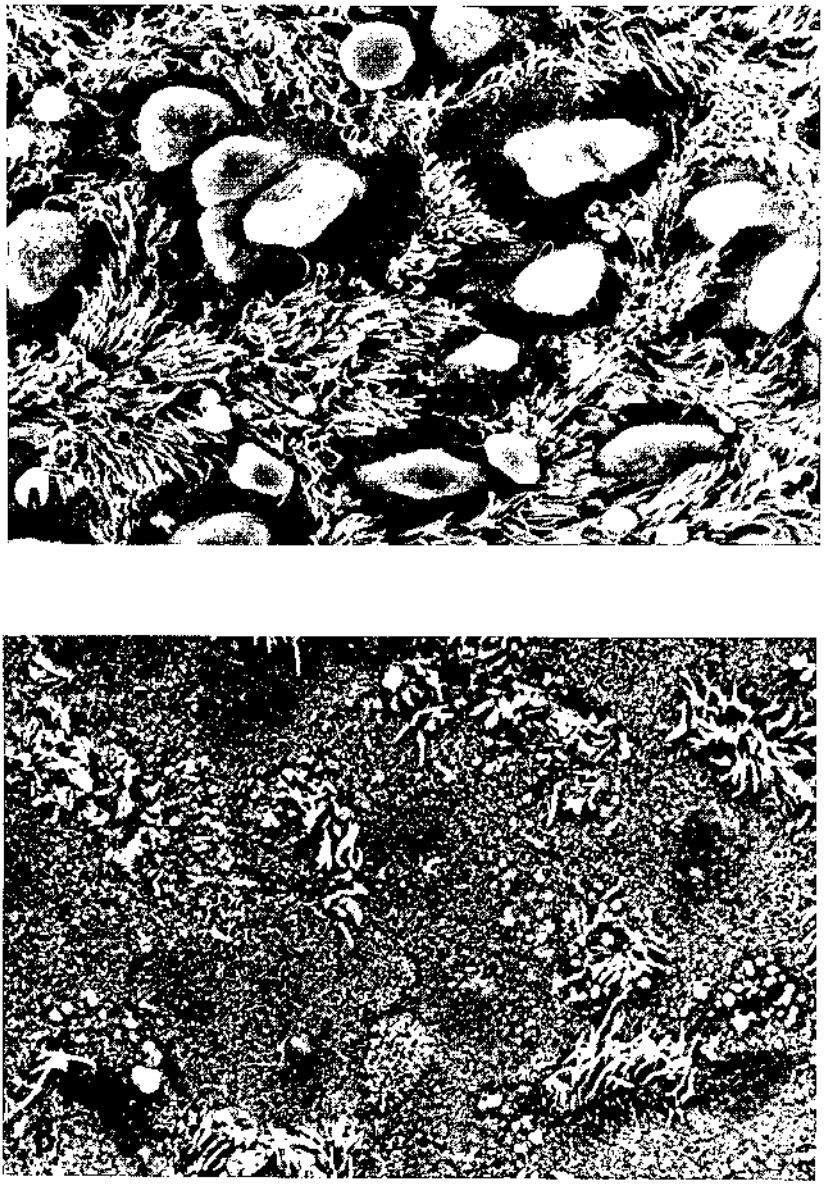

Figure 6 *A.* Highly magnified SEM view of normal terminal bronchiolar epithelium in the rat, 2800 X. *B.* In contrast, note the loss of surface projections of nonciliated bronchiolar (Clara) cells and the shortening and reduced density of cilia in a rat exposed to 0.8 ppm ozone for 7 days, 2300 X.

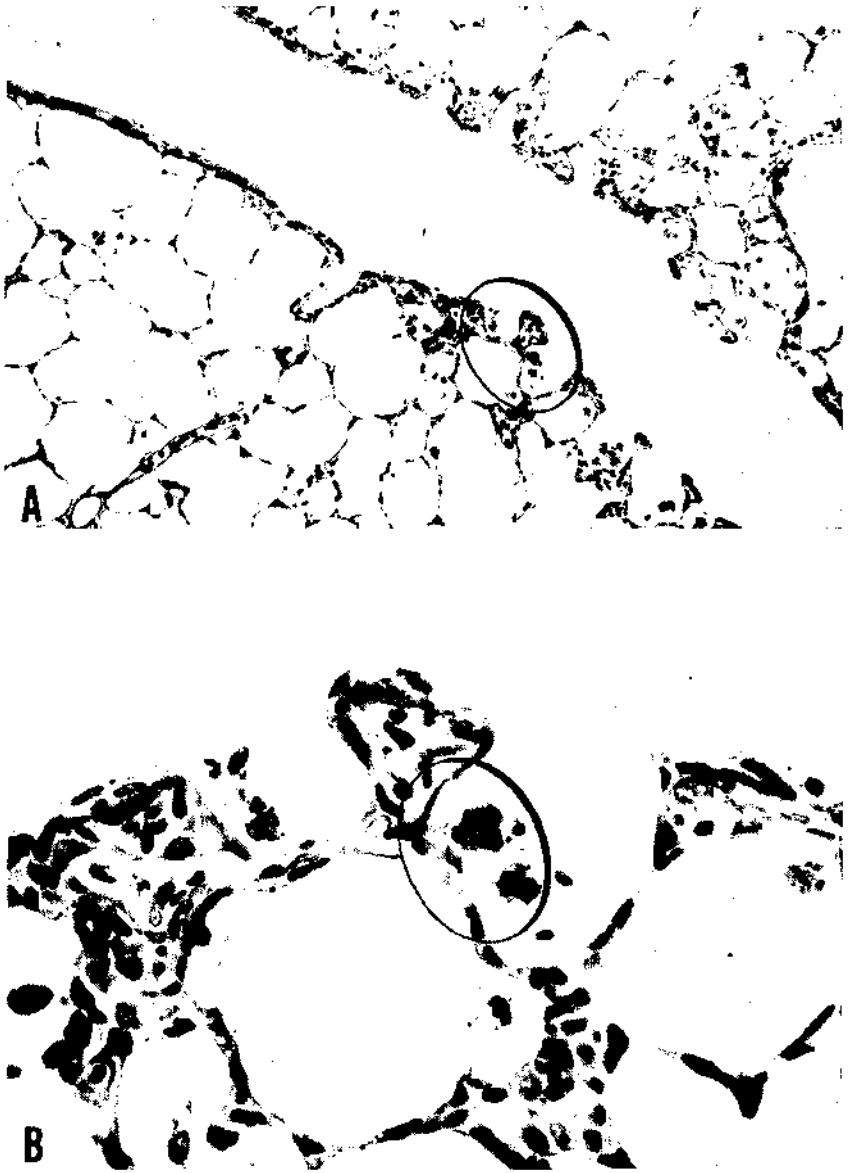

Figure 7 *A.* One micron section from plastic-embedded lung of a rat exposed to 0.8 ppm ozone for 2 days provides good resolution of cellular detail by LM and localization of specific region of interest (*circled*). Richardson trichrome, 111 X. *B.* Higher magnification of area circled in *A,* 500 X.

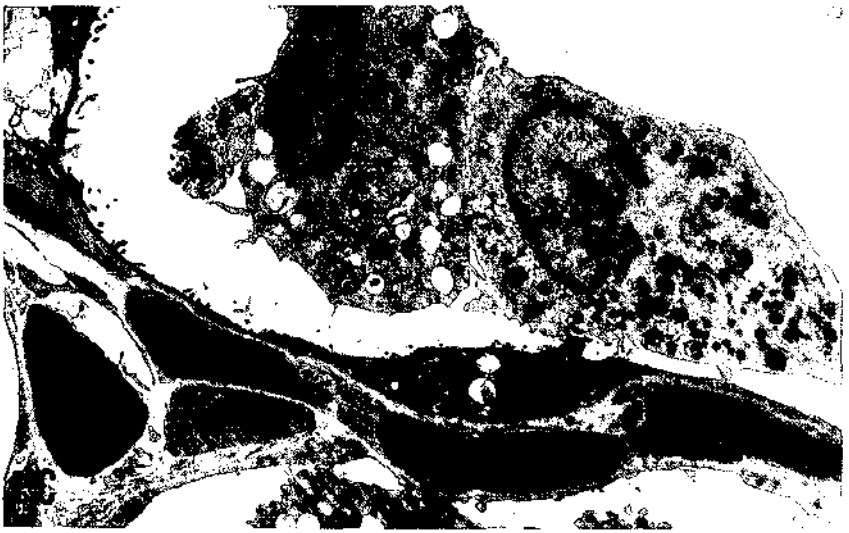

Figure 7 *C.* Using the mesa technique, a portion of the lesion, such as circled in *B*, can be selected and thin sections from the same region of the block examined by TEM. Uranyl acetate and lead citrate, 3750 X.

SPECIAL METHODS FOR GROSS AND SUBGROSS EVALUATION

Whole Lung Sections

The technique of preparing whole sections from human lungs was first described by Gough & Wentworth (25) and was used in their studies of emphysema in man. The sections can be useful as permanent records or illustrations of whole lung involvement in certain types of disease processes. Subsequent developments of the technique and their use in the measurement or grading of emphysema in human lungs is briefly discussed in a report by Dunnill et al (3). Preparation of lung macrosections and their permanent mounting by their lamination between sheets of transparent plastic film has also been described (26, 27).

Vascular Injection Technique

A technique using thin slices of lungs in which the vessels have been injected with multicolored latex has been used in studies of the comparative subgross pulmonary anatomy of a variety of mammals (28, 29). A major focus of attention in these studies was the comparative anatomy of the vascular tree. Vascular injection and casting have been used in investigations of the vascular changes accompanying emphysema in man (30).

Airway Casting

This is useful for development of mathematical models for behavior of inspired gases and particles (31) and for the study of airway disease (32) and the pathogenesis of emphysema (33).

Replica casts of airways down to and including alveoli can be prepared in situ for large and small animals (31). The method involves replacement of air by cyclic ventilation with CO_2, filling of the lungs with degassed saline, and slowly injecting silicone rubber through the trachea while allowing saline to drain from the thorax via slits between ribs. After curing (2–20 hr) the organ is removed from the thorax and the tissue digested away. Morphometric measurements that may be made on such casts include branching angles and dimensions of airways and alveoli. In some cases alveolar pores can be seen and their relative sizes determined via the scanning electron microscope.

The major limitation in using airway casts is that all but the simplest measurements made on them may require considerable time, effort, and skill. On the other hand, a replica cast captures and preserves the entire airway structure, allowing precise determination of the spatial and structural distribution of lesions.

SPECIAL METHODS OF MICROSCOPIC EVALUATION

A variety of investigative methods is needed in the search for pathogenetic mechanisms underlying disease processes in the lung, as it is for any organ. These methods are relevant to studies of both cellular biology and pathobiology, and unavoidably investigations into the one have considerable impact on the other. The techniques in question have for the most part either been in use for a relatively short time, or are in the process of being explored. Only an introduction to these topics is therefore provided.

Histochemistry

Histochemistry and cytochemistry are essential for the full elucidation of the pathogenesis of toxic changes in inhomogeneous organs such as the lung, because it is necessary to localize biochemical changes to the specific cells or cell populations involved.

Many specialized methods of tissue preparation and incubation are required for the broad spectrum of histochemistry. Enzyme histochemistry and histochemistry for certain other cellular components are best done on cryostat sections. As with all sections of pulmonary parenchyma, distended cryostat sections are much easier to evaluate and provide more useful information. Usually the tissue is distended with a cryostat embedding material, commonly 4% gelatin, as originally described by Tyler & Pearse (34). This embedment has the double advantage of distending the lung and also providing a medium that permits much more complete sections than can be obtained if the lung is handled like other organs or tissues. Without such embedding, only fragments of sections are obtainable. These are extremely difficult to evaluate in terms of total distal airway and parenchymal morphology. Freezing

of the gelatin-infiltrated section is generally accomplished using Freon 22® cooled to near its freezing point of –160°C. Freezing directly in liquid nitrogen is considerably slower and frequently distorts the tissue blocks. While most cryostat sections tend to be thicker and therefore provide less resolution than paraffin sections, with appropriate equipment it is possible to cut serial frozen sections at 5 or 6 μm. Such sections are suitable for a wide variety of histochemical procedures, which are commonly applied to the serial section in order to obtain correlated biochemical and morphological information at the cellular level.

Histochemical procedures have been diversified significantly in recent years (35) and include methods for many enzymes as well as cell inclusions and intercellular material. Many of these procedures can be applied at both the light and electron microscopic levels of observation of the lung (36–40) and some are suitable for automated image analysis (41). The studies of Spicer et al (42) and Lamb & Reid (43, 44) concerning toxic effects of inhaled gases on respiratory mucopolysaccharides are especially noteworthy. Another example is the fluorescent amine technique of Falck that has been used in studies of serotonin-producing cells of neuroepithelial bodies present in respiratory mucosa (45).

A promising new area of chemical analysis that can be applied to the respiratory system is that of analyzing X rays, cathodoluminescence, or back-scattered electrons generated by the interaction of the electron beam of an SEM or TEM with the atoms of cellular components, inclusions, or histochemical final reaction products in situ, thus providing elemental analysis of endogenous or foreign materials in cells and tissues (46–49).

Autoradiography

Autoradiography has been used to determine the cytokinetics of pulmonary cells responding to damage caused by toxic environments, such as in the demonstrations that alveolar type 2 epithelial cells are the precursors of type 1 epithelial cells (50, 51). The second major use of autoradiographic techniques is for tracing the intracellular pathways traversed by radiolabeled precursors of known or hypothesized cell products (52–54). A third use is in studying the deposition and fate of inhaled particles (55).

Morphometry

Morphometry is necessary for precise correlation of structure and function in both normal and diseased organs. It can provide accurate measurement of the severity of damage in diseased organs, and it is the only means of confirming, by statistical methods, the existence of significant subtle lesions in a particular treatment group of experimental animals.

A systematic approach to a quantitative morphologic analysis of the architecture of the pulmonary system using manual methods has been provided by Dunnill (56), Weibel (57), and Thurlbeck (58). Those authors established the formulae and methods necessary to obtain statistically reliable quantitative values for the pulmonary system. Recently quantitation of the pulmonary system has been automated by use of computed pattern recognition techniques (59) and automated measuring micro-

scopes (60). The greatest application of automation has been with automated measuring microscopes. They have been used to quantitate selected features of conducting airways in normal and experimental bronchitis (61) and of distal airspaces in normal (62), emphysematous (63), and experimental, pollutant-damaged lung (12, 64). Pattern recognition techniques have also recently been used to classify and measure the distal airways on an automated measuring microscope (65).

Freeze-Fracture

Freeze-fracture is a method of looking at replicas of fractured surfaces at very high resolution using TEM. Like the SEM, it provides a view of surfaces rather than cross sections. Thus for low magnification and low resolution of natural or fractured surfaces, the SEM is the most appropriate instrument, whereas for high magnification, high resolution freeze-fracture or freeze-etch is the most appropriate technique.

Freeze-fracture procedures avoid the necessity for including chemical interactions, which may cause artifacts in the preparation of tissue, and reveal an en face view of membranous surfaces. In the pulmonary system, the method has been used for study of cell organelles, particularly during secretion and phagocytosis (66), for visualization of the alveolar lining layer (67), and in the examination of endothelial cells relative to their capability for metabolizing circulating vasoactive agents (68). The method is also necessary for the study of normal and abnormal cell junctions (69).

Tracer Techniques

These have been used in studies of the permeability of the pulmonary vasculature in both normal and edematous lungs. Horseradish peroxidase, hemoglobin, microperoxidase, ferritin, and colloidal particles have been used (70–74). The investigations on pathways of clearance of inhaled iron oxide aerosols (10) or intratracheally instilled ferritin or colloidal carbon (9) referred to earlier also involved the use of tracers.

Thick Histologic Sections

These were principally used in the study of human emphysema (75). To some extent they have been superseded by SEM, but they still have an important role in documenting the pattern of collagenous and elastic fibers in interalveolar septa and determining their abnormalities during the pathogenesis of diseases such as emphysema.

ADDITIONAL SPECIAL METHODS OF FIXATION

No one fixation procedure is appropriate for all investigative purposes. To the extent that considerations of methods of fixation are intimately related to the techniques of evaluation for which they are to be employed, common fixation techniques have already been discussed. There remain several, however, that have special indications to be matched with the specific aims of the investigator.

Vapor Fixation

Methods have been developed for the use of formalin vapor (76, 77) or formalin steam (78) but have little to offer in the way of advantages and nothing at all in convenience. Air fixation likewise has no usefulness other than to provide a convenient gross anatomical reference. A recent method of vapor fixation using osmium tetroxide suspended in cooled fluorocarbon has been briefly reported by Kilburn & McKenzie (79). The mixture was injected intratracheally into breathing hamsters to fix the lungs while inflated and to lessen the chance of translocation of cells and particles on luminal surfaces of airways.

Vascular Perfusion

The primary use of this method has been in the demonstration of extracellular lining layers of alveoli and bronchioles by electron microscopy (80, 81). Translocation of cells and particles should be less than by intratracheal perfusion, which may make this method useful for localization of these components.

Rapid-Freeze Method

This method was developed by Staub & Storey (82). It provides an accurate representation of the morphologic state of the lung "frozen" at a point in time in its cycle of dynamic events. The animal's lungs are frozen while it is alive, at the desired phase of the respiratory cycle. The procedure does require thoracotomy with good exposure of the lungs. Carefully controlled ventilation is required to maintain physiological state with the ability to momentarily hold the lung at the desired degree of inflation or vascular perfusion. Freon 22 cooled to near its freezing point of −160°C or propane cooled to −175°C is used as the cryogenic agent for rapid freezing as each of them absorbs significantly more heat per unit volume of weight than liquid nitrogen, which rapidly absorbs heat, then boils, forming an air interface which effectively reduces the transmission of additional heat from the specimen to the cryogenic agent. Only the first few millimeters of tissue under the pleura are extremely rapidly frozen; deeper tissue is frozen considerably more slowly. Tissues frozen in this manner may be freeze-dried or freeze-substituted for subsequent critical point drying followed by evaluation in the SEM (6) or followed by embedding in paraffin or plastic for light microscopy (23) or TEM. In the SEM, the general architecture of the pulmonary tissues is well preserved and available for evaluation, but the surface detail of the cells is obscured by the mucous coat or alveolar lining layer in the airways or alveoli respectively.

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