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# Immunohistochemical Techniques and Their Applications in the Histopathology of the Respiratory System

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**Subsequent to the first report in the 1940s on incubation of tissue sections with fluorescein-conjugated antibodies for localization of antigens, a great number of modifications were introduced to improve the validity of immunohistochemistry which has become a growingly popular tool. The use of immunoenzymatic techniques eliminates the need for expensive fluorescence microscopy equipment, the lack of permanency of preparations and the lack of electron density required in ultrastructural localization of antigens. Regardless of the technique, it is also important to choose a correct fixation which allows the proper preservation of antigens and morphology and the penetration of antibodies through the entire thickness of the preparation. A variety of immunohistochemical techniques have been applied to study several components of the lung, such as collagen, surface active material, lung specific antigens, and enzymes and the detection of tumor markers, immunoglobulins and infectious agents in the respiratory system which is reviewed. The large surface area and the multiplicity of cell types provided by the respiratory tract epithelium of humans for exposure to microbial as well as toxic substances in the environment make this organ system very vulnerable but a good early indicator of adverse health effects. Immunohistochemistry provides valuable information complementary to the immunochemical and biochemical characterization of this barrier.**

## Introduction

In recent years immunohistochemistry (IHC) has become an increasingly popular and effective tool in research and diagnostic laboratories. The manifest success of this method is due to technological improvements, increased availability of purified antigens, antisera, and other reagents, and the accumulation of practical experience in a variety of applications. IHC combines the advantages of the high specificity and affinity of antibodies in recognizing tissue antigens with the high topographical resolution of light and electron microscopy. It can thus provide information which would be difficult or impossible to obtain by other techniques (*1–6*).

Applications of IHC have contributed significantly to our knowledge about the distribution and pathological reactions of several components of the lung such as collagen, surface active material, lung-specific antigens, and enzymes; the method has also been applied to the detection of tumor markers, immunoglobulins, and infectious agents in the respiratory system. The first part of this review will deal with general principles of IHC techniques, while the second part will discuss particular applications relevant to pulmonary physiology, pathology and toxicology.

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## Immunohistochemical Techniques

### Development of Immunohistochemical Techniques

The origin of the IHC techniques goes back to the early 1940s when Coons et al. (*7*) reported that fluorescein-conjugated antibodies were useful in demonstrating bacterial antigens in infected tissue. The simple principle of this "direct" technique is schematically illustrated in Figure 1*A*. The method involved a single incubation of the tissue preparations with the fluorescein-conjugated antibodies directed against the antigen to be localized. Subsequently this original technique underwent many modifications, all aimed at improving its practicability and efficiency. The first major innovation (*8*) eliminated the need for conjugating each individual antiserum with the fluorescent dye by using unconjugated antibodies in the first step, followed by a secondary conjugated antibody directed against the

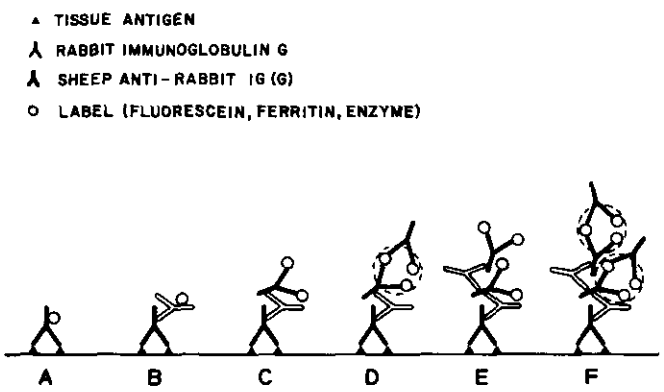


FIGURE 1. Immunohistochemical techniques: schematic representation of their development. (*A*) The direct technique with labeled primary antibody (*7*). (*B*) The indirect, sandwich, or two-layer technique (*8,12*) with labeled anti-IgG in the second layer. (*C*) The three-layer immunoglobulin-enzyme bridge (*11,21,22*) with unlabeled antibodies; anti-IgG in the second layer forms the bridge between first- and third-layer antibodies; binding sites of the third-layer antibody (usually anti-HRP) are free to bind marker enzyme. (*D*) The PAP (*23*) method; preformed peroxidase–antiperoxidase complexes (dashed circle) are used in the third layer. (*E*) The double bridge (*27*); incubations with second- and third-layer antibodies are repeated, in this order, followed by a single incubation with HRP. (*F*) The double PAP technique (*28*); second- and third-layer components are applied repeatedly (more than twice if necessary). For additional explanations, see the text.

immunoglobulins of the species from which the first or primary antiserum was derived (two-layer, sandwich or indirect technique) (Fig. 1*B*). For example, rabbit antiserum to the antigen is followed by fluorescein-conjugated sheep anti-rabbit gammaglobulin. Since the latter antibodies recognize determinants common to all rabbit IgG molecules (Fc region), a single conjugated reagent can be used for the localization of any number of tissue antigens as long as suitable rabbit primary antiserum is available. In addition, since several molecules of the labeled antibodies can bind to one molecule of primary antibody (a feature not shown in Fig. 1), substantial amplification of the staining is achieved. This technique is still used today with good success in many laboratories throughout the world. However, difficulties in preparing effective conjugates, the need for expensive fluorescence microscopy equipment, frequent incidences of disturbing background fluorescence, the lack of permanency of the resulting preparations, and the lack of electron density of the fluorescent label have all combined to stimulate the search for more convenient and versatile labels. The first such "substitute" label was ferritin (*9*), which is still popular since it permits high resolution at the electron microscope level. Ferritin can be used as a label chemically conjugated to the primary or secondary antibodies instead of fluorescein, as in Figure 1*A* or 1*B*. It can also be used as part of a more efficient ferritin bridge (*10*), to be discussed later. Several enzyme labels were suggested and among these horseradish peroxidase (HRP) emerged as the most popular (*11,12*). This enzyme, when used with suitable substrates (*13,14*), produces colored reaction products which are properly localized, stable, and electron dense, permitting ordinary light microscopy as well as electron microscopy. Other enzymes are also being used with good results with the various methods represented in Figure 1: acid phosphatase (*15*), alkaline phosphatase (*11*), glucose oxidase (*16,17*), and smaller hemelike molecules with peroxidase activity (*18–20*). These labels eliminate most of the disadvantages of immunofluorescence except the inconvenience and the loss of potency due to chemical coupling of the label to the antibodies. This last problem was solved in 1969 when Avrameas (*11*), Mason et al. (*21*), and Sternberger and Cuculis (*22*) independently described the so-called unlabeled antibody or bridge methods. In these techniques, antibodies directed against HRP (and derived from the same species as the primary antibody) are used as a third layer. Thus, all chemical labeling is eliminated, since HRP will bind to the third-layer antibodies by immunological mechanisms as an antigen (three-layer, bridge, or unlabeled antibody-enzyme techniques) (Fig. 1*C*). The ferritin bridge introduced by Willingham et al. (*10*) works on the same principle, using affinity-purified antiferritin antibodies in the third layer. The bridge technique afforded further amplification over the two-layer sandwich as well as the additional benefit of avoiding chemical labeling of antibodies altogether. In a further improvement, Sternberger et al. (*23*) demonstrated that HRP and its antibodies form stable soluble complexes (Peroxidase-Anti-Peroxidase or PAP complexes) and these can be applied as a single reagent in the third layer (Fig. 1*D*). Effective measures improving the binding of the primary antibody to tissue antigenic sites (extended incubation times), preventing nonspecific binding (blocking with normal sera, use of high dilutions of primary antisera), and optimizing the conditions for the enzyme–substrate reaction have resulted in a degree of efficiency never before possible in IHC staining (*24–26*). Furthermore, it has been shown (*27,28*) that additional amplification (and greater sensitivity) can be achieved by extending the length of the bridge through repeated incubations with the second- and third-layer components (double bridge techniques, Figs. 1*E* and 1*F*).

Protein A (*29*) may be used instead of the anti-IgG as the second-layer component (*30*), either to bear a suitable marker or to form a bridge with anti-HRP antibodies. In recent years, colloidal gold particles have been used as markers with good advantage (*31–34*). Additional variants of the bridge principle rely on the avidin-biotin system instead of immunological binding (*35,36*). These methods are very promising and seem to offer certain advantages over the "classical" bridge procedures; however, it remains to be established whether or not the use of protein A, avidin or biotin as IHC reagents creates any unexpected problems with regard to specificity.

## Preparation of Tissue

It has been shown, especially with the more efficient enzyme labels and multilayer techniques, that many endogenous and exogenous tissue antigens are remarkably resistant to the effects of denaturing (alcohols, acetone) and crosslinking (aldehyde) fixatives and routine paraffin embedding procedures. For lung tissue, frozen sectioning after filling of the alveoli with optimal cutting temperature (OTC) embedding compound (Tissue-Tek II, Miles Laboratories, Elkhart, IN) to ensure expanded state of the lung parenchyma, followed by acetone fixation was found excellent for immunofluorescent studies (*37*). Frozen sectioning and the concomitant loss of structural integrity may be replaced by sectioning fixed tissue blocks in a special instrument, the Vibratome (Oxford Instruments, Columbia, MD). Sections of up to 100 μm thickness can be prepared and, after permeabilization (see below), stained with any of the available peroxidase techniques. After evaluation with the light microscope, the stained sections or parts of them can be embedded in plastic and further processed for electron microscopy. Such "pre-embedding" staining is one of the most powerful approaches available today (*38–42*). More detailed guidelines for the selection of fixation and embedding procedures are given by Sternberger (*5*) and Pearse (*4*). Following the suggestion of the latter author, it is recommended that a suitable series of methods be tested whenever a new problem is being considered. This should include: routine formaldehyde fixation, paraffin embedding; routine Bouin's fixation, paraffin embedding; cold ethanol or acetone fixation, paraffin embedding; any of the above fixatives, frozen or Vibratome sectioning; fresh frozen sections, any suitable fixative. Fixation must be followed by thorough washing in phosphate-buffered saline (PBS) to remove excess fixative and thus facilitate optimal immunostaining.

Penetration of the antibodies through the entire thickness of the section is essential for best results. The organic solvents used during paraffin processing appear to render such sections permeable; however, when fixed frozen or Vibratome sections are used, treatment of the sections with detergents (*38,43,44*) or ethanol (Petrusz, unpublished) is essential before application of the primary antibody.

## Unmasking of Antigens in Tissues with Proteolytic Enzymes

The detection of many tissue antigens is facilitated by treatment of the sections with proteolytic enzymes prior to IHC staining. Such treatment results in both intensification of specific staining and reduction of nonspecific background. This is well documented for immunoglobulins (*45–48*) and various infectious agents (*49–51*), and promising results were observed with neuropeptides (*52*) and pituitary hormones (Petrusz, unpublished). Trypsin (*49*), pepsin (*53*), and pronase (*47*) have all been recommended, and the required conditions seem to vary with both the methods of tissue processing and the enzyme used. As pointed out by Curran and Gregory (*46*), pretreatment with proteolytic enzymes permits the use of much higher dilutions of the primary antisera, thus reducing nonspecific background and staining of unwanted antigens (see below).

# Criteria of Validity

Performing IHC staining is relatively easy, but correct interpretation of the results presents some unusual problems. Interpretation should be based on generally accepted criteria of validity. Such a set of criteria, including efficiency, accuracy, precision, sensitivity and specificity, have been proposed by Petrusz et al. (*54*). These criteria have been defined and procedures have been outlined whereby IHC staining can be improved and the results interpreted in terms of these criteria. Among the proposed criteria of validity only two, efficiency and specificity, will be discussed here, since these are the most essential ones and have direct bearing on the performance and interpretation of IHC staining. For further details, the reader is referred to other publications (*2,4–6,54,55*).

## Efficiency

Efficiency in IHC staining is defined as the signal-to-noise ratio in a stained preparation. Clearly, the improvements represented in Figure 1 from *A* to *F* are all aimed at maximizing the signal, i.e., the quantity or intensity of label attached to a given antigenic site in the tissue. Efficiency may also be improved by facilitating the binding of the primary antibody to the tissue antigen (extended incubation time, low ionic strength buffer, close to neutral pH detergents in buffer) and by taking effective steps to reduce any possible background staining. The latter can be achieved by saturating nonspecific binding sites with proteins from normal sera, by using purified antisera if necessary, and in general by adjusting the conditions of all incubations to favor specific but to prevent nonspecific binding. Efficiency may be improved by treating sections with proteolytic enzymes prior to immunostaining. Optimal efficiency in a given system is best found by titration, determining the dilution of the primary antiserum which produces maximum specific staining without nonspecific background. Naturally, a prerequisite of this procedure is prior rigorous standardization of all other steps involved in the particular technique used. Such standardization and titration should precede all definitive IHC work.

## Specificity

Two fundamental sources of nonspecifi city have been defined in IHC staining: method nonspecificity and

antibody nonspecificity (*5,55*). This approach to specificity is useful because it is generally valid for all IHC techniques and it offers relatively simple practical guidelines to solve or prevent actual problems of nonspecificity.

Method nonspecificity essentially includes all staining that commonly would be described as "artifact." It may arise from binding to the tissue of any other staining component than the primary antibody (e.g., protein-protein interactions) (*56*) or from generation of a false signal through mechanisms unrelated to the IHC staining (e.g., endogenous peroxidase, tissue autofluorescence). The use of the highest possible dilution of the primary antiserum and omission of individual steps (reagents) from the staining sequence will be very helpful in identifying and eliminating some of these problems. As a perplexing example, mast cells have been constantly reported (*57–62*) to bind antibodies in their granules by immunofluorescence and immunoperoxidase techniques. Many naturally occurring substances (e.g., collagen, porphyrins, cartinoids) show autofluorescence. In addition, exogenous substances such as drugs (acridines, certain antibiotics, salicylic acid derivatives) and other chemicals can be detected by fluorescent microscopy and thus can be the sources of false positive results in immunofluorescent studies (*60*). With immunoperoxidase techniques, endogenous peroxidase activity may be particularly disturbing in lung tissue where peroxidaselike enzymes are present in macrophages (*60,63*), in erythrocytes and leukocytes trapped in the rich vascular system (*64*), on surfaces of alveolar cells (*64*), and in epithelial cells lining the bronchial tree (*64,65*). Unless proven otherwise, it should be assumed that such enzymatic activity survives the fixation and embedding procedures commonly employed. Endogenous peroxidase can be (1) tolerated if it is known and not disturbing, (2) inactivated by any of several available methods before immunostaining (*1,59,66–68*), or (3) reacted with a substrate producing different color than that used for the immunostaining (*69*).

The ultimate question to be answered in every IHC staining is the question of antibody specificity: how confident can one be concerning the identity of the tissue antigen localized. From this point of view, IHC does not differ from other immunochemical systems: the specificity of the antibodies responsible for a given IHC staining can only be defined by studying the interaction of these antibodies with known antigens of high purity. However, IHC methods *per se* are not based on competitive interactions such as, e.g., radioimmunoassay (RIA). In RIA only those antibodies will be operative which recognize (bind) the radioactive tracer; all other antibody populations present in the antiserum can be excluded from consideration. In IHC, all antibody populations which find a complementary antigenic site in the tissue will bind and produce staining. Consequently, inhibition of the IHC staining with known and pure antigens (absorption) is the only direct method to gain information on the specificity of the staining, i.e., on the identity of the antigen localized. This information is not absolute, since it evidently refers only to the antigenic site and not to the entire molecule of the antigen. When an antigenic site occurs as a partial sequence in two or more larger molecules (a frequent possibility), it is clear that the absorption test alone will not be sufficient for identification of the tissue antigens stained. (It should be noted that this as well as most of the previous arguments apply to both conventional and monoclonal antibodies.) In such cases, one possible recourse is to search for antisera which might recognize a different portion of the antigen not shared with other molecules. Otherwise IHC results must be supported by physiological or biochemical data concerning the identity of the antigen localized.

Absorption or "blocking" is usually done by combining known amounts of the antigens with working dilutions of the primary antiserum. If the antigen binds to the sites that are critical for immunostaining, subsequent staining will be blocked or inhibited. Since the tissue antigen is in insoluble phase, it will be favored by the antibodies versus the antigen added in soluble form; therefore, it may be necessary actually to remove antibodies from the solution, either by creating suitable conditions for the formation of an insoluble precipitate, or by using antigen coupled to insoluble carrier particles. Similar liquid or solid phase systems may also be used to assess cross-reactivities of antisera and to purify antisera by removing unwanted antibody populations. Further theoretical and practical questions concerning specificity and other criteria of validity have been discussed elsewhere (*54,55*).

## Interpretation of Staining

The great popularity of IHC techniques leaves no doubt about their usefulness and practicability. However, for correct interpretation of the results it is extremely important to follow certain routine rules of standardization and to perform all necessary controls. In our view, method specificity in most cases should be practically absolute, with only very few exceptions (e.g., peroxidaselike activity in blood cells when specific staining is clearly separated from the vascular compartment). The method, including the enzyme–substrate reaction, should be standardized so that conditions are optimal and do not vary from one staining to the next. This will ensure reproducible results when identical materials are used. Titration should establish the optimal dilution of the primary antiserum and no staining should be present at extremely high dilutions (method specificity). Antibody specificity should be evaluated by at least one of the available absorption procedures and addition of excess antigen should inhibit the staining. Moreover, any additional other evidence (physiological, biochemical, pathological) should be sought and evaluated to support the validity of the conclusions drawn from IHC results.

# Immunohistochemistry of Specific Components of the Lung

## Collagen

In much of the early immunochemical work on extracellular sites of collagen and reticulin, which is not reviewed here, it was difficult to assess the role of contaminating antigens, particularly serum proteins. However, in 1969 Nagasawa and Shibata (*70*) found immunofluorescence in human alveolar capillary basement membrane with fluorescein-labeled antiserum to human glomerulal basement membrane. Engel and Catchpole in 1972 (*71*) demonstrated immunofluorescence in alveolar walls and in the lamina propria of the airways in the rat using a specific antiserum against rat tail collagen. The antibody reacted with several forms of isolated collagen but not with rat serum. Basement membranes and reticular fibers of other organs were also stained.

Subsequently, purified antibodies to distinct types of interstitial collagens and procollagens (types I, II, and III) were used for a qualitative survey on distribution of various collagens in many IHC studies on healthy as well as diseased tissues (*72*). Purified antibodies against both human and mouse type IV collagen reacted in indirect immunofluorescence tests with basement membranes in various human and mouse tissues including the lung (*73*). The findings indicated that various basement membranes might contain related or identical collagenous proteins with a high degree of interspecies homology. All types of collagen (types I, II, III, IV and $AB_2$ or V) have been found in the lung (*74*). In an immunofluorescence study of pulmonary fibrosis with affinity-purified antibodies in 1980, Madri and Furthmayr (*74*) assessed the relative amounts of collagens in different locations. Type I collagen was markedly increased in alveolar septae, type III was markedly reduced and observed only perivascularly, type V was considerably increased in the interstitium and located in areas of smooth muscle cell proliferation. No change was noted with type IV collagen. These IHC results were consistent with the results obtained with various other methods such as dissection, isolation and compositional analysis.

The previously cited IHC work on collagen of the lung was performed on fresh frozen material with fluorescein label. But there is cumulative evidence that at least collagen type IV antigenicity is preserved through conventional formaldehyde-fixation and paraffin processing (*75,76*). Ultrastructural localization of a soluble collagen antigen (*77*) and type IV antigen (*78*) was obtained in the trachea of chick embryos and endothelial cells of gums, respectively, by a post-embedding immunoperoxidase technique. At the time of preparing this article immunoelectronmicroscopical studies on collagen synthesis in the lung were not available. In the future they may provide useful information on the pathogenesis of pulmonary fibrosis which composes 20% of the noninfectious diseases of the lung, and is often associated with drugs or toxic substances.

## Surface-Active Material

Following the discovery in 1958 of the surface-active lipoprotein layer, so-called surfactant, that forms the interface between gas and liquid in the alveoli (*79*), the demonstration of the material was dependent upon the surface tension lowering effects of lung extracts until an IHC study by Craig (*80*). In 1964 he obtained an antiserum to washed foam from autopsied human lungs that did not cross-react with human serum proteins and was localized by fluorescence microscopy on the alveolar walls of human lungs. Since all commonly used fixatives dissolved the antigen from specimens, he mainly used unfixed cryostat sections for his study on the distribution of surface-active material in the lungs of infants with and without respiratory distress. He described the progression from intracellular to both intracellular and alveolar, and then to solely alveolar localization, of the specific antigenic protein during the gestation period.

In a number of immunochemical analyses, different preparations of surface-active material were found also to contain serum protein antigens (*81*). Ultrastructural demonstration of plasma proteins, namely autologous albumin and IgG in the alveolar lining material of the normal rat lung was reported by Bignan et al. (*82–84*) with an immunoperoxidase technique: fibrinogen was not found under normal conditions. Deposits of $C_3$, IgG, fibrin and occasionally $C_4$, factor B and IgM were demonstrated by immunofluorescence also in hyalin membranes of infants who died with group B streptococcal sepsis or idiopathic respiratory distress syndrome (*85*). Thus it was suggested that immunologic processes might contribute to the pathogenesis of certain acute lung injuries in infants.

In 1973, Klass (*81*) used absorbed rabbit antibody against dog pulmonary surface-active material that did not cross-react with dog serum. Specific staining in cryostat sections of dog lung was observed in the alveoli and also in the walls and mucosal surfaces of the large airways but was lost from the alveolar surfaces after fixation and processing of the tissue. An RIA of pulmonary surface-active material in sheep lung was developed in the same laboratory (*86*) and offered for evaluation of the maturity of the lung. In 1977, Sueishi et al. (*87*), using immunoelectron microscopy after pre-embedding immunoperoxidase staining of glutaraldehyde fixed cryostat sections, were able to demonstrate surface active material in the alveolar lining layer, close to tubular myelin figures and in lamellar bodies of Type II pneumocytes in rabbit lung. No reaction product was detected inside the epithelial cells of airways. Their antibody was raised in ducks against a protein extracted from saline endobronchial washings from normal rabbit lungs (*88*), and absorbed with rabbit serum (*89*). Although ultrastructural morphology was

compromised, immunohistochemically the results were convincing. In 1981, Williams and Benson (*90*) improved ultrastructural localization of the major surfactant protein with an antibody to purified material from rat lung. In adult rat lung, the antibody labeled the rough endoplasmic reticulum and Golgi apparatus of Type II cells only, suggesting that these cells synthesize the protein.

Katyal and Singh (*91*) studied surfactant apoprotein first in the lungs from adult, newborn, and fetal rats by the use of IHC. With antiserum specific for primate surfactant, they demonstrated in 1980 that the extent and intensity of staining for apoprotein was markedly increased in reactive Type II pneumocytes in nonmalignant pulmonary disorders when standard formalin-fixed paraffin sections were used (*92*). The same antisera were useful in IHC diagnosis of some bronchioloalveolar carcinomas which consisted of Type II pneumocytes (*93*). According to Bhattacharyaa et al. (*94–96*), a specific glycoprotein of molecular weight 36,000 appears to be a component both in lung lavage and lamellar bodies of various normal animals as well as in alveoli of patients with alveolar proteinosis (Fig. 2).

## Studies with "Lung-Specific" Antibodies

Preparing an organ-specific antibody for the lung to be used as a tool in IHC studies on fetal development or tumor formation may seem attractive. The fact that the lung can be composed of as many as 40 different cell types presents an inherent problem. Ten Have-Opbroek (*97–99*) prepared an antiserum specifically recognizing the antigenic determinants of adult mouse lung tissue. With IHC, the antigen appeared to be localized in Type II cells. Akeson (*100*) demonstrated the presence of lung tissue-specific (NL-1) antigen(s) in normal lung tissue with IHC using a selected antiserum against a lung tumor cell line.

# Immunohistochemistry of Enzymes in the Lung

## Lysozyme

IHC investigations (*101*) have demonstrated lysozyme in a number of different cell types, including serous cells of bronchial glands (*102*), pointing to the role of the enzyme as an antibacterial agent (*103*). In respiratory tissue, lysozyme was localized with the immunoperoxidase technique on formalin-fixed paraffin sections (*92*, *104*) in alveolar macrophages, Type II pneumocytes, ciliary layer of the trachea and in secretory cells in laryngotracheal glands in various rodents and man. Specific staining was obtained with rabbit anti-rat lysozyme or anti-human urinary lysozyme antibodies but not with antibodies against egg white lysozyme. Interestingly, the respiratory system of the hamster, which is a useful experimental animal due to its relatively low pulmonary infection rate (*105*), seemed to be devoid of the lysozyme based on immunohistochemical as well as biological activity studies (*101*). As an interpretative caution, Spicer et al. (*101*) tested different fixatives and the specificity of the IHC staining in lung. In their thoroughly controlled system, laryngotracheal serous cells still possessed an unexplained, selective property of (nonimmune) binding of immunoglobulin. Such binding was also noted in mast cells (*61*). However, Bowes et al. (*106*) reported specific ultrastructural localization of lysozyme in serous cell

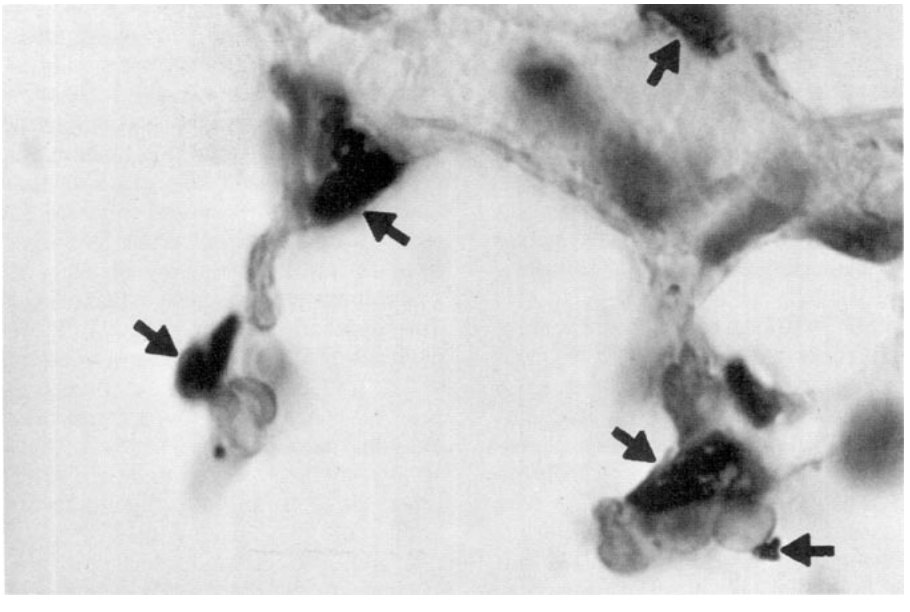

FIGURE 2. Localization of a unique glycoprotein in the alveolar Type II cells of the rat lung (immunoperoxidase-bridge method) (*26*). Arrows point to dark precipitate in the alveolar walls associated with a specific antibody (a gift from Dr. S. N. Bhattacharyya) to the 36,000 molecular weight glycoprotein which was purified from lamellar bodies (*94–96*). A formalin-fixed 6 μm thick paraffin section counterstained with toluidine blue ×1300.

granules in human bronchial glands. In comparative studies of lysozyme between conventionally reared and germ-free rats (*107*), IHC was of limited value, but proved to be very useful in an ontogenic study of the enzyme (*108,109*).

## Pulmonary Monooxygenase Enzymes

The cytochrome P-450–dependent monooxygenase system, which metabolizes endogenous as well as foreign compounds, is also present in the lung (*110*), although at a lower concentration than in the liver. Recent IHC localization of cytochrome P-450 isoenzymes and NADPH-cytochrome P-450 reductase in specific cell types of the lung such as in Clara cells and Type II pneumocytes probably reflects the fact that these enzymes are present in cells in high enough concentrations to be involved in organ specific carcinogenesis or toxicity (*111–114*). The antigenicity of cytochrome P-450 (Fig. 3) is retained after paraffin embedding of paraformaldehyde-fixed tissues (*113,114*). IHC provided valuable information complementary to the immunochemical and biochemical characterization of pulmonary monooxygenase system (*115*). With regard to sensitivity, IHC techniques are likely to be superior to conventional histochemistry, allowing better cellular and subcellular resolution. However, the method requires at least partial purification and characterization of the enzyme of interest, which is not always feasible. Also, immunoreactivity does not necessarily reflect the presence of biologically active enzyme.

## Angiotensin-Converting Enzyme in Pulmonary Endothelial Cells

Angiotensin-converting enzyme is a mammalian peptidase which metabolizes circulating bradykinin and catalyzes the change of angiotensin I to angiotensin II, the vasoactive agent of the renin–angiotensin system. Bradykinin and angiotensin I are metabolized during their passage through the vasculature of the lung. Ryan et al. (*116*) demonstrated with IHC that the converting enzyme was actually localized along the luminal surface of pulmonary endothelial cells. For pre-embedding ultrastructural localization the authors used the anti-converting enzyme antibody coupled to microperoxidase on glutaraldehyde or, preferably, paraformaldehyde-picric acid-fixed tissue (*117*). The strategic location suggested that the enzyme might help regulate the entry of bradykinin and angiotensin II into the systemic arterial circulation. Furthermore, this regulation could easily suffer from diseases that specifically damage the pulmonary endothelium as demonstrated with IHC techniques in Rocky Mountain spotted fever (*118*).

The converting enzyme, localized by immunofluorescence, appeared as a good marker for studying the development of the pulmonary vascular bed in rabbits (*119*) or for identifying of pulmonary arterial endothelial cells from calves *in vitro* (*60*). Besides the converting enzyme, other antigens (*120–122*) have also been localized in the endothelial cells by IHC thus providing possible tools for further studies on these metabolically important cells of the lung.

## Other Enzymes

Endogenous peroxidases have been histochemically demonstrated in various alveolar and bronchiolar cells, including macrophages (*123,124*), and we have already discussed the implications in regard to nonspecific staining with immunoperoxidase techniques. Furthermore, endogenous peroxidases may hamper the use of IHC to demonstrate exogenous horseradish peroxidase,

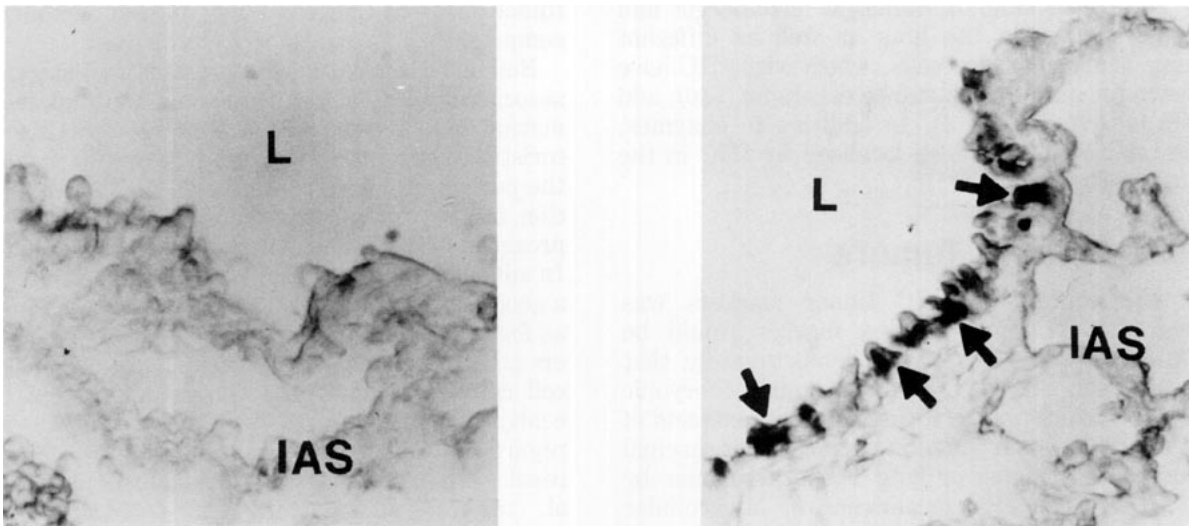


FIGURE 3. Localization of cytochrome P-450$_I$ in the apices of nonciliated epithelial cells of a terminal rabbit bronchiole (PAP-technique) (*5*): (*A*) control, treated with normal serum; (*B*) arrows point to the reaction product associated with the specific antiserum which was diluted 1000-fold and located in Clara cells (*113–115*); L, lumen of a bronchiole. IAS, interalveolar septa. These photomicrographs courtesy of Dr. C. J. Serabjit-Singh. ×620.

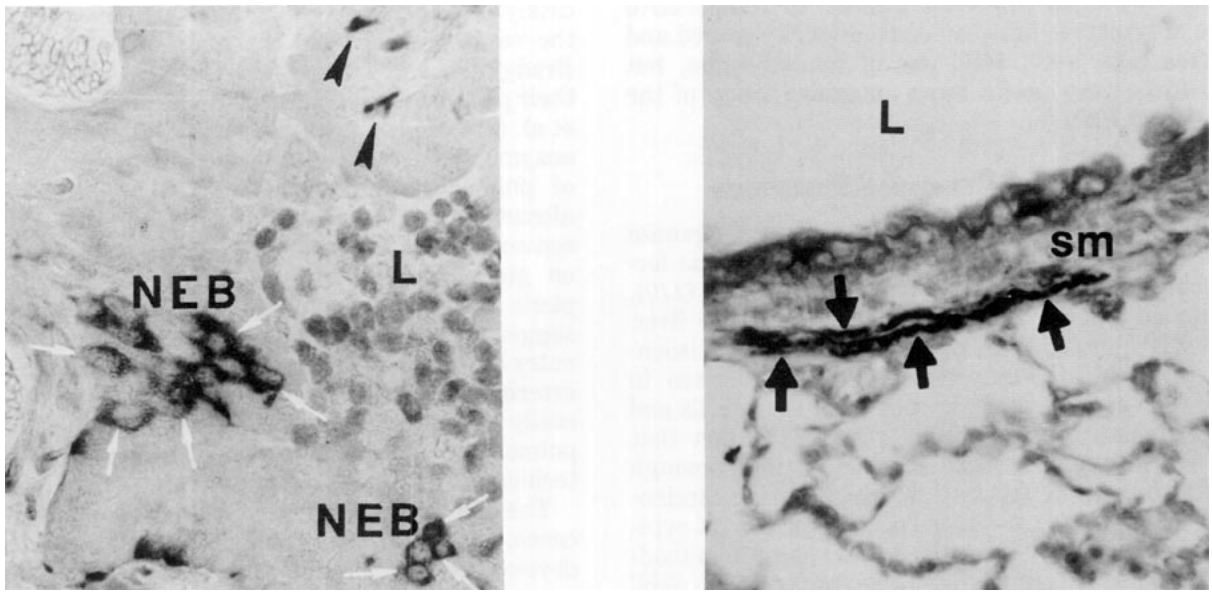


FIGURE 4. Photomicrographs of neuron-specific enolase (NSE) (*127–129*) in the lung (immunoperoxidase-bridge method, (*26*). (*A*) Localization of NSE in neuroepithelial bodies (NEB) and in solitary cells (arrowheads) of the bronchial epithelium of the guinea pig. Note that bronchial lumen (L) is filled with erythrocytes which contain endogenous peroxidase, ×440. (*B*) A thick bundle of NSE-containing nerve filaments (arrows) under a bronchial smooth muscle (sm) of the hamster; L, lumen of a bronchus ×470. The antibody to NSE was a gift from Dr. P. Marangos.

which has been used as a marker for capillary (*125*) or epithelial (*126*) permeability.

A glycolytic neuron-specific enolase isoenzyme, which was originally extracted from bovine brain (*127*), was recently demonstrated in neuroendocrinelike cells of fetal lung using IHC (*128*). Neuron-specific enolase has also been found in other endocrine (APUD or "amine precursor uptake and decarboxylation") cells of the central and peripheral divisions of the diffuse neuroendocrine system (*129*). This isoenzyme can provide the capability of using the same IHC marker to delineate various autonomic (e.g., adrenergic, cholinergic and peptiderdic) nerves in the lung as well as different pulmonary neuroendocrine cells, which with IHC have been shown to contain for instance calcitonin (*130*), and bombesin (*131,132*) (Fig. 4). In addition to enzymes, protease inhibitors have been localized by IHC in the respiratory system (*133,134*).

## Markers of Lung Tumors

When the concept of IHC tumor markers was generated, it was hoped that a marker would be identified for each histologic type. Unfortunately, that goal has not been realized (*135,136*). Carcinoembryonic antigen (*137*), which was first discovered in extracts of colonic tumor (*138*), was found in 20 to 30% of bronchial squamous cell carcinoma or lung adenocarcinomas by IHC (*139,140*). The IHC localization of intracellular keratin appeared helpful in establishing the epithelial nature of poorly differentiated neoplasms, but in a preliminary survey, primary lung tumors could not be classified by keratin content (*141*).

Human chorionic gonadotropin and immunoreactive growth hormone were localized in bronchogenic carcinomas with immunofluorescence (*142,143*). These hormones have been associated mainly with large cell carcinomas and adenocarcinomas of the lung (*144*). In an IHC study of 29 oat cell carcinomas of the lung, Deftos (*145*) demonstrated the presence of calcitonin, β-endorphin or ACTH in all but three tumors. The IHC localization of histaminase (*146*) as well as selected polypeptide hormones, such as bombesin (*147*), in small cell carcinoma of the lung revealed that within each tumor different cells contained different amounts of the compound.

Bell used IHC to demonstrate an antigen highly associated with oat cell carcinoma and undetectable in normal adult tissue (*148*). The antigen was also characteristic of certain normal neural crest-derived cells in the peripheral nervous system (*149*). The IHC localization of the antigen was further correlated with the presence of cytoplasmic neurosecretory granules (*150*). In addition to this, neuron-specific enolase appeared as a good marker for oat cell carcinomas of the lung as well as for other neuroendocrine tumors (*151*). These markers allow advance morphological investigations on small cell carcinomas and other lung tumors that have recently demonstrated to be quite heterogeneous with regard to cellular composition (*152,153*). So far the results are in concert with the suggestions of Bensch et al. (*154–156*) and Hattori et al. (*157–159*), that small cell carcinoma and carcinoid tumor of the lung may originate from the pulmonary neuroendocrinelike cell (Kultschitzky-cell).

In summary, IHC techniques have been useful and

are well established in the field of tumor markers (*160–164*), although the search for better markers is still warranted. Moreover, the IHC results of Katoh et al. (*165*) demonstrated that blood group antigens were good markers of normal human bronchial epithelial cells and that the loss of blood group antigens might be indicative of carcinogen-induced transformation of human bronchial epithelial cells.

# Localization and Roles of Immunoglobulins in the Lung

## Immunoglobulins in the Lung

Part of the defense system of the lung against micro-organisms is attributed to immunoglobulins. In IHC studies, plasma cells and other immunoglobulin-containing cells were found mostly in the submucosal glands but were also present in the lamina propria of the human tracheal and bronchial epithelium. The greatest number appeared in the main bronchi. The cells were occasionally present around small bronchi and bronchioles and were virtually absent from alveolar walls (*166*). Cells containing IgA were much more numerous than those containing IgG, IgM, or IgE. The study was performed with direct immunofluorescence on unfixed cryostat sections which can be unsatisfactory in resolution of anatomical details. IgA was demonstrated in epithelial cells of intramural glands of the human tracheobronchial tree and at the luminal margin of associated ducts near their origin (*167,168*). Mogi (*167*) improved the localization by using alcohol-fixed paraffin sections according to the method of Hamashima and Kyogoku (*169*). Since secretory IgA differs in chemical and immunological properties from serum IgA, Mogi emphasized that individual antisera specific for the secretory component and/or secretory IgA should be used. A careful summary of IHC studies on various aspects of glandular immunoglobulin transport in man was presented by Brantzaeg (*170*). There was a striking preponderance of IgA-producing immunocytes adjacent to glands of the respiratory tract, and serous glandular cells selectively transported dimeric IgA. The epithelial occurrence of IgG, most of which was serum derived, was less conspicuous and restricted to the interstices. The same epithelial cells were demonstrated to produce a glycoprotein, the secretory component, which was characteristically associated with IgA in apical parts of the cells. Pentameric IgM was handled by the glands in a way similar to dimeric IgA, but local synthesis of IgM was normally negligible in the respiratory tract. For satisfactory morphology the author used a modified Sainte-Marie (*171*) method with direct fluorescence. Requirements and comments for reliable immunohistochemistry of immunoglobulin-containing cells were also given (*170*). It is important to realize that epithelial cells and eosinophilic granulocytes are most prone to become nonspecifically stained in glandular sites (*172,173*) as we pointed out previously.

Rudzik et al. (*174*) performed an interesting experiment where bronchial and intestinal lamina propria of rabbits was repopulated with IgA-containing cells after transfer of homologous bronchial and Peyer's patch lymphocytes. Direct fluorescence on formalin-fixed cryostat sections was used as described by Bienenstock et al. (*175*). The immunoperoxidase technique on alcohol-fixed paraffin sections was applied in studies of the respiratory tract immune system in the pig (*176,177*) and in sheep (*178,179*). In sheep, IgM was the main immunoglobulin seen by immunofluorescence and peroxidase techniques in the nasal and bronchial glands before suckling; after suckling also IgG was found at all levels of respiratory tract. In adult sheep, IgA became the major locally appearing immunoglobulin of the respiratory system. Alley et al. (*178*) suggested that immature epithelial cells such as those found in proliferative areas of diseased airways would be actively engaged in immunoglobulin transport.

## Response to Infections

Immunoglobulin production in lungs of hamsters experimentally infected with *Mycoplasma pneumoniae* was studied by Fernald et al. (*180*) with indirect immunofluorescence in acetone-fixed cryostat sections. In control lungs only IgA-containing plasmacytes were found, but during the infection varying numbers of cells which also contained other immunoglobulins, infiltrated the peribronchial spaces. Non-immunoglobulin-stainable lymphocytes increased in number, too. However, it is important to recognize clinically silent infections such as caused by *Mycoplasma pulmonis* in rats, since they may significantly change the results of IHC studies on respiratory system immunity in laboratory animals (*181*).

## Immunological Reactions

With direct immunofluorescence on unfixed cryostat sections obtained from asthmatic patients through autopsy or surgery Gerber et al. (*182*) demonstrated that IgE was present in the bronchial epithelium and along the basement membrane. IgE was observed in the bronchial glands of all patients with asthma and to a lesser degree of many patients with chronic bronchitis or normal bronchi. IgE was not detected in mast cells. The presence and distribution of other immunoglobulins did not differ from that in normal bronchi. In patients with lung cancer Zeromski et al. (*183*) found large amounts of immunoglobulins in plasma cells with a predominance of the IgA and IgG classes.

Type II immune reactions have been thought to be involved in several so-called diffuse lung diseases, where the presence of immune complexes was verified by IHC. In procainamide-induced systemic lupus erythematosus specific nuclear fluorescence with deposition of

either IgG or IgM and also $C_3$ was found in pleura by direct immunofluorescence (*184*). Churg et al. (*185*), using formaldehyde-fixed paraffin sections, found IgG in alveolar walls and vessels of a patient with systemic lupus erythematosus. This was in agreement with studies of Inoue et al. (*186*) who were able to demonstrate granular deposits of IgG, complement ($C_3$) and DNA in the alveolar walls and in the alveolar capillary walls in systemic lupus erythematosus pneumonitis. In rheumatoid lung both IgM and IgM-rheumatoid factor complexes as well as IgG were localized in alveolar walls, vessels and adjacent to rheumatoid nodules (*187,188*). In IHC studies on Goodpasture's syndrome, which is characterized by development of antibodies to alveolar and glomerular basement membrane antigens, IgG and complement ($C_3$) were localized both in alveolar walls in the lung and in glomerular basement membranes of the kidney (*189–191*). An experimental model sharing these features and thus resembling Goodpasture's syndrome was developed by injecting rabbit anti-rat lung serum into normal rats (*192*). Based on immunofluorescence, Levy et al. (*193*) reported an example of autoimmune disease characterized by the presence of antitubular and antialveolar basement membrane antibodies associated with immune-complex glomerulonephritis. In this study both human and rat lungs were used in order to demonstrate indirect immunofluorescence with the patient's serum.

Farmer's lung or allergic alveolitis also belongs to diffuse lung diseases which involve type II and possibly Arthus-type reactions. The main source of sensitizing agents for this disease are *Miropolyspora faeni* and *Thermoactinomyces vulgaris*, and the IHC studies with patients' antisera were able to include the localization of the causative agents (*194*). Although the most consistent finding was a diffuse interstitial pneumonitis, the walls of the bronchioles appeared rich in antigen when the immunofluorescence technique on acetone-fixed cryostat sections was used (*195*). The lungs showed great numbers of fluorescing histiocytelike cells with anti-$C_3$ complement, suggesting the antecedent presence of antigen–antibody complexes. In an immunofluorescent study on bovine hypersensitivity pneumonitis, also caused by *M. faeni*, Wilkie et al. (*196*) found that pretreatment of frozen sections with pH 2.8 glycine-HCl buffer was a necessary prerequisite for specific staining for the antigen. The authors felt that the buffer "unmasked" the antigen by effectively eluting antibodies bound to the antigen *in vivo*. Thus, routine immunofluorescent staining of *M. faeni* antigen in tissues of individuals with specific serum antibody titers may be inhibited by prior combination with the antibody. Antigens of *M. faeni* were located in apparent association with alveolar macrophages. In order to elucidate the pathogenesis of farmer's lung, a number of animal models were recently developed (*197*). Schällibaum et al. (*197*) exposed rabbits intratracheally to enzyme I, which is a highly immunogenic esterase isolated from *M. faeni*. After repeated exposures, intracellular antigen, immunoglobulin and complement could be demonstrated in histiocytes by direct immunofluorescence on alcohol-fixed cryostat sections. The authors suggested that direct enzyme action might contribute to the farmer's lung type pneumonia in eliciting tissue damage.

## Diagnosis of Diffuse Interstitial Lung Disease

So far immunofluorescence has found little application in *diagnostic* histopathology of lung tissue (*198–200*) though it has been very valuable in renal disease. Hogan et al. (*201*) studied small needle biopsy specimens from 30 subjects with signs of diffuse interstitial lung disease; immunofluorescence examination of biopsies from six cases yielded positive results. The authors evaluated their study critically and in their summary three points received general attention: (1) Eosinophils and macrophages frequently demonstrated nonspecific staining (*45*). To avoid such pitfalls, it was necessary to identify positively any particular fluorescing cell by staining the same section with hematoxylin and eosin after immunofluorescence was completed. (2) An alternative to ascertain that the antibody deposits were specific would be to demonstrate deposits of complement or *antigen* in relation to antibody. However, such an approach would be applicable only to a small group of uncommon pulmonary diseases (other than infections) with known causative agents. (3) The small proportion of positive results obtained in their study might indicate that immunologic processes were involved at only one stage in the progression of disease or of response to a wide range of injuries that often have no humoral immune background. Thus Hogan et al (*201*) concluded that the artifacts encountered in lung IHC render it difficult to obtain consistent results. Furthermore, the way in which the specimens are obtained may contribute to the difficulty. A needle biopsy, as in the study by Hogan et al. (*201*) may not provide enough proper material of the affected area. On the other hand, in autopsy material the amount of autofluorescence is often increased. Fortunately, in most of the cases, satisfactory material can be obtained through open lung biopsy.

Based on direct immunofluorescence on fixed and unfixed cryostat sections of idiopathic interstitial pneumonias, Schwarz et al. (*202*) suggested that immune complex deposition might play a role in pathogenesis and that once mural fibrosis supervened, these complexes (IgG and $C_3$) were no longer demonstrable. Physiologic abnormalities appeared to correlate with the presence of fibrosis, which leads one to emphasize the results of the previously mentioned IHC studies on collagen (*74*). In this regard it was not surprising that Warvik et al. (*203*) demonstrated evidence of antibody and complement deposits in alveolar capillaries in only 6 instances out of 33 cases of fibrosing alveolitis with a similar immunofluorescence technique. In 17 cases

there was evidence of antibody in plasma cells or germinal follicles.

Application of IHC in diffuse lung diseases is challenging and important since diffuse lung diseases form a large group in pulmonary pathology and are on many occasions associated with previous exposure to toxic agents (dusts, asbestos) or drugs.

# Immunohistochemical Approach to Infections of the Lung

## Microorganisms Pathogenic to Man

Presently IHC applications in diagnostic pathology of infections of the human lung are scanty despite the vast literature on the IHC of various microorganisms. In selected cases where other methods either failed or were not practical, IHC techniques were used. A good example is Legionnaires' disease pneumonia (*204*), in which final diagnosis requires identification of the organism in the tissue, sera or both. The causative organism has been positively identified by immunofluorescence in tissue sections from biopsies or autopsies and in secretions from the lung (*205–207*). As an alternative to fresh or fresh-frozen material, formalin-fixed paraffin sections can be used (*205,208*). Recently Suffin et al. (*208*) identified *Legionella pneumophila* in tissue sections by a new immunoenzymatic (glucose-oxidase) procedure (*17*), that can be adapted to routine diagnostic practice.

*Rickettsia rickettsii* is typically difficult to demonstrate with histochemical stains (*209,210*) in tissue sections because of its small size, poor staining contrast between the organisms and tissues, and the affinity of the stains for normal tissue structures such as mast cell granules which, because of their size and morphology, can be mistaken for rickettsiae. Using direct or indirect immunofluorescence, Hall and Bagley (*211*) succeeded in identifying *R. rickettsii* in formalin-fixed paraffin sections after trypsin digestion. The morphology in these sections was far better than in frozen sections. Distribution of rickettsiae and the vasculitis of the pulmonary microcirculation coincided according to the immunofluorescence study of Walker et al. (*118*). These authors speculated that the striking invasion of the pulmonary vascular endothelium affects important nonrespiratory (e.g., metabolic) functions of the lung, contributing to the pathophysiology of Rocky Mountain spotted fever. The organisms were never observed within the alveolar lining cells or bronchial epithelium, and thus person to person aerosol transmission of the disease appeared unlikely.

If microorganisms cannot be cultured, IHC techniques provide a valuable tool for pathologists, as demonstrated by immunofluorescent identification of *Pneumocystis carinii* on ethanol-fixed cryostat sections of the human lung (*212*). Bacteriologic culture of specimens taken at autopsy usually gives negative results, but this may be due to the organisms being nonviable, instead of not being present. Danielson et al. (*213*) performed direct immunofluorescence studies on *Haemophilus influenzae* and *Neisseria meningitidis* in postmortem formalin-fixed, paraffin-embedded tissues including the lung. The identification of the bacteria was facilitated by counterstaining with lissamine rhodamine B (RB-200)-labeled antistaphylococcal globulins to counteract nonspecific staining and tissue autofluorescence. This may be of importance in demonstrating the organisms in the lung which is often invaded also by staphylococci via the respiratory tract after death. Immunofluorescence studies on *H. influenzae* in the respiratory tract by means of a direct method on acetone-fixed cryostate sections failed to demonstrate any correlation between the presence of these bacteria and the atopic state (*214*). In addition to this, there are a few scattered reports of immunofluorescence techniques in identifying viruses or mycoplasma in the human lung (*215,216*).

The use of immunofluorescence is deemed necessary when it is difficult to identify various pathogenic fungi by conventional histopathologic methods. IHC techniques add an attractive dimension of serologic specificity. Kaplan and Kraft (*50*) demonstrated how five different fungi could be identified by the direct fluorescent antibody method in trypsin digested, formalin-fixed paraffin sections as well as in sections that had been previously stained by the hematoxylin and eosin, the Brown and Brenn or the Giemsa procedures. The beneficial effect of trypsin treatment was also demonstrated after the mounting of organisms on egg albumin-coated slides in comparison with the results on clear slides. In tissue sections Kaplan and Kraft (*50*) recommended 1% trypsin for 1 hr at 37°C as a most favorable digestion method.

## Experimental Infections in Animals

In experimental work, IHC techniques have been often used to localize various infectious agents in the airways and lung in order to investigate in detail the routes of spread of specific micro-organisms. This has been correlated with conventional histopathological findings. In immunofluorescence studies on the pathogenesis of infection with influenza A virus in mice following exposure to aerosolized virus, Yielma et al. (*217*) came to the conclusion, in agreement with Lemercier et al. (*218*), that viral replication happened earlier in the bronchi than alveoli. This appeared to be in disagreement with the findings of Denk and Kovac (*219,220*) who claimed that after intranasal inoculation the replication started closer to alveolar sacs and later spread to the bronchi. Acetone fixed cryostat sections (*217*), ethanol-fixed paraffin sections (*218*) and unfixed cryostat sections (*219,220*) were used. Tada and Ishida (*221*) inoculated mice intraperitoneally with the PR8 strain of influenza virus and localized the virus with a different immunofluorescence technique (*222*). In brief,

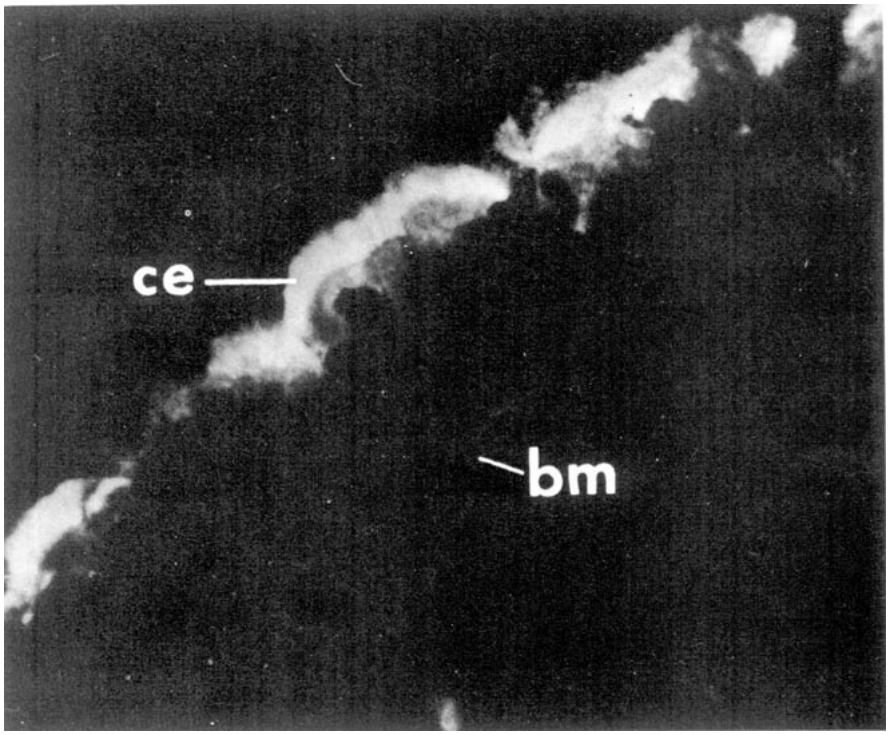


FIGURE 5. Localization of *Mycoplasma pneumoniae* in human fetal trachea infected in organ culture (indirect immunofluorescent technique). Specific fluorescence 48 hr postinfection is limited to ciliated epithelium (ce); (Bm) basement membrane. Reproduced with permission from Collier and Clyde (*236*).

their method required antivirus antibody to be applied with fresh non-immune serum (containing complement) on acetone-fixed cryostat sections, and subsequent incubation with a fluorescent antibody against complement. Initial spread of virus, which was first located in reticular cells in the lung, was found to differ from that after intranasal inoculation of influenza virus (*223–226*).

The course of herpesvirus infection was studied in rabbits (*227*) and in infant mice (*228*) by direct immunofluorescence on acetone-fixed sections. The influence of the route of administration of Newcastle disease virus on host response in chickens was studied by Beard and Easterday (*229*) with immunofluorescence. In domestic animals fluorescent antibody studies on parainfluenza 3 virus infections in lambs (*230*) and in enzootic pneumonia of pigs (*231*) have been useful. Hill (*32*) used peroxidase technique on ethanol-fixed paraffin sections to demonstrate mycoplasmas in animal tissues. Single organisms could be demonstrated and there was no nonspecific staining in the lung.

In an effort to further examine host response to infection, immunofluorescence was used to demonstrate the Newcastle disease virus in the brain and trachea, as well as globulins and specific antibody against the virus in mononuclear cells, mucous glands and mucus in tracheal mucosa of chickens which had been serially sacrificed during 1 to 120 days after infection (*232*). A similar study on chronic pneumonia in swine with experimentally induced African swine fever was performed with antisera against viral antigen, porcine immunoglobulin, complement and fibrinogen on serial unfixed frozen sections (*233*). The same approach was also applied to human autopsy material (*142*). Localization of immunoglobulins in the lung has been discussed in the preceding section of our review.

## *In Vitro* Models for Environmental Research

The large surface area provided by the respiratory tract epithelium of humans for exposure to microbial as well as toxic substances in the environment makes this organ system very vulnerable but a good early indicator of adverse health effects. However, the complexity of pulmonary defense mechanisms complicates definition of the interactive effects of pollutants and infectious agents. As a model, tracheal organ culture obtained from human fetuses as well as animals was utilized to maintain organized differential respiratory epithelium *in vitro* (*234*). In this system Collier (*234*) used immunohistochemistry as a major tool for investigating the epithelial injury by *Mycoplasma pneumoniae* (Fig. 5), respiratory syncytial virus or parainfluenza virus type 3. A similar system was used in studies of murine cytomegalovirus infection (*235*). These experiments demonstrated that individual infectious agents can invade the organized epithelium in different ways. The

information was useful in defining the susceptible cell type for different pathogens, and can be further applied to explore the injury produced or modified by different pollutants and toxic agents.

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