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Respiratory Tract

By far and away the most important pulmonary diseases in humans are related to the smoking of tobacco. However, occupational lung diseases caused by inhalation of industrial chemicals, particulate matter and antigens, are also important causes of morbidity and mortality. For this reason, considerable effort has been directed to the examination of airborne pollutants over recent years, including study of their effects in laboratory animals when administered by the inhalation route. Extensive study has shown that a complex array of defensive mechanisms protects the lung against the adverse effects of airborne substances and pathogenic organisms. Aerodynamic factors prevent access of particles larger than 10μm diameter for these are deposited on the walls of the nasal passages. Particles measuring between 2 and 10μm diameter tend to be trapped by the mucus-covered ciliated epithelium lining the bronchial tree and removed by mucociliary transport aided by the cough reflex. Smaller particles may reach the alveoli where they are ingested and transported by pulmonary macrophages.[1] Considerations of airborne delivery to the lungs are also important in the development of therapies to be administered via the respiratory tract. Whilst the inhalation route has been used for many years for volatile anaesthetic gases, the respiratory tract is increasingly being employed for delivery of therapy in not only for asthma and other lung diseases but also as a means of systemic delivery of polypeptides such as insulin.

In contrast to the adverse pulmonary effects of cigarette smoke and industrial pollutants, therapeutic agents remain a relatively minor cause of pulmonary toxicity in humans although actual incidence is difficult to ascertain. However, drug-induced pulmonary disease appears to be an increasingly frequent clinical problem and the drugs associated with parenchymal pulmonary injury in humans continue to increase.[2]

Although it is difficult to assess in the context of the underlying disease process, it has been suggested that about 10% of patients receiving well-established anticancer drugs develop various forms of pulmonary toxicity.

Some novel antineoplastic therapies may have a similar liability.[3] Drug-induced toxicity usually occurs after exposure of lung tissue via the circulation to parent drug or metabolites, although increasingly the adverse effects of direct administration of drugs to the lungs needs consideration in preclinical studies.

In patients a number of drugs have been associated with pulmonary toxicity which can occur through different mechanisms and take different forms.[2–4] Through their specific pharmacological action drugs can produce excessive effects on bronchial calibre or pulmonary function. Drugs mediate allergic reactions in the bronchi or lungs. They may also produce a variety of obscure, diffuse pulmonary alveolar conditions including a pulmonary syndrome resembling systemic lupus erythematosis. As the respiratory tract is a major route by which microorganisms gain entry into the body, opportunistic pulmonary infections with bacteria, viruses, fungi or protozoa are consequences of immunosuppression or broad-spectrum antibacterial therapy. As in other organs, drugs that disturb coagulation may precipitate pulmonary thromboembolism or haemorrhage. Localized lung lesions also result from accidental, diagnostic or therapeutic inhalation of xenobiotics. Mucociliary clearance is also sensitive to therapeutic agents that affect the secretion of mucus and fluid, ciliary activity and transport.[5] Treatment with antacids or histamine H_2 blockers can also increase the risk of pneumonia developing in patients in intensive care units through increasing gastric pH, which leads to an overgrowth of gram-negative bacteria in the stomach and retrograde pharyngeal colonization.[6]

In preclinical safety studies, pathology of the respiratory system can be the result of intercurrent disease or be induced by drugs administered systemically. Intranasal or inhalation modes of therapy pose particular challenges in terms of the formulations and the technologies required to administer drug. The different anatomical and physiological characteristics of the airways also influence drug toxicity, disposition and metabolism. The development of drugs to be administered by inhalation or intranasal routes is particularly difficult because of the perceived risks of high local drug concentration in respiratory tissues and their use in potentially vulnerable patients with pulmonary disease.[7]

Inhalation toxicology

A complex technology has been developed to support the assessment of the adverse effects of inhaled substances in rodent and non-rodent species and the extrapolation of the experimental findings to humans.[8,9] In order to administer drugs by inhalation, it is necessary to generate aerosols (suspensions of particles in a gas) with a well-defined composition, particle size and shape. They must be delivered to the respiratory tract of laboratory animals in a way that parallels the likely human exposure. In case of therapeutic agents, this should avoid non-respiratory pathways through the skin and food.

When aerosols are inhaled, various fractions of the particles are deposited at different locations in the respiratory tract. Site of deposition depends primarily on particle size, but variability in the sites of deposition occurs among

different laboratory animal species and humans by virtue of the differences in the size and shape of the respiratory passages as well as breathing patterns.[10] In addition, there are different types of inhalers used in human therapy to consider in the assessment, which can deliver different materials to the lungs, for example nebulizers, propellant-driven metered dose inhalers and dry powder inhalers for asthma treatment.[11]

The subsequent fate of inhaled particles depends not only on their size but also their shape, chemical nature, and solubility in body fluids. Soluble substances are absorbed into the blood stream and are removed by the pulmonary circulation. They may also undergo metabolism by enzymes present in the cell populations of the respiratory tract and reactive metabolites may cause local pulmonary damage. Insoluble, inert particles are removed primarily by the mucociliary transport system of the trachea and bronchi or through phagocytosis by macrophages. Overload of the lung by even relatively inert, non-fibrous particles such as titanium dioxide or carbon black may impair alveolar macrophage-mediated particle clearance.[12] This may lead in turn to accumulation of dusts over time with eventual fibrotic and tumorigenic responses, particularly in rats.[13]

Measurements of respiration rate, tidal volume, airway resistance, pulmonary gas exchange and the disposition of the inhaled substances have an important place in the evaluation of chemically induced lung damage in laboratory animals.[14,15] However, the key component of the evaluation of the adverse effects of inhaled substances is careful morphological assessment of the fixed tissues. Even though there are novel and very sensitive physiological methods for the characterization of oedema following lung injury in rodents, light and electron microscopy of lung tissue provides vital qualitative evidence of the nature of injury.[16]

NOSE, NASAL SINUSES, NASOPHARYNX AND PHARYNX

The nasal chambers are the structures which are first to be subjected to the effects of inhaled substances, whether microorganisms or chemical substances. Although these chambers are not usually examined in great detail in conventional toxicity studies in which substances are administered orally or by parenteral routes, they are carefully examined histologically when drugs are administered by inhalation.

Study of nasopharyngeal silicone rubber casts has shown considerable species differences in the anatomy of this part of the airway.[17–20] Relative to total nasal length, the nasopharynx is longest in rats and shortest in humans with the dog in an intermediate position. Maxilloturbinates are relatively simple structures in man and non-human primates but highly complex in dogs and rodents. As a consequence, regional nasal airflow and disposition patterns vary considerably and this influences the distribution of lesions produced by inhaled xenobiotics in the nasal cavity.[21]

However, comparison of the nasal cavity of rhesus monkey and humans using magnetic resonance imaging and nasal casts have shown that many similarities in structure exist in these species.[22]

The anterior nares are lined by stratified squamous epithelium. In other zones the sinuses are covered either by respiratory or olfactory epithelium with a zone of transitional epithelium at the junction between the two types. Respiratory epithelium is similar to that found elsewhere in the respiratory passages being composed of ciliated cells, serous and mucous cells, brush cells, intermediate cells and progenitor basal cells. It represents a cellular system engaged in mucociliary clearance carrying surface secretions to the nasopharynx to be cleared by swallowing. Although this epithelium is similar to that lining the other large airways, key differences are the particularly rich complement of secretory cells and the complex vasculature of the nose which can modulate capillary, arterial and venous blood flow through the mucosa.[23] Mucins may be particularly important. It has been postulated that they not only have a physical protective function but also possess antioxidant properties by virtue of the scavenging behaviour of their high proportion of sugar groups.[24]

The proportion of the nose lined by olfactory mucosa is variable between species, being disposed over a much larger area in dogs and rodents than in primates.[18] However, it is structurally similar in humans and rodents. It is located in more dorsal or posterior regions of the nasal passages out of the direct line of airflow during normal respiration. Olfactory mucosa is a pseudostratified columnar epithelium composed of basal cells, sustentacular cells and sensory cells with mucus-secreting Bowman's glands situated in the lamina propria. Basal cells are composed of two distinct types, light and dark cells. The light type represents the primitive, stem cell population. Sustentacular or supporting cells are non-ciliated, columnar cells possessing microvilli that extend into the overlying layer of mucus.

Cell bodies of olfactory sensory neurons are situated in the middle layer of the epithelium between sustentacular and basal cells. Their dendritic processes extend above the epithelial surface to end in a ciliated expansion referred to as the olfactory vesicle that is believed to be the receptor of odour perception. Olfactory axons extend from the cell body, penetrate the basement membrane in bundles to become surrounded by Schwann cells and eventually join with the olfactory bulb.

The olfactory system is of importance in toxicology for it can be selectively damaged by xenobiotics, including therapeutic agents, presumably as a result of its high metabolizing capacity. The superficial location of neural cells in the olfactory epithelium also provides a model system for the study of the effects of xenobiotics on neural cells.

Submucosal mucous glands have been well characterized in the rat, hamster and dog, where they are divided into lateral nasal glands and maxillary recess glands. These are both situated in the posterior parts of the nasal cavity and composed of mucus-secreting cells.[25–27]

Immunocytochemical study using antisera raised against the major isoenzymes of rat hepatic microsomal cytochromes P450 induced by β-napthoflavone, 3-methylcholanthrene, phenobarbitone and pregnenolone-16-α-carbonitrile as well as NADPH-cytochrome P450 reductase, epoxide hydrolase and glutathione S-transferases B, C and E, has shown their presence in rat nasal mucosal cells.[28] *CYP2A* enzymes appear to be expressed at high levels in the respiratory tract mucosa.[29–33] This suggests that the nasal mucosa not only has a capacity for metabolizing and activating xenobiotics by oxidation, but also for hydration and inactivation of potentially toxic epoxides and conjugating electrophilic, reactive metabolites with reduced glutathione. It has been shown that the distribution of immune-reactive enzymes is different in olfactory and respiratory mucosa.[34] Xenobiotics can be metabolized within both olfactory and respiratory mucosa but the olfactory regions appear to possess greatest capability for oxidative metabolism. Consequently, regional differences in nasal toxicity and tumour formation from inhaled materials may not only be a response to different water solubility and deposition patterns but also differences in the formation of reactive metabolites.[34] Another feature of this metabolizing activity is that it can be induced by systemically administered xenobiotics and this can alter the distribution of enzyme activity in the nasal mucosa.[28] Studies of the mouse olfactory mucosa have shown that whilst typical hepatic inducers of *CYP2A5* do not significantly change its expression in the mucosa, olfactory toxicants can alter the pattern of enzyme distribution.[31,33]

Like many other tissues exposed to external environmental agents, the nasal mucosa possesses aggregates of lymphoid tissue in the underlying lamina propria. In rats these areas, characterized by follicles containing both T and B cell areas, are located in the ventral aspects of the lateral walls of the nasal airways at the opening of the nasopharyngeal duct.[18,35] Like the gut-associated lymphoid tissue, these nasal follicles have been shown in the rat to be covered by specialized epithelium with islands of cells with microvilli, so-called M or membranous cells. Little is known of any toxicity occurring in this tissue despite its strategic position in the respiratory tract.[36]

Technical approaches

In rodents, the relatively small size of the nose and nasal sinuses facilitates histological examination. Usually this area is sectioned transversely into several standardized blocks following decalcification.[37] There have been a number of detailed publications describing the histological preparation and assessment and recording of pathology of the rodent nasal cavity.[37–41] Careful standardized histological sections, careful recording of lesions with the use of diagrams of the rodent nasal cavity are useful in the assessment of lesions in the nasal cavity found in inhalation studies.[42] In larger species, particularly dogs and primates, sectioning and blocking is more complex. Although dissection is required, a similar procedure following decalcification can be adopted. Careful examination of haematoxylin and eosin stained sections remains paramount in the assessment of the nasal cavity, although special stains may

be helpful. Examination of cytokeratin expression in the respiratory mucosa has been used as a marker of epithelial differentiation in the respiratory tract.[43]

A test system that relates to the innervation of the nasal mucosa is that proposed by Alarie.[44] The trigeminal nerve endings in the nasal mucosa of mice mediate the response to sensory irritants and this can be measured by a decrease in respiratory rate. It has been shown that a good correlation exists between the decrease in respiration rate in mice exposed to airborne chemicals and the nasal irritancy potential of the chemicals in humans.[45] This enables the detection of airborne sensory irritants and the prediction acceptable levels of exposure to the upper respiratory tract in people.

Degeneration, inflammation, ulceration (rhinitis, sinusitis)

Microbial pathogens

Infectious agents cause inflammation in the nose and nasal sinuses and this may be associated with inflammation in the conjunctiva, middle ear and oral cavity. Murine pathogens may cause alterations in the respiratory tract that can confound the assessment of changes induced by xenobiotics.[46] In rats, microbiological agents implicated in the development of rhinitis and sinusitis include *Corynebacterium kutscheri* (pseudotuberculosis), *Streptococcus pneumonia*, *Pasteurella pneumotropica*, *Klebsiella pneumoniae*, *Mycoplasma pulmonis* and the sialodacryoadenitis virus or rat corona virus.[47] Rats infected with the sialodacryoadenitis virus show inflammation and necrosis of the upper respiratory epithelium as well as damage to salivary and lachrymal glands. The Sendai virus, a paramyxovirus, also has marked tropism for the respiratory tract, including the nasal cavity, and is associated with systemic effects that can compromise studies in laboratory rodents. Occasionally, fungal infections of the airways with *Aspergillus fumigatus* are reported.[46]

A variable that has been shown to influence the severity of the rhinitis produced by *Mycoplasma pulmonis* is the strain of rat. Following housing of Lewis and Fischer 344 strains together to eliminate microbial and environmental differences it was shown that the Lewis strain developed a more severe rhinitis following inoculation with *Mycoplasma pulmonis* than Fischer 344 rats, although the reason for the difference was unclear.[48]

Rats exposed to ammonia, a common pollutant of the air in laboratory animal cages, have also been shown to develop lesions of the dorsal meatus, dorsal nasal septum and prominence of the turbinates.[49] These lesions are characterized histologically by swelling or mild degeneration of the epithelium. It appeared that ammonia exposure potentiated the acute inflammatory response of the nasal cavity to microbiological pathogens.

A microorganism reported in the nasal cavity of rhesus monkeys employed in inhalation studies is the nematode of the genus *Anatrichosoma*.[50] Sections of this nematode are found in the squamous epithelium of the nasal vestibule

and are associated with acanthosis and hyperkeratosis of the epithelium and a multifocal or diffuse granulomatous inflammation in the submucosa.

Xenobiotics – inhalation administration

Administration of toxic or irritant substances to laboratory animals by the inhalation route produces degenerative, inflammatory and reactive changes in the nasal mucosa. The range of histological features is similar to those found in other mucosal surfaces damaged by other exogenous agents. Whilst therapeutic agents administered by the inhalation route do not usually produce a severe degenerative or inflammatory responses in the nasal mucosa, at least at therapeutic doses, the simple categories proposed by Hardisty and colleagues in recording of degenerative and reactive lesions following exposure to volatile chemicals are useful.[42] Categories suggested are: *degeneration*, *regeneration*, *atrophy* (postdegenerative), *respiratory metaplasia* and *basal cell hyperplasia* and *inflammation*.

Degeneration is usually the earliest morphological change characterized by loss of sensory and sustentacular cells resulting in a thinner mucosa. Bowman's glands and nerve bundles, individual cell necrosis may be seen in more severe cases. *Regeneration* is characterized by proliferation of basal cells associated with an epithelium that loses its regular structural features. *Post-degenerative atrophy* usually follows severe damage and is characterized by loss of sensory and sustentacular cells. *Respiratory metaplasia* is a process whereby the normal olfactory mucosa is replaced by pseudostratified epithelium of respiratory type often with cilia. *Basal cell hyperplasia* represents a longer term effect where the proliferating basal cells form a distinct layer of cells below the respiratory epithelium.

An example of the type and distribution of the degenerative and inflammatory conditions which can be induced by inhaled irritants is provided by the study in which Swiss–Webster mice were given various irritants by inhalation for periods of 6 hours per day for 5 days at concentrations that produced a 50% decrease in respiratory rate (Alarie test). Although the degree of histological changes varied with different agents, the changes were broadly similar in type and distribution.[51] Most agents examined produced little or no alteration in the squamous mucosa lining the anterior part of the nose apart from some mild increase in thickness of the squamous layers. Principal sites of damage were shown to be the anterior respiratory epithelium adjacent to the vestibule and the olfactory epithelium of the dorsal meatus. There was a distinct decrease in severity in posterior regions. Histologically, the lesions in respiratory epithelium ranged from mild loss of cilia and small areas of epithelial exfoliation to frank erosion, ulceration and necrosis of the epithelium and underlying tissues including bone. Variable polymorphonuclear cell infiltration was also found. In some cases, early squamous metaplasia developed on the free margins or the naso-maxillo-turbinates. Changes to the olfactory epithelium varied from focal to extensive loss of sensory cells associated with damage to sustentacular cells. In severe cases, complete loss of olfactory epithelium

occurred. Although the degree of histological change was shown to vary with different agents, lesions induced by the more water-soluble chemicals tended to remain localized in the anterior part of the nasal cavity whereas agents with relatively low water solubility produced lung lesions in addition. It was suggested that these findings demonstrated the powerful 'scrubbing' action of the nasal cavity for water soluble, airborne xenobiotics.[52]

Inflammatory alterations have been induced in the nasal cavity of rodents treated with therapeutic agents at high doses by inhalation. Significantly irritant substances do not make viable therapies. However, the precise relevance of such changes for human therapy by the inhalation route are sometimes questionable when the nasal damage is limited to high doses and it is not associated with alterations in other parts of the respiratory tract.

In the case of tulobuterol, a β_2-adrenergic receptor agonist, it was argued that the nasal inflammation induced in rats in a one month inhalation toxicity study was the result of a particularly high exposure of the nasal epithelium to drug, not representative of the likely human exposure to tulobuterol by inhalation, where little or no nasal exposure would occur.[53] RP73401 [3-cyclopentyloxy)-*N*-(3,5-dichloro-4-pyridy)-4-methoxybenzamide], a novel type IV phosphodiesterase inhibitor which was being developed for the treatment of asthma and rheumatoid arthritis, was also reported to produce degeneration of the olfactory epithelium in rats but neither dogs nor mice after single and repeated oral doses and by inhalation.[54] Histologically, the olfactory epithelium showed necrosis of the superficial epithelial layers including the sustentacular and sensory cells, with sparing of the basal cell layer. There was also damage to Bowman's glands. The development of proliferative lesions and ultimately tumours of principally neuroectodermal origin followed chronic treatment. As RP73401 was highly metabolized and the nasal lesions could be inhibited by treatment of rats with metyrapone, a non-specific inhibitor of cytochromes P450, it was postulated that the changes were the result of P450-mediated activation in the olfactory tissues, not linked to its pharmacological phosphodiesterase activity.[54]

Nasal epithelial degeneration and necrosis has also been reported in both rats and dogs treated with another candidate anti-inflammatory drug CI-959 by the intranasal route. This agent affected olfactory epithelium more than respiratory mucosa, suggesting that metabolism was important in the generation of this toxicity.[55]

Xenobiotics – other routes of administration

Although the nasal cavity has not been often examined histologically in great detail in toxicity studies conducted on drugs administered orally or by parenteral routes, damage to the nasal mucosa can be induced by drugs administered by these routes. One example is methimazole, a thioureylene antithyroid drug used in clinical practice where oral doses of 0.2–2 mg/kg/day are employed and abnormalities of taste and smell have been described.[56] Administration of methimazole at relatively high doses to Long–Evans rats by single oral (50 mg/kg)

or intraperitoneal (25 mg/kg) routes was shown to produce damage to the sustentacular and sensory cells with sparing of the basal cells and basement membrane.[57] Bowman's glands were also involved. Methimazole is metabolized by the flavin-containing monooxygenase system and it is employed as a model substrate for this enzyme *in vitro*. The presence of flavin-containing monooxygenase isoforms in olfactory mucosa of Long–Evans rats suggested that reactive intermediates may be responsible for the nasal toxicity.[57] Similar changes have been reported in mice where depletion of glutathione in the olfactory mucosa has been demonstrated also suggesting formation of local reactive metabolites.[58]

Histological examination has also shown that intravenous administration of a single dose of vincristine to mice damages the olfactory epithelium.[59,60] Vincristine is a vinca alkaloid derivative used in cancer therapy which has antimitotic activity and binds to tubulin. Cell death was noted in olfactory cells 2–5 days after dosing with a peak of cell proliferation at 5 days and repair after about 10 days. These features resemble those that can be seen in other proliferating tissues after single doses of antimitotic drugs.

The risk of damage to human olfactory cells from agents with these effects in rat nasal mucosa often remains uncertain because an understanding of relative exposure and metabolism in different species and a better understanding of the metabolic potential of human olfactory mucosa is required.

Inclusions of the nasal mucosa

A particular response of the rodent nasal mucosa to some irritant substances, including pharmaceutical agents, is the formation of rounded eosinophilic inclusions in the cytoplasm of sustentacular cells of the olfactory epithelium and to a lesser extent in respiratory and glandular epithelial cells.[52,61] These inclusions are PAS-negative and ultrastructural examination shows that they are membrane-bound, ellipsoid bodies containing homogenous electron dense matrix. Their significance remains uncertain.

Proliferative lesions of the nasal mucosa

A consensus classification for the variety of proliferative, non-neoplastic changes and atypical epithelial lesions and neoplasms found in the rat nasal cavity has been defined by Schwartz and colleagues.[62] The classification of the *International Agency for Research on Cancer* provides a similar perspective for rats and mice.[63,64]

Proliferative lesions may be occasionally seen in untreated rodents in carcinogenicity studies but are much more commonly induced by administration of xenobiotics in inhalation carcinogenicity studies. Spontaneous nasal tumours are uncommon but most often squamous in type in rats whereas in mice

spontaneous squamous tumours are extremely rare and haemangiomas and respiratory adenomas predominate.[65,66] The generally agreed categories are described below:

Mucous (goblet) cell hypertrophy and hyperplasia affects the nasal respiratory epithelium and are characterized by the presence of enlarged mucus-filled goblet cells, some of which form clusters suggestive on intraepithial glands.

Squamous cell hyperplasia is seen in the stratified squamous epithelium of the nares and is characterized by a focal increase in the number of cell layers. Cells may show atypia with irregular enlarged, pleomorphic nuclei and nucleoli.

Squamous metaplasia occurs to respiratory epithelium under conditions of chronic damage. It is characterized histologically by the presence of three or more layers of epithelial cells with eosinophilic cytoplasm and clear cell boundaries whereas advanced lesions show typical keratinization and formation of intercellular bridges. Cellular atypia may also be seen and should be characterized when found.

Respiratory epithelial metaplasia (of the olfactory epithelium) represents atrophy and degeneration of the olfactory epithelium with loss of sensory cells and in advanced cases loss of sustentacular cells with replacement by ciliated and non-ciliated respiratory epithelium. It may be seen as a spontaneous focal lesion in aged rats.

Epithelial hyperplasia with cellular atypia (atypical hyperplasia, basal cell hyperplasia, dysplasia) is a term used to embrace proliferative lesions in the respiratory and olfactory mucosa in the nasal cavity in which there is varying degrees of altered differentiation and atypia. There is perturbation of the growth pattern of the epithelium such that the changes are not those found in the normal regenerative response to transient mucosal damage.

Adenomas (polypoid or villous adenoma, adenomatous or villous polyp) usually develop in the anterior part of the nasal cavity and are usually exophytic lesions that develop from respiratory epithelium or nasal glands. Adenomas of respiratory epithelium may be papillary in form but are, by definition, well circumscribed with minimal cellular pleomorphism and atypia. They may very occasionally occur spontaneously in aged rats.[65] Adenomas of nasal glands usually show an acinar pattern.

Squamous cell papillomas develop in the squamous epithelium of the nares or in areas of squamous metaplasia in respiratory or olfactory epithelium. They are exophytic lesions with limited connective tissue stroma. They may develop spontaneously in aged rats.[67]

Carcinomas of either squamous or glandular differentiation develop in the nasal mucosa. Histologically, they have similar characteristics to those in other epithelial tissues. They are rare spontaneous lesions in aged laboratory rodents but may be induced by xenobiotics administered by inhalation, orally or by the parenteral route. Squamous carcinomas have been reported to develop in a small number of untreated Fischer 344 rats used in carcinogenicity studies in association with point mutations in the c-H-*ras* and c-K-*ras* gene.[67]

Olfactory neuroblastoma (ethesioneuroblastoma, olfactory neuroepithelioma, olfactory neuroepithelial carcinoma) show olfactory differentiation and arise from olfactory epithelium. They do not seem to occur as spontaneous lesions in rats or mice and only rarely induced.[62,65] Cells are arranged in lobules or in solid sheets with scanty stroma. Cells are relatively uniform with scanty cytoplasm with round or oval hyperchromatic nuclei. True rosettes with lumens or pseudorosettes are also seen. Poorly differentiated tumours of this type may require ultrastructural study for diagnosis. Olfactory neuroblastomas typically show the presence of electron-dense neurosecretory granules, neurofilaments or axons. As there is no detailed understanding of the biological behaviour of these neoplasms in laboratory rodents, the generic term olfactory neuroblastoma is usually preferred. They are almost always invasive tumours.[65]

Olfactory carcinomas forming glands, follicles and rosettes have been occasionally reported in aged Syrian hamsters.[68,69]

Mesenchymal neoplasms may be seen in the nasal cavity, particularly after exposure to potent carcinogens. Their histological features are similar to those in the soft tissues and bone elsewhere in the body (see Chapter 2).

LARYNX AND TRACHEA

The mucosa lining the larynx and trachea becomes involved as part of an upper or lower respiratory tract infection. For instance, in rats, an acute laryngitis or tracheitis has been shown to accompany experimental infection with *Mycoplasma pulmonis* and the sialodycroadenitis virus.[48,70] A spontaneous degenerative condition of tracheal and laryngeal cartilage of uncertain pathogenesis associated with granulomas has been reported in Fischer 344 rats.[71] The condition increases in severity and incidence with advancing age although it is seen in rats as young as 6 weeks of age. Tracheal cartilage rings may also show alterations in genetically engineered animals, such as the C57BL/6J-TgN(C3-1-TAg)cJeg (TAg) mice that have generalized defects in cartilage development.[72]

The larynx of rodents is also susceptible to the effect of inhaled substances, notably tobacco smoke but also pharmaceutical agents and propellants.[61,73] In view of the localized nature of induced lesions in the larynx, standardized

histological sectioning techniques have been proposed for rats, mice and hamsters using anatomical landmarks.[74–77]

The target site is located on the ventral floor of the larynx near the base of the epiglottis cranial to the ventral laryngeal diverticulum. Lesions tend to occur in the ventrolateral region, which is covered by respiratory epithelium and the inner aspect of the arytenoid processes which is lined by squamous mucosa. The larynx responds to inhaled irritants by inflammatory, degenerative and regenerative changes in a manner similar to other regions of the respiratory tract. These include disruption of the epithelial cells, inflammatory cell exudates and infiltration, goblet cell hyperplasia and squamous metaplasia.[73] However, these changes are not specific to inhaled irritants but also occur as a response to natural respiratory tract pathogens in conventionally housed rats.[78]

The pseudostratified ciliated and non-ciliated mucosa of the trachea may also show pathological alterations in inhalations studies, although sites at the bifurcation (carina) are those often first affected. Consequently, the carina should be systematically included in examination of the respiratory tract for induced lesions.[62,77]

Neoplasia

As in the nasal passages a range of proliferative lesions including squamous hyperplasia, mucous cell hyperplasia, as well as papilloma, carcinoma and mesenchymal tumours are occasionally reported in the airways in laboratory rodents.

BRONCHI AND LUNGS

In humans and laboratory animals, the trachea terminates at the bifurcation giving rise to two main bronchi which serve left and right lungs. Depending on species, the main bronchi subdivide into further branches that enter the different lobes. Various forms of branching are recognized. Bronchi may arise as side-branches from a parent or stem bronchus (*monopodial*). The parent bronchus can divide into two equal daughter bronchus (*dichotomous*) or several daughter bronchi (*polychotomous*).[79] Study of silicone rubber casts of the respiratory tract has shown that the bronchial trees of humans and non-human primates are essentially dichotomous, in contrast to the monopodial pattern of rodents.[20] The comparatively long trachea of the dog gives rise to dichotomous upper airways but monopodial branching develops peripherally within each lobe.

The size of the lungs is generally dependent on size and weight of the different species. Allometric studies have shown that lung volume, alveolar surface area and diffusing capacity increase proportionally with body weight across a

broad range of mammalian species, although cell size and surface area appear to be more determined by cell function rather than species size.[80] Dogs have comparatively smaller body mass and higher airway dimensions compared to humans.[20] The number of lobes is species-dependent. The human lung possesses an upper and lower left lobe and an upper, middle and lower right lobe. This contrasts with the upper, middle and lower left lobes and a fourth, azygos right lobe in rhesus monkeys and baboons.[20] The dog has three lobes on both right and left sides. Rats, mice and hamsters show cranial, middle, caudal and postcaval right lobes with a single, left lobe in mice and rats and a superior and inferior lobe on the left side in hamsters.

Cell types lining the bronchi are generally similar between species.[5] The majority of cells are the ciliated cells that are accompanied by variable but relatively smaller proportions of basal cells, intermediate cells, mucous or goblet cells, serous cells, neuroendocrine and brush cells. In addition, mucous cells line the adjacent bronchial glands.[81] Unlike the tracheal mucosa, which is pseudostratified, the mucosa of intra-pulmonary bronchi is non-stratified.

Ciliated cells are tall, columnar cells attached to basal and intermediate cells by desmosomal junctions. Tight junctions exist between adjacent specialized cells at the apex. Each cell possesses 200 or more cilia that are engaged in mucociliary clearance.[82] The superficial cell surface also shows a pronounced glycocalyx. The cytoplasm of ciliated cells contains scattered profiles of rough endoplasmic reticulum, a supranuclear Golgi and numerous mitochondria particularly near the apex where a prominent cytoskeleton is also found. Mucous or goblet cells are typical mucus-secreting cells representing about 10% of the bronchial mucosa cell population in man but less than 1% in pathogen-free rats.[5] The serous cell is a cylindrical or pyramidal cell containing small, round, closely packed serous granules.[81] Basal cells are compact, pyramidal cells resting on the basement membrane. They are believed to be progenitor stem cells with the intermediate cells representing an intermediate stage of cell differentiation.

The mucus-secreting and ciliated cells form the cellular basis for the mucociliary clearance mechanism of the main conducting airways. The epithelium is covered by a mucous blanket that is fairly complete in humans and rabbits but patchier in rats.[5] The mucous layer is segregated into an upper layer or gel phase separated from epithelial cells by a serous layer or sol phase. The complex carbohydrates of the glycocalyx and secreted mucosubstances show species-related differences in their sugar residues, which can be demonstrated histochemically by the use of labelled-lectins.[83]

Mucociliary clearance mechanisms are sensitive to the effects of many therapeutic agents, particularly those that alter mucins, fluid or electrolyte balance and ciliary activity. Anaesthetic gases, barbiturates, narcotics and alcohol depress clearance function. By contrast, topical, oral or parenteral administration of β-adrenergic agonists, isoprenaline and adrenalin, produce a dose-dependent stimulation of mucociliary transport by an effect on ciliary beat frequency, probably mediated by increasing levels of cyclic adenosine

monophosphate in ciliated cells rather than through vascular changes. Although basal mucociliary function is dependent on normal vagal tone, parasympathomimetic agents can affect mucociliary transport. Acetylcholine and cholinergic agents stimulate ciliary activity whereas anticholinergic drugs, atropine and hyoscine, inhibit ciliary activity and mucociliary transport. These substances may alter deposition of inhaled particles in the lung.[5]

Clara cells or non-ciliated bronchiolar cells located in the bronchiolar epithelium, first described by Clara in 1937, are small and cylindrical in shape with highly infolded nuclei, surface microvilli, well developed Golgi, abundant endoplasmic reticulum and characteristic oval, homogeneous electron-dense granules in the apical cytoplasm. In rats, rabbits and humans the granules are PAS-positive, although they are usually considered PAS-negative in hamster and mouse.[81] Clara cells have high metabolic activity. They contain cytochrome P450-dependent enzymes and secrete a variety of proteins.[84–86] Clara cell secretory protein is the major component of their cytoplasmic granules and they have been shown to produce mucin following antigen challenge.[86]

In most laboratory rodents, the conducting airways terminate abruptly at the non-cartilaginous terminal bronchiole that opens directly into an alveolar type airway, the alveolar duct which in turn communicates with the alveoli.[87] Squamous epithelial or type I cells form only about 10% of all lung cells but they line over 90% of the alveolar surface, by virtue of extremely long cytoplasmic extensions. The principal gas exchange takes place across this cell. In the rat, the typical thickness of this barrier is 20 nm for a cytoplasmic extension of a type I pneumocyte, 90 nm for basal lamina and 90 nm for an endothelial cell.[81] The type I cell contains juxtanuclear mitochondria and the long smooth cytoplasmic extensions contain many ribosomes and pinocytotic vesicles. The anatomical configuration and function of type I cells render them highly vulnerable to inhaled gases and particles.

The other main alveolar lining cell is the granular pneumocyte or type II cell which constitutes about 10% of all lung cells, but which covers only about 5% of the alveolar surface.[88] This cell does not possess the long cytoplasmic processes typical of type I cells and it shows many microvilli on its luminal surface. The cell cytoplasm contains rough endoplasmic reticulum, Golgi apparatus, some mitochondria and characteristic oval, osmiophilic lamellar inclusions. Surfactant, a microaggregate of phospholipid and protein which modifies alveolar surface tension at low inflation volumes, is secreted by type II alveolar cells. Ultrastructural immunocytochemistry has shown the presence of surfactant apoproteins in the synthetic organelles and in the lamellar bodies of these cells, in agreement with the concept that the surfactant apoproteins are synthesized in the rough endoplasmic reticulum, glycosylated in the Golgi and are stored in lamellar bodies.[89] Type II cells are more resistant to the damaging effects of xenobiotics and unlike type I cells they retain the ability to undergo mitotic division. Following damage to type I cells, increased numbers of mitoses are evident in type II cells which results in the appearance of large

undifferentiated epithelial cells which ultimately differentiate into type I and type II cells.[81]

The lung also contains a dense neural network and a population of endocrine-like cells believed to be important in lung function.[90] These neurosecretory cells (Kultschitsky or APUD cells) are scattered sparsely in the epithelial surface of the larynx, trachea bronchi, bronchioles and alveoli. These cells are oval or cuboidal with oval nuclei, and argyrophilic cytoplasm which electron microscopic examination shows to contain dense core granules. The role of neuroendocrine cells in the lung is uncertain but immunocytochemical study has shown them to contain a number of neuroendocrine substances including neurone-specific enolase, synaptophysin, chromogranin and a variety of other peptides similar to vasoactive intestinal peptide, bombesin, calcitonin, serotonin, leu-encephalin, β endorphin and ACTH.[90,91]

Cells lining the bronchi, bronchioles and alveolar walls are capable of metabolizing xenobiotics. Immunocytochemical study has shown the presence of immune-reactive cytochromes P450, NADPH cytochrome P450 reductase, epoxide hydroxylase and glutathione S-transferase in bronchial epithelial cells, ciliated bronchiolar cells, Clara cells, type II and possibly type I pneumocytes in the rat lung.[28] Different cell populations contain different amounts of enzymes, Clara cells containing the greatest concentrations of the phenobarbitone-inducible isoenzyme of cytochrome P450, NADPH-cytochrome P450 reductase and epoxide hydrolase. Studies of microsomal enzyme activities suggest that lung tissue contains fewer P450 isoenzymes than liver, principally forms *CYP1A1, CYP2B1, CYP3A2* and *CYP4B1*.[92] Whereas P450 enzyme activity is highly concentrated in specific cell types, overall microsomal enzyme activity is low compared with liver on the basis of microsomal protein weight.[92]

Other important cells are the pulmonary alveolar macrophages and lymphocytes. Lymphocytes are found in the epithelium of the airways, in the interstitium of alveoli and as part of follicles in bronchial walls. Pulmonary macrophages form part of the specific immune defence system of the lung, involving, as elsewhere in the body, antigen presentation. In the rat and mouse, distinctive populations of pulmonary macrophages have been described based on enzyme activities and reactivity to monoclonal antibodies against monocyte and macrophages surface determinates.[93,94] Bronchus associated macrophages in rat and mouse have more acid phosphatase and less non-specific esterase activity than the populations found in the pulmonary alveoli and interstitial tissues.

An important aspect of the immune system is the *bronchus-associated lymphoid tissue* or BALT, which forms part of the mucosal lymphoid system found in other epithelia. The morphology of BALT is a useful guide to the nature and degree of immune stimulus in the lung. BALT is organized in a way that is characteristic of other peripheral lymphoid organs. It is structurally similar in the laboratory rat, mouse, rabbit, guinea pig as well as in man but its size and prominence is species and strain-dependent as well as a function of the degree of antigenic stimulus[94–96].

In the rat, the BALT is composed of lymphoid aggregates or follicles located mostly between a bronchus and artery with a zone of lymphocytes situated immediately under the bronchial epithelium. As in other peripheral lymphoid tissue, BALT is organized into B and T cell zones but in no predetermined manner. Immunocytochemical staining has shown that B and T lymphocyte zones differ in location from one aggregate to another. There are about two T lymphocytes for every three B cells compared with a ratio of 2:5 in rat Peyer's particles.[97] The ratios may be different in other species. Quantitative observations of T cell subsets using monoclonal antibodies have also shown that rat BALT normally contains twice as many T-helper as T-suppressor/cytotoxic lymphocytes.[97] The T cells are confined to one or two discrete zones with a light scattering of T cells within the B cell zones and immediately under the bronchial epithelium. In common with lymph nodes, interdigitating cells are also found. The epithelium overlying BALT shows anatomical modifications. It is composed of ciliated and non-ciliated cells covered by microvilli.

In conventional, untreated laboratory rats, BALT shows little activity and germinal centres are usually absent, although BALT may be more prominent in some rat colonies in association with non-specific inflammatory lesions in lungs.[98,99] In one colony of young Wistar rats germinal centres were not seen in BALT in untreated animals but they developed following the administration of a single intratracheal dose of lipopolysaccharide, a T cell-independent antigen.[100] Single intratracheal doses of T cell dependent antigens such as horseradish peroxidase, bovine serum albumin and BCG have been shown to produce only minor morphological changes which include expansion of the zone of lymphocytes immediately under the epithelium and infiltration of the bronchial epithelium overlying BALT by lymphocytes.[101] In addition, perivascular, peribronchial or alveolar infiltrates of small and large lymphocytes and macrophages were observed in the lungs of rats given BCG.

Immunocytochemical study of the rat BALT following intratracheal challenge with horseradish peroxidase showed that the majority of cells that infiltrated the bronchial epithelium were T helper (CD4 positive) lymphocytes.[101] Furthermore, Ia antigen expression of the epithelial cells overlying the BALT was shown to increase, associated with an increase in the number and size of microvilli, a more pronounced glycocalyx and a decrease in number and size of cilia.

Immunocytochemical study of the BALT tissue in C57B1/6 mice using monoclonal antibodies to lymphoid and macrophage populations has demonstrated quite similar arrangements of cells to those in the rat with the majority of T cells belonging to the T helper (CD4 positive) class.[94]

The pulmonary lymphatic system drains into mediastinal or cervical lymph nodes. Although among rat strains differences in the location of lymph nodes and their drainage occur, tracer studies in the Fischer 344 rat using colloidal carbon have shown that the lung lymphatics drain mainly into posterior mediastinal lymph nodes and those in the tracheal wall drains primarily to the internal jugular and posterior cervical nodes.[102]

Structural evaluation

Although a variety of fixation, embedding and staining procedures are available for light and electron microscopic examination of lung tissue, there is no substitute for initial, careful visual inspection of the lungs at autopsy. Uneven collapse of lungs on opening the thoracic cavity, discoloration or alteration in texture of the pleural or cut surface, congestion and presence of fluid in the larger airways may indicate structural damage. In rodent lungs, small pulmonary adenomas may be detectable by inspection in good light.

Fresh lung weight is also a helpful measure in lung assessment, although passive vascular engorgement can significantly affect this value. Nevertheless, studies in the normal Fischer 344 rat have shown that after exsanguination, wet lung weights show a close relationship to body weight and that dry weight of lungs consistently represents about 20% of the wet weights regardless of age or body weight.[103] An increase in wet weight over dry weight appears to be a good index of pulmonary oedema.[16]

Various methods of fixation have been employed although simple immersion fixation in formalin for conventional light microscopy has the virtue of simplicity and it avoids the risk of translocation or removal of exudates from airways and alveoli. Mixtures of formaldehyde, paraformaldehyde and glutaraldehyde are used in initial fixation for electron microscopy.[38] The best overall appreciation of lung architecture is achieved by instillation of fixative via the trachea under an appropriate constant pressure or by perfusion fixation of the pulmonary arteries that is less liable to dislodge intra-alveolar exudate. In a review of methods employed routinely in rodent toxicity studies, instillation of fixative via the trachea was the preferred method in most laboratories because its advantages were seen to outweigh its disadvantages.[75]

The sampling procedure is an important aspect of histological examination of the bronchi and lungs, particularly those of large laboratory animals. The extent of histological sectioning in conventional toxicity studies should be modulated to take account of lesions found by macroscopic examination, the type of study and the nature of the test substance. The bronchi should be carefully sampled to allow assessment of any alterations in bronchial epithelium.

Morphometric analysis represents a sensitive tool of value in the evaluation of drug-induced lung changes, but it requires particularly rigorous sampling and evaluation procedures.[104,105] A tiered, multiple stage or cascade sampling technique is normally considered the most appropriate for morphometric studies.[104] This involves dividing the lung into a series of homogeneous compartments or strata from which randomly selected samples can be examined by appropriate light or electron microscopic techniques.

Conventional special stains for reticulin and collagen as well as PAS and alcian blue for mucins are helpful in the characterisation of lung damage and changes to the respiratory epithelium. Immunocytochemistry and enzyme cytochemistry are also useful in the study of the heterogeneous cell population of the lung. Xenobiotic metabolizing activity can be studied both by enzyme cytochemical methods as well as by immunocytochemical techniques

using antisera specific for pulmonary monooxygenases and related enzymes.[33] Important structural components, particularly collagen and laminin can be studied both at light and ultrastructural level with immunocytochemical methods.[106] Cytokeratin immunocytochemistry can be used as a method for the characterization of changes to epithelial cells.[43] Clara cells can be localized by the presence of Clara cell secretory protein and ciliated cells by the presence of tubulin.[86] Endocrine cells are visualized by immunocytochemistry using antibodies to general neuroendocrine markers such as chromogranin and synaptophysin or regulatory peptides.[90] Other useful antigens, which can be demonstrated in the lung, include surfactants, lysozyme, immunoglobulins and those of microorganisms that infect the lung.[107]

Electron microscopy is particularly useful for the detailed characterization of injury to the cells of the alveolar epithelium and endothelium (Figure 6.1).

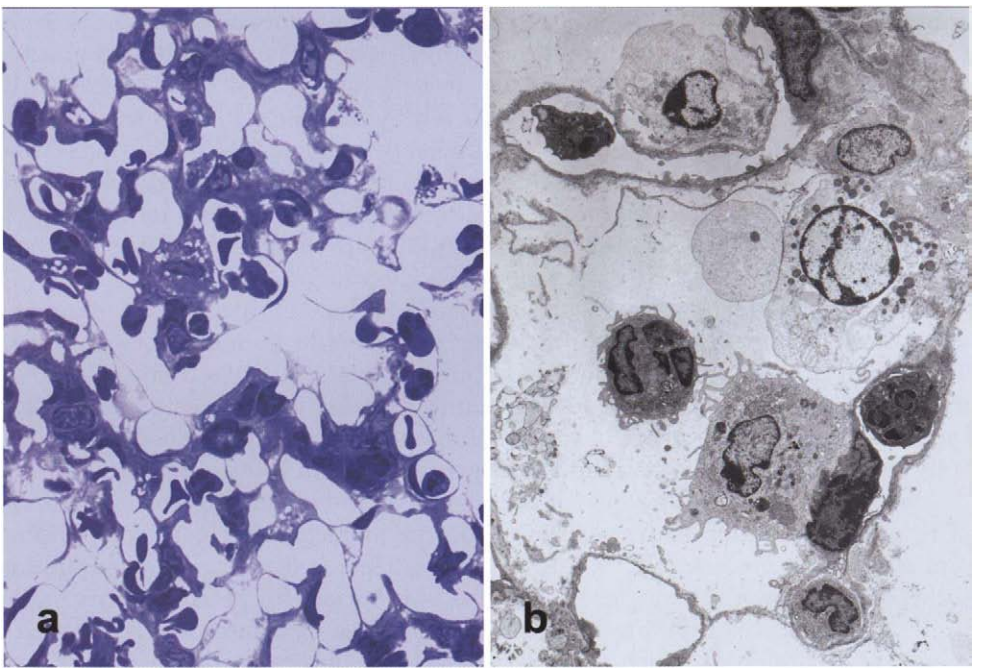

Figure 6.1 Guinea pig lung 12 hours after a single intraperitoneal injection of 35 mg/kg of paraquat. *Panel a:* An example of the high definition obtained from plastic-embedded material. It shows intra-alveolar oedema, alveolar wall thickening and increased numbers of inflammatory and degenerate cells (methylene blue ×550). *Panel b:* Electron micrograph illustrates the degenerative changes in type I and type II pneumocytes along with cellular debris and macrophages in the alveoli (×1100). Illustrations by courtesy of Dr N.G. Read

Oedema

Pulmonary oedema is a component of many inflammatory conditions of the lung, including those induced by infectious agents. However, the term oedema is reserved for a poorly cellular exudate characterized by the presence of pale, homogenous eosinophilic material in the alveoli, sometimes associated with a similar exudate in the lung septae and perivascular connective tissue.

It occurs in a number of spontaneous conditions such as in congestive cardiac failure, metastatic pulmonary neoplasms or as an agonal change in association with pulmonary congestion and haemorrhage. Drugs may induce cardiogenic pulmonary oedema as a consequence of pulmonary hypertension or impaired ventricular contractility. Cardiogenic oedema is often associated with vascular congestion and red blood cells and haemoglobin may leak into airspaces. This can give rise to the presence of haemoglobin crystals within the oedema fluid in formalin-fixed tissue sections.

Most importantly, pulmonary oedema may be a manifestation of acute lung injury. Inhalation or systemic administration of toxic chemicals may produce acute pulmonary oedema (see Figure 6.1). Some substances such as phenylthiourea and α-naphlythiourea produce massive pulmonary oedema in laboratory animals when administered orally, principally as a result of damage to the endothelium of pulmonary capillaries and venules.[108] Over 30 drugs have been reported to produce non-cardiogenic pulmonary oedema in humans, either directly or through poorly understood immunogenic mechanisms.[2]

Another form of pulmonary oedema involves the main airways. Allergic reactions in sensitized airways of asthmatic individuals is believed to result from cross-linking of IgE and activation of mast cells that degranulate and release inflammatory mediators.[109] This has been reproduced in the main airways of rats sensitized to ovalbumin and then challenged with ovalbumin by the intratracheal route.[110] This treatment leads to rapid accumulation of bronchial exudate, degranulation of mast cells and the development of mucosal oedema, most marked immediately below the respiratory epithelium.

Congestion and haemorrhage

Congestion and haemorrhage is a frequent finding in the lungs of laboratory animals, where it is usually related to certain modes of death. It can be associated with administration of drugs and chemicals that have adverse effects on cardiac function or on the coagulation system. Administration of heparin to rats produces a characteristic extravasation of blood into the air spaces.[111]

Inflammation due to infections and infestations

Lower respiratory tract infection is generally not a major health hazard among laboratory animals but it is nevertheless an ever-present threat that can

cause overt respiratory disease within a colony or develop following administration of xenobiotics. Subclinical pulmonary infections and infestations can also produce histological alterations in the bronchial airways or pulmonary parenchyma which mimic changes induced by inhaled irritants or systemically administered drugs.[98,99] Furthermore, some respiratory pathogens alter immune defences and exacerbate the effects of inhaled substances.[112]

A range of bacterial and viral pathogens may produce inflammatory lung changes.[46] Typically, bacterial pathogens such as *Steptococcus pneumoniae* produce acute bronchitis associated with a variable degree of acute inflammation of the lung parenchyma (bronchopneumonia) or a confluent lobar pneumonia. Viral agents are generally associated with histological features of bronchiolitis and interstitial pneumonia, characterized by an increase in mononuclear cells in the respiratory bronchioles and alveolar septa. The histological features are variable for they depend on the particular pathogen, species and strain, immune status, presence or absence of secondary infection and the particular stage at which the infection is examined. Respiratory infections are frequently mixed. Changes due to secondary bacterial infection are frequently superimposed on those induced by viruses.

Sequential histopathological examination of the lungs of laboratory animals following inoculation with respiratory tract pathogens has been able to characterize the evolution of pathological changes produced by individual organisms. For instance, following inoculation with *Mycoplasma pulmonis*, one of the more important respiratory pathogens among laboratory rodents both Lewis and Fischer 344 rats were shown to develop upper and lower respiratory tract inflammation. In the Lewis strain this was characterized after 28 days by a variable acute inflammatory exudate in bronchi and bronchioles with focal bronchiectasis, inflammation and hyperplasia of the epithelium with a predominantly macrophage infiltration of the alveoli and alveolar walls.[48,113] These changes were associated with marked hyperplasia of the bronchus-associated lymphoid tissue (BALT), which extended down the airways and blood vessels towards the periphery of the lungs. Although the lymphoid hyperplasia was also found in inoculated Fischer 344 rats, it was less marked and accompanied by little or no mucopurulent exudate or active inflammation of the bronchial walls. This disparity in response suggested that differences were related to the degree of lymphocyte activation in the two strains, an imbalance in regulation of lymphocyte proliferation in Lewis rats, or both.[113]

Other studies have been conducted in both rats and mice infected with another important respiratory pathogen of laboratory rodents, the Sendai virus (parainfluenza type 1). Sequential studies showed that the initial damage to bronchial and bronchiolar epithelium is associated with polymorphonuclear and lymphocytic inflammation (bronchiolitis). Immunocytochemical and ultrastructural studies revealed the presence of viral antigen in the mucosa.[112] Hyperplastic and multinucleated syncytial epithelial cells develop in the hyperplastic terminal bronchiolar epithelium and the inflammatory process extended to involve peribronchial or peribronchiolar parenchyma

with infiltration of alveolar walls by mononuclear cells, macrophages and neutrophils. A similar cell population accompanied by cell debris and oedema fluid develops in air spaces. Pulmonary arteries show only minor involvement with inflammatory cells and focal reactive hyperplasia of the endothelium. Immunocytochemistry and ultrastructural examination suggested that virus replication takes place in alveolar type I and type II epithelial cells and macrophages but not in endothelial or interstitial cells of the alveolar septae.[114] It was shown that when repair occurs there may be residual distortion of bronchiolar and alveolar walls by collagen and hyperplastic cuboidal epithelium may line the thickened alveolar septa. Air spaces may also contain enlarged macrophages with pale vacuolated cytoplasm.[115] Strain differences in susceptibility have also been demonstrated to this virus. There is differential pulmonary interleukin 12 (IL-12) gene expression between virus-susceptible Brown Norway rats and resistant Fischer 344 rats and IL-12 treatment provides protection from virus-induced chronic airway inflammation and remodelling. Moreover increased tumour necrosis factor α (TNFα) expression has been shown to be an important regulatory factor in the development of Sendai virus-induced bronchiolar fibrosis in infected rats.[116] Virus-inoculated Brown Norway rats had increased TNFα pulmonary mRNA levels and increased numbers of bronchiolar macrophages and fibroblasts expressing TNFα protein compared with virus-inoculated F344 rats.[117]

The Corona virus, which causes sialodacryoadenitis in many rat colonies, also produces lower respiratory tract inflammation. This is characterized by acute bronchitis and bronchiolitis with focal extension into lung parenchyma. Thickened oedematous, hypercellular alveolar walls infiltrated by monocytic cells are found.[70] Immunocytochemistry has shown the presence of viral antigen in bronchial and bronchiolar epithelial cells. There is also peribronchial lymphocytic infiltration and increased prominence of BALT. Ultimately complete resolution occurs.

Viruses remain a potential source of spontaneous respiratory disease in laboratory dogs. Canine adenovirus type 2, parainfluenza SV5, canine herpes virus, coronavirus and parvovirus have all been isolated from laboratory dogs developing respiratory disease.[118]

The syndrome of visceral larva migrans also incites focal inflammation, granulomas and fibrosis in the lungs of species such as dog and primate in which parasites are prevalent. The syndrome of visceral larva migrans is usually defined as that which results from the migration of nematode larvae into the viscera. It has been well described in the beagle dog lung where it results from the larvae of toxocara species or metastrongyloid nematodes.[119,120] The precise identification of parasites is not always possible in tissue sections. Histological appearances of infested lungs are highly variable. Nematodes surrounded by granulomas and granulomatous inflammation, mostly in a subpleural location, may be visible in sections. In affected lungs there may be perivasculitis and active arteriolitis, bronchiolitis and peribronchiolitis. Pleural involvement by the inflammatory process can be marked, particularly in regions

overlying granulomas. Scarring develops and pleural and sub-pleural fibrosis is frequently associated with epithelial hyperplasia and squamous metaplasia of the associated airways (Figure 6.2).[120] The lesions may sufficiently severe to resemble those induced by high doses of anticancer drugs such as bleomycin (see below).

Pulmonary acariasis is a common infestation of many species of non-human primates caused by various species of the mite *Pneumonyssus*. Reproduction of the mites appears to take place in the terminal bronchioles. *Pneumonyssus simicola* is the recognized form found in rhesus monkeys.[121] Although it is most prevalent in wild caught primates, the disease is not easily eliminated during breeding in captivity.[122] Even when eliminated by ivemectin the lesions of chronic bronchiolitis, bronchiectasis and pigmentation may persist as an incidental finding.[123] As the mite can produce significant destructive pulmonary pathology and render animals susceptible to secondary pulmonary bacterial

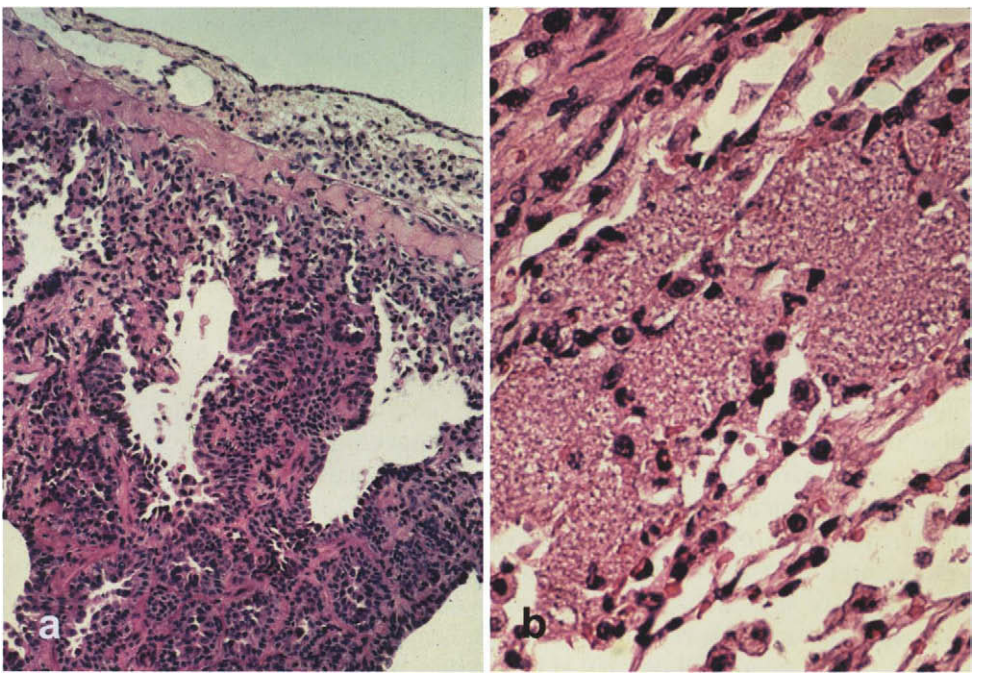

Figure 6.2 This shows two spontaneous lung conditions that can affect safety studies. *Panel a:* Lung from a young control beagle dog showing fibrous scarring, epithelial proliferation and thickening of the parietal pleura, probably due to previous infection. Features resemble those induced by bleomycin (H&E ×45). *Panel b:* Lung from an immune deficient (nude) mouse showing the typical granular, eosinophilic appearance of *Pneumocystis* within the air spaces. There is no evidence of an inflammatory reaction as expected in an immune deficient state (H&E ×190)

infections, it can disrupt or confound the interpretation of toxicity studies performed in primates. The lesions are located most frequently in cranial lobes and are characterized by the presence of bullae distending the pleural surface, parenchymal cysts, nodules and scar tissue.[121,122] Histologically, there is a wide range of inflammatory activity. Fully developed lesions are characterized by granulomatous bronchiolitis and peribronchiolitis with involvement of immediately adjacent alveoli. Cystic lesions involving the bronchiolar walls develop around the parasites giving rise to the appearance of walled-off cysts composed of highly cellular granulation tissue, associated with neutrophils, lymphocytes, macrophages, multinucleated giant cells and various pigments (see below). In less active lesions, dilated, cystic airways with walls composed of thick bands of smooth muscle and lined by squamous or cuboidal epithelium are found.

Pneumocystis carinii is an important cause of pneumonia in patients with the acquired immunodeficiency syndrome (AIDS) as well as in other immunocompromised patients, including those treated with immunosuppressive drugs.[124] The natural habitat of *Pneumocystis carinii* is pulmonary alveoli and it is widely encountered in the human population without being associated with overt disease. Both clinical and experimental evidence suggests that impaired cellular immunity is much more important as a predisposing factor than impaired humoral immunity.[124] As in humans, laboratory animals may have latent pneumocystis infection that becomes clinically evident following immunosuppression. It has been shown in the rat that chronic administration of various regimens of adenocorticosteroids, low protein diets, cyclophosphamide and other immunosuppressive drugs with concomitant antibiotic administration to prevent other infections gives rise to typical pneumocystis pneumonia.[125] Rodents with genetically deficient cellular immunity also develop pneumocystis pneumonia. The importance of pneumocystis pneumonia in toxicology is that it can be considered as a sentinel of chronic immune depression.

In haematoxylin and eosin stained sections, pneumocystis pneumonia is characterized in both humans and rodents by the presence of alveoli filled with foamy eosinophilic material containing a few macrophages and indistinct nuclei of pneumocystis (Figure 6.2*b*). Ovoid or crescent-shaped structures of the organisms become clearly visible with Gomori methenamine silver or toluidine blue stains. Ultrastructural study of rats with pneumocystis pneumonia shows that trophozoites attach themselves most frequently to type I pneumocytes by altering their morphology to the contours of the pneumocytes rather than by a process of invasion.[126]

Drug-induced inflammation

Systemically administered therapeutic agents may produce histological changes within the lung parenchyma that mimic components of the normal

response to respiratory pathogens. However, there is no sharp separation between agents that produce pulmonary oedema with those that are associated with acute inflammatory changes and histological features overlap because an acute inflammatory process is often accompanied by exudate within airspaces.

An example of drug-induced pulmonary inflammation in laboratory animals and humans is reported following the administration of interleukin 2 (IL-2). IL-2 is a glycoprotein lymphokine, molecular weight 15kDa, which is normally produced by activated T cells and mediates immunoregulatory responses. It has been produced in large quantities by recombinant DNA technology for use in tumour immunotherapy. However, high doses have been associated with a number of adverse effects, notably the *'vascular leak' syndrome*, characterized clinically by pulmonary oedema, pleural effusions and ascites.[127]

The vascular leak syndrome has been reported in laboratory animals given high doses of this agent. Histological examination of the lungs of B6D2F mice developing this syndrome following administration of IL-2 showed infiltration of the alveolar walls with large lymphocytes and intra-alveolar proteinaceous exudate containing large lymphocytes, macrophages and red blood cells.[128,129] Pulmonary venules and arterioles showed the presence of lymphocytes attached to or lying beneath the endothelium, infiltrating vessel walls or in a perivascular location where they were accompanied by oedema fluid or red blood cells. Similar, but less severe changes have been demonstrated in rats given IL-2.[128] In addition, treated rats showed an infiltration of pulmonary vasculature with eosinophils probably secondary to an eosinopoietic cytokine produced by IL-2 stimulated lymphocytes. Immunocytochemical evaluation of the lymphoid infiltrate in mice showed that most of the cells were Thy 1.2-positive (CD90) lymphocytes. Furthermore, co-administration of asialo GM1 (ganglio-n-tetrosyl-ceremide) with IL-2 not only abrogated the clinical signs but also reduced the number of asialo GM1-positive lymphocytes in the tissue sections.

As lymphoid cells expressing Lyt-2 (CD8, suppressor/cytotoxic T cells) were unaffected by asialo GM1 treatment, it was postulated that the vascular leak syndrome (but not antitumour efficacy) in these mice was mediated by an endogeneous subset of IL-2 stimulated lymphocytes or lymphokine-activated killer cells.[129] Corresponding changes were also observed in liver and lymphoid tissue. Immunocytochemical and detailed electron microscopic studies in rats have supported the concept that IL-2 induces cytotoxic vascular damage that is mediated both directly by lymphokine-activated killer cells and cytotoxic T lymphocytes with secondary release of inflammatory cytokines.[130]

As in humans, severe chronic pulmonary inflammatory disease in laboratory animals may compromise pulmonary function and lead to secondary alterations in other organs. Although the mechanisms were not explored in detail, a diffuse interstitial pulmonary inflammatory process with lung haemorrhage was induced in rats treated for two years with prizidilol (SKandF 92657-A2), an antihypertensive agent with both vasodilator and β adrenoceptor blocking properties.[131] Affected animals developed dyspnoea associated with reduction

in lung volume, deformity of the thoracic spinal column and marked cardiac hypertrophy.

Granuloma, granulomatous inflammation

Inflammation with granulomas develops in the lungs of laboratory animals under a variety of different circumstances, which have been alluded to above. A common cause in rodents is granulomatous pulmonary inflammation resulting from aspiration of stomach contents or food particles (aspiration pneumonia). This is sporadically observed in aged rodent where it is associated with general ill health, particularly resulting from pressure effects of large pituitary adenomas and subsequent disturbance of pharyngeal or laryngeal reflex mechanisms.[132] Histologically, the lungs show peribronchial and peribronchiolar granulomatous inflammation with macrophages and foreign body cells associated with fragments of refractive vegetable matter. The associated bronchial mucosa may also show reactive changes including goblet cell hyperplasia in long-standing cases.

As dogs and primates are more liable to be infested by parasites, granulomatous inflammation in response to pulmonary larvae is more common in these species. Pulmonary tuberculosis represents a potential problem among non-human primate colonies in view of its insidious onset and its liability for transmission from monkeys to humans.[133] Pathological findings are similar to those so well known in the human disease. The disease is characterized by the presence of granulomas in lung parenchyma and lymph nodes. In florid cases there may be caseation surrounded by epithelioid and multinucleated giant cells and variable numbers of lymphocytes, plasma cells and fibroblasts. Diffuse granulomatous pneumonia as a result of tuberculosis is also reported in non-human primates. Granulomatous pneumonitis is also produced in laboratory animals by the intravenous injection of BCG. Twenty-eight days following intravenous injection of bacille Calmette–Guérin (BCG), the lungs of C57B1/6 mice contained numerous granulomas composed of histiocytes and round cells which were surrounded by alveoli with thickened walls and associated with mild interstitial pneumonitis.[134] These histological changes were associated with an increase in the number of Thy 1.2-positive (CD90) cells, especially Lyt-1 (CD5) positive lymphocytes. The histological changes were abrogated by treatment with cyclosporin A suggesting an important role for CD5-positive lymphocytes in the development of the granulomas.

Discrete granulomas occur in the lungs of experimental animals in response to intra-tracheal or intravenous injection of certain relatively insoluble substances (Figure 6.3). Intra-tracheal administration of insoluble polymerized dextran and latex micro-particles to mice showed that the morphology and the systemic effects of granulomas depends on the nature of the injected substances. It has been shown that large granulomas develop rapidly in the pulmonary parenchyma around dextran particles that subsequently regress

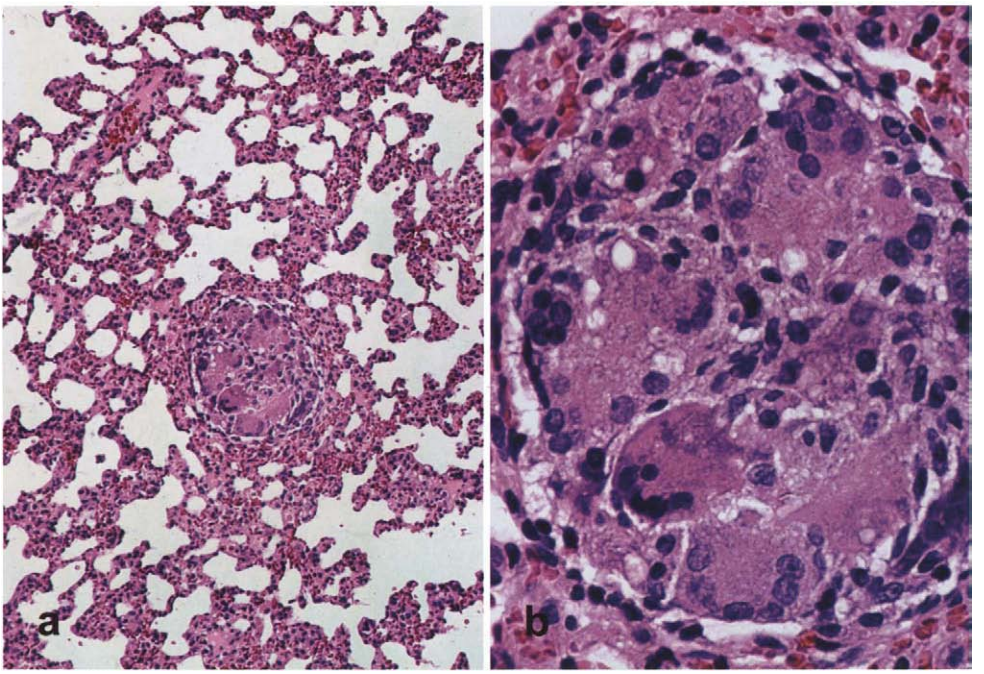

Figure 6.3 Lung of a Sprague–Dawley rat given repeated intravenous doses of a soluble synthetic polymer that produced angiocentric foreign body granulomas. *Panel a:* Low power view (H&E ×45). *Panel b:* High power view (H&E ×190)

quickly, whereas latex particles produce small, discrete stable granulomas.[135] Although both forms of granulomas are of foreign body or non-immunological in type, those produced by dextran but not latex beads, are associated with anergy-like immunosuppression, probably caused by release of soluble factors from the granulomas.

It has been reported that granuloma formation after instillation of sephadex beads is associated with increases in the interleukin 1- (IL-1) like activity in the lung.[136] Studies comparing the effects of inhaled crystalline silica and titanium dioxide have shown a correlation between the release of the macrophage derived cytokine IL-1 and granuloma formation, suggesting that IL-1 might be a useful biomarker for granuloma formation.[137]

Localized, angiocentric granulomas of foreign body type, clustered around pulmonary arteries and arterioles and occasionally alveolar capillaries and venules also develop following intravenous injection of relatively insoluble polysaccharides or other polymers.[138] Characteristic epithelioid and large, foreign body type giant cells efface the smaller vessels although overt necrosis is not usually observed (Figure 6.3).

Pigment

Haemosiderin-laden macrophages accumulate in the alveoli of laboratory animals in association with chronic pulmonary congestion and haemorrhage. Similar changes occur in patients in congestive cardiac failure where the haemosiderin-laden macrophages are termed *'heart failure'* cells.

The lungs of non-human primates are especially liable to contain alveolar, perivascular and peribronchial aggregates of macrophages laden with various brown pigments. Iron-containing pigments have been associated with the inflammatory changes produced by simian lung mites (*Pneumonyssus simicola*) which are prevalent in many non-human primates. In addition, lungs from some primate colonies may show perivascular and peribronchial collections of brown-grey macrophages containing highly refractive spicules and plates composed of high concentrations of silica.[139,140] It has been shown that in Old World primates including rhesus and cynomolgus monkeys, this pigment contains fossil diatomaceous material, compatible with the concept that the animals inhale dusts containing diatoms and other silicon fragments to which they are exposed in their semi-arid, natural habitats.[139]

Fibrosis

Chronic lung injury from a variety of different causes is frequently associated with the development of pulmonary fibrosis characterized by the replacement of the normal pulmonary structure by a thickened collagenous matrix with consequent reduction in the capacity for gas exchange. Regardless of the inciting agent, the fibrogenic process appears to be generally characterized by disruption of the normal alveolar-capillary structure, leakage of exudate from the vascular compartment into the airspaces, subsequent invasion by inflammatory cells and fibroblasts associated with excess matrix formation. Studies in laboratory animals with different fibrogenic agents as well as in humans have suggested that central to pulmonary fibrogenesis is increased production of TNFα by macrophages.[12,116,141–143] This cytokine is a not only a mitogen for fibroblasts but also a potent activator and chemo-attractant for macrophages, capable of stimulating release of other cytokines and inducing expression of adhesion molecule expression on endothelial cells. Moreover, it has been shown that TNFα receptor knockout mice appear protected from the fibroproliferative effects of inhaled asbestos.[144]

Pulmonary fibrosis is a common sequel of chronic lower respiratory tract inflammation. It may be associated with, or preceded by interstitial pneumonitis, characterized by infiltration of lymphocytes, plasma cells and macrophages with scattered polymorphonuclear cells.[145] Focal pulmonary fibrosis occurs spontaneously in laboratory animals, although this is usually most prevalent in dogs and non-human primates as a response to chronic infestation by parasites, which are not easily eliminated during breeding.

In humans, conditions leading to pulmonary fibrosis vary widely. They include infections, shock lung syndrome, ionizing radiation, inhalation of irritant particulate matter, exposure to antigens or excessive amounts of oxygen as well as the results of the toxicity of paraquat and a range of both cytotoxic and non-cytotoxic therapeutic agents which cause pulmonary parenchymal injury.[2,146]

Fibrosis induced by therapeutic agents

The principal therapeutic agents that produce pulmonary fibrosis in both humans and laboratory animals are anticancer drugs. Bleomycin, a glycopeptide preparation derived from *Streptomyces verticillus*, is the best known example but pulmonary fibrosis is also associated with the clinical use of a number of other anticancer agents, including 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU or carmustine), cyclophosphamide, busulphan, mitomycin C and methotrexate.[2,108,147–150]

The precise mechanisms involved in the induction of pulmonary fibrosis by antineoplastic drugs in humans are poorly understood. The true incidence for a particular drug is difficult to estimate because of confounding factors in cancer patients, such as concomitant administration of several drugs, radiation and oxygen therapy, diffuse pulmonary cancer and opportunistic infections. It is probable that drug-induced fibrosis is accentuated by concomitant administration of several antineoplastic agents, radiation therapy, hyperoxia, pre-existing pulmonary damage and age of the patient. Severity is often related to total dose of drug received.[108] Novel antineoplastic drugs may also produce lung toxicity.[3]

Bleomycin is associated with the development of interstitial pneumonia and pulmonary fibrosis in clinical use and this can be reproduced in experimental animals. The histopathological appearances of bleomycin-induced pulmonary fibrosis in patients are in many instances different from those seen in laboratory animals because the lungs of patients treated with bleomycin are modified by the primary neoplastic disease, smoking, multiple drugs, radiation therapy and secondary pulmonary infections, interstitial pneumonitis and fibrosis.[151] It has been postulated that TNFα is an important mediator in the development of bleomycin-induced fibrosis.[142]

In the preclinical evaluation of bleomycin beagle dogs were given cycles of drug by the intravenous route for periods of up to 26 weeks.[152] Dogs developed anorexia, weight loss, a variety of epithelial lesions as well as focal interstitial pneumonia and fibrosis. The focal lung lesions were characterized by increased elastic fibres, reticulin, collagen and acid mucosubstances. The lesions were situated predominantly in the pleural and subpleural zones, suggestive of a potentiating effect of friction between the pleural surfaces. Histologically the lesions resembled those produced by larvae migrans in the dog (see Figure 6.2*a*).

Similar histological changes have also been described in both rats and mice treated with bleomycin by both the intravenous and intratracheal route.[153,154] As fibrosis is such a consistent change, bleomycin-treated rodents have been

extensively employed as a model for pulmonary fibrosis. Early changes include mild, diffuse increases in interstitial lymphocytes, macrophages, polymorphonuclear cells and perivascular or interstitial oedema. After about a week, interstitial infiltrates also comprise fibroblasts with early collagen deposition, associated with proliferation of macrophages and type II pneumocytes.[153,154] Subsequently, the amount of interstitial collagen increases, with eventual scarring and collapse of lung tissue in proportion to the cumulative dose given.[155] Immunohistochemical and ultrastructural study of rats and mice treated with bleomycin shows a large accumulation of immune-reactive laminin and reduplication of the basement lamina within the thickened alveolar walls.[145] In bleomycin-treated rats three-dimensional scanning electron microscopy shows drug-induced capillary remodelling comprising irregular alveolar and pleural capillaries with increased diameter and decreased branching.[156] Certain strains of mice have been shown to possess greater sensitivity to bleomycin fibrogenesis. The C57BL/6 strain produces a greater fibroblastic response than DBA/2 and Swiss mice and the BALB/C strain demonstrates a particularly poor fibroblastic response.[157]

Therapeutic use of cyclophosphamide is also occasionally associated with the development of pulmonary interstitial fibrosis.[148,149] It appears to be associated with two forms of pathology: an early-onset pneumonitis and a late onset progressive pulmonary fibrosis.[149] Similar changes have been less easy to reproduce in laboratory animals. When mice were sequentially examined for periods of up to one year after a single intravenous dose of 100 mg/kg of cyclophosphamide, only slight pulmonary interstitial thickening and hypercellularity was observed in association with progressive multifocal accumulation of intra-alveolar macrophages.[158] However, these changes were also accompanied by a progressive increase in pulmonary hydroxyproline content and a decrease in pulmonary compliance with time in treated animals compared with controls. The changes were amplified by exposure to 70% ambient oxygen.

The bronchiolitis, alveolar septal inflammation and fibrosis induced by gold therapy in patients with rheumatoid arthritis is probably immune-mediated. This condition is associated with peripheral eosinophilia and drug-induced alterations to the immune system.[146]

Emphysema

Emphysema is characterized by abnormal, permanent enlargement of airspaces distal to terminal bronchioles, accompanied by destruction of their walls without obvious fibrosis. Three principle types, *centriacinar*, *panacinar* and *distal acinar* emphysema, are recognized in humans. Enlargement of air spaces as a result of congenital factors or fibrous scarring are grouped separately and not regarded as emphysema.[159]

Emphysema has been reported as an age-related spontaneous change in laboratory rats.[160] However, several experimental rodent emphysema models have

been developed, using intratracheal instillation of proteolytic enzymes papain, pancreatic and neutrophil elastase. This gives rise to histological appearances resembling panacinar emphysema in humans.[159]

Irritant gases, notably oxides of nitrogen, are also capable of inducing changes in the lungs of laboratory rats and hamsters following long term exposure which resemble mild human, centrilobular emphysema.[161,162]

Phospholipidosis (lipidosis, 'pulmonary alveolar proteinosis')

A variety of different names have been applied to membrane-bound, acid phosphatase-positive cytoplasmic inclusions with a lamella or crystalloid ultrastructural matrix. These include *myeloid bodies*, *myelinoid bodies*, *myelin figures* or *myelinosomes*. These lysosomal inclusions are seen in small numbers in a variety of normal cell types but they accumulate in various organs in laboratory animals following administration of a wide variety of drugs of diverse therapeutic classes.[2,163–167]

The generalized accumulation of these lysosomal cytoplasmic bodies is generally called *phospholipidosis*, a term coined to describe the tissue accumulation of phospholipids.[168] At the light microscopic level, phospholipids are characterized by the increase in the number of foam cells in the airspaces. Examples of drug-induced phospholipidosis include the anorectic drug chlorphentermine, tricyclic antidepressants, inhibitors of cholesterol biosynthesis such as triparanol, the antihistamine chlorcyclizine and its analogues, the selective oestrogen receptor antagonist tamoxifen, chloroquine and the cardiovascular drugs amiodarone, 4,4′diethylamino-ethoxyhexestrol and perhexiline.[164,169,170] Many tissues and organs may develop the cytoplasmic inclusions, including lymphoid cells, liver, pancreas, endocrine tissue, nervous system, muscle cells, eyes and particularly lungs. Aminoglycoside antibiotics may produce laminated phospholipid inclusions in the renal tubular cell and imidazol antifungals in hepatocytes (see under Liver and Kidney, Chapters 9 and 10).

Many drugs that induce phospholipidosis usually share structural features, notably a hydrophilic cationic side chain, a primary, secondary or tertiary amine and a hydrophobic region that is usually an aromatic ring or ring system. As this structural pattern renders these molecules amphiphilic, these drugs probably bind with polar lipids by means of electrostatic and hydrophobic forces.[164] This leads to formation of drug–lipid complexes which are poorly degraded by lysosomal enzymes and which accumulate in the cell cytoplasm to form the inclusions described above. As the binding is not covalent, its reversibility depends on the dissociation rate constant under the particular intracellular conditions and drug concentration achieved. Predictions of this activity based on molecular structure have shown reasonably good correlation with the ability of compounds to produce phospholipidosis in cultured rat peritoneal macrophages. More recently other cell based systems have been proposed for screening for phospholipidosis.[171,172] However these perform less well in

the prediction of *in vivo* potency, presumably because of differences in drug disposition in blood and tissues.

It should be underlined that the accumulation of foamy macrophages in the alveolar spaces may also be a spontaneous change in laboratory animals. It has long been recognized as a spontaneous alteration in ageing rats.[173] It may also be found in lung tissue distal to bronchial lesions that impede clearance mechanisms. In contrast to drug-induced changes, the spontaneous lipidosis characterized by accumulation of alveolar foam cells occurs sporadically in older rats and is observed in both controls and treated animals. Drug-induced phospholipidosis occurs within a period of several months during which lungs of control animals remain fairly free of foam cell accumulation.

The lungs appear especially vulnerable to drug-induced phospholipidosis, possibly because macrophages are in very close proximity to blood-borne agents. Phospholipidosis is also more clearly visible microscopically in alveoli whereas it can be easily overlooked in other organs. The continuous uptake of phospholipid-rich surfactant material from the alveoli by macrophages leads to excessive accumulation of phospholipids when their catabolism is impaired.[164,165] The fact that lungs are commonly affected is a potentially useful diagnostic feature because in many organs phospholipidosis can be extremely difficult to recognize in haematoxylin and eosin stained sections. Although the changes in the lungs are not specific for drug-induced phospholipidosis, an increase in the number of lipid-containing lung macrophages in treated animals compared with controls is relatively easy to detect and provides a simple way for the pathologist to screen for this effect.

In severe generalized phospholipidosis in rats, the lungs show irregular pale grey or yellowish patches of discoloration of the pleura and parenchyma. This is a result of patchy or confluent aggregates of large, pale, foamy macrophages. They may be free lying or packed in alveoli and accompanied by granular, extracellular material. Their abundant cytoplasm shows a vacuolated appearance in which fine eosinophilic granules are sometimes visible. The nuclei are rounded and centrally located structures of variable size (Figure 6.4). Multi-laminated cells are also occasionally seen, as are vacuolated cells firmly attached to alveolar walls, probably pneumocytes. These foamy cells stain typically for phospholipids (e.g. acid haematin), although neutral lipids may also be present and stain with oil red O.

Semi-thin plastic-embedded sections stained with toluidine blue allow better characterization of phospholipidosis in all organs, including the lungs. The macrophages in the air spaces contain unmistakable dense, dark round cytoplasmic inclusions of variable size, some over 5 mm diameter.[174] Plastic-embedded sections also show the inclusions in other pulmonary cells including pneumocytes attached to the alveolar walls, from which they can be seen discharging into the alveolar spaces.

As in other organs affected by phospholipidosis, ultrastructural examination reveals dense, multi-lamellar membranes and numerous heterogeneous dense bodies of lysosomal origin (Figure 6.4). These bodies need to be distinguished

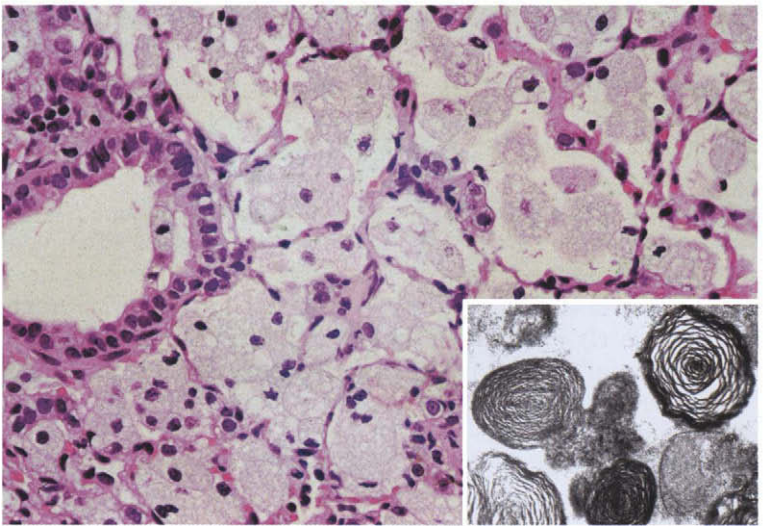

Figure 6.4 Lung from a Wistar rat given tamoxifen for over one year. The alveoli contain large macrophages with pale granular cytoplasm typical of phospholipidosis. There is no evidence of inflammation or parenchymal damage (H&E ×250). *Inset:* Electron micrograph showing typical phospholipid lamella inclusions

from membranous bodies that form as a result of fixation for ultrastructural study. Lipids tend to leach out and become hydrated to form myelinoid membranes during glutaraldehyde fixation. These structures are subsequently fixed by osmium to give rise to electron-dense membranous figures both outside and inside cells, particularly in mitochondria where they may be mistaken for pathological lesions.[175]

The lamella patterns seen in phospholipidosis may be simple alternating dense and clear lines spaced at 4–5 nm, or more complex arrangements of clear and dense lines. The other typical crystalloid inclusions of hexagonal aggregates of tubular subunits seen in other organs are not usually found in the lungs. The significance of these various forms is uncertain but they probably represent the various phases in which phospholipids exist and are influenced by proportions of lipids present. Electron microscopic examination reveals that not only are pulmonary macrophages affected by these changes but that inclusions may be present in pneumocytes types I and II, pulmonary capillary endothelial cells, smooth muscle cells, bronchiolar epithelium and occasionally neutrophils.[176–178] The changes are typically still visible several weeks after withdrawal from treatment with the offending agent.

Although the extent of pulmonary phospholipidosis in the lungs varies between dosage regimen and animal species, studies with chlorphentermine, 4,4′-diethylaminoethoxyhexestrol and amiodarone indicate that similar cytological and ultrastructural changes occur in most laboratory animal species studied including rats, mice, hamsters, guinea pigs, rabbits and dog.[176,177,179,180]

Safety assessment – amphiphilic drugs

What are the implications for humans of drugs that induce phospholipidosis in laboratory animals? Novel compounds continue to be found that possess the property of producing phospholipidosis in laboratory animals with varying degrees of severity.[181,182] Although not all drugs that produce phospholipidosis in animals have been studied in humans, only very few drugs that produce phospholipidosis in animals have been shown capable of inducing significant phospholipidosis in human clinical practice.[2] Agents such as chloroquine, 4,4'-diethylamindethoyhexestrol and amiodarone, which have been shown to produce phospholipidosis in patients, can also induce cellular damage in the same organs. However the phenomenon of phospholipidosis where phospholipids are packaged behind lysosomal membranes may not be causally related to cellular damage in humans. Indeed the weight of evidence suggests that drug-induced phospholipidosis *per se* is an adaptive phenomenon and does not in itself have functional or deleterious consequences unless excessive.[183] Hence, the finding of phospholipidosis in animal studies with a novel drug requires careful assessment on a case by case basis with respect to its implications for the safety of humans.

An example of this issue is the iodinated benzofuran derivative amiodarone, a potent antiarrhythmic drug effective against ventricular arrhythmia. Lung toxicity continues to be a problem in patients treated for cardiac arrhythmias with this drug.[184] Not only does phospholipidosis occur in a wide variety of organs in laboratory animals treated with amiodarone,[180,185] but also in liver, peripheral nerve cells, skin, lymphoid cells and lungs in patients at therapeutic doses.[177,186,187] Although pulmonary interstitial fibrosis occurs in association with phospholipidosis in patients, amiodarone-induced phospholipidosis in rodents is not associated with pulmonary fibrosis or significant functional alterations. Several theories have been proposed for the pulmonary alveolitis and interstitial fibrosis in humans. The weight of evidence to date suggests that the accumulation of lipid-laden histiocytes is *not* causally related to the alveolitis or pulmonary fibrosis.[188] Indeed, overall there is little evidence that the mere presence of phospholipidosis is deleterious to the organism.[183] Cytotoxicity, possibly through the metabolite desethylamiodarone, has been proposed and an immune-mediated mechanism has been postulated, possibly favoured by the binding of drug to components of pulmonary tissue.[187] It might also involve free radical formation or indirect influences on inflammatory mechanisms.[184] It is also possible that pulmonary disease results from an interaction of several mechanisms and metabolic factors unique to particular patients.[188]

Despite undoubted differences in tissue and species sensitivity to development of phospholipidosis, dose, drug disposition, metabolism and elimination and the degree of tissue exposure to drug are important considerations in safety assessment of drugs that produce phospholipidosis in laboratory animals. Although phospholipidosis is more likely to occur at high doses employed in toxicity studies than at lower therapeutic doses used in patients, it has been

suggested that this may be offset by faster elimination of the drug, characteristic of small laboratory animals.[164] The potential for drugs to accumulate in critical tissues such as eye and heart is especially important when drugs are administered for long periods of time, particularly as tissue/plasma ratios of some amphiphilic drug may exceed 100, following repeated administration.[189] Consequently, although phospholipidosis may not have functional consequences, any implications for humans of drugs that induce phospholipidosis in laboratory animals can only be assessed on a case by case basis, with due consideration of mechanism, drug disposition and clinical risk–benefit analysis.

Safety assessment – other drugs

It is important to underline that similar morphological changes due to the increased presence of phospholipids in lysosomes can also result from treatment with compounds that are *not* cationic amphiphilic structures. Mechanisms include direct or indirect inhibition of lysosomal enzyme activity. This reenforces the need to understand the mechanism of any chemically induced increase of phospholipids in the lungs of laboratory animals.

For example, it has been shown that when glycosaminoglycans accumulate in inherited human lysosomal disorders they inhibit other lysosomal enzymes, thereby inducing lysosomal phospholipid inclusions.[190] This is reflected by administration of high doses of the trypanocidal drug suramin to rats which induces intracellular storage of glycosaminoglycans associated with phospholipid inclusions in diverse organs including lungs.[191] Although at light microscopy clear vacuoles are typically seen, electron microscopic examination shows the presence of both clear vacuoles containing glycosaminoglycans and lamellar phospholipid inclusions. A similar effect seems to have been produced in rats by Elmiron®, a semi-synthetic heparin-like macromolecular carbohydrate derivative, chemically and structurally similar to glycosaminoglycans used clinically for anticoagulant effects and interstitial nephritis.[192]

Another example is the induction of lysosomal inclusions in the lungs of rat and dogs by the macrolide antibiotic erythromycin.[193] Collections of foam cells were described in the lungs and lymphatic tissues of dogs and rats treated with high oral doses. Foam cells in the lung showed a pattern of small whorls in vacuoles similar to that seen with other drugs that induce phospholipidosis. *In vitro* studies have suggested all macrolide antibiotics have the potential to cause phospholipidosis. Biochemical studies suggest that drug binds to phosphatidylinositol-containing liposomes and inhibits activity of lysosomal phospholipase in close correlation with the number of cationic groups carried by each of the drugs.[194]

Hook reviewed other agents, such as oxidant gases and insoluble particles including silica, that can also increase phospholipid levels and histological appearances of phospholipidosis in the lungs.[195] Some of these agents inhibit phospholipid catabolism in the lungs giving rise to accumulation of surfactant protein A and surfactant lipoproteins and a clinico-pathological picture similar to *pulmonary alveolar proteinosis* in humans. Studies from humans have

shown that three clinically distinct forms of this condition occur: *congenital*, *secondary* or *acquired*. The congenital disease is caused by a diverse range of mutations in the genes encoding surfactant proteins or the β_c chain of the receptor of granulocyte-monocyte colony stimulating factor (GM-CSF). The secondary form occurs in association with conditions where there is functional impairment or reduced numbers of alveolar macrophages, such as in haematological cancers, following immune suppression or inhalation of silica or toxic fumes. Acquired or idiopathic alveolar proteinosis that accounts for over 90% of all cases (0.37 per 100000 persons) has been an enigma until recently. Patients are at risk from infections, particularly *nocardia*, and the 5 year survival rate appears to be about 75%. Studies from transgenic mouse models and in humans have shown that autoantibodies against GM-CSF are important in the development of the acquired form of the disease as this antibody causes a defect in macrophages function which impairs the catabolism of surfactant lipids and proteins.[196]

In this context it is of interest to note that treatment with imatinib, a tyrosine kinase inhibitor of the Bcr-Abl tyrosine kinase constitutively expressed in Philadelphia chromosome positive myeloid leukemia, has been associated with the accumulation of lamellar inclusions in pulmonary macrophages in a leukaemia patient, although this might have been a result of the primary disease.[197] Studies in mice have suggested that imatinib mesylate actually inhibits the fibrogenic activity of transforming growth factor β and prevents fibrosis induced by bleomycin.[198]

Hyperplasia

Various forms of hyperplasia are found in the airways and lungs of laboratory animals. The mucosal surface of the bronchi may show hyperplasia of the goblet cells, squamous hyperplasia or metaplasia. The cells lining the terminal bronchiole and alveolus may also show hyperplasia and squamous metaplasia. Standard classifications for the characterization of these changes in histological sections have been developed for use in rodent studies.[62–64]

Goblet cell hyperplasia, goblet cell metaplasia (mucous cell hyperplasia)

Goblet cell hyperplasia is a well-recognized response of the mucosa of conducting airways to chronic inflammation and inhalation of irritant substances such as cigarette smoke and sulphur dioxide.[64,73,199,200] The degree of goblet cell hyperplasia is dictated by the severity and duration of the irritation or inflammatory process. Florid cases of goblet cell hyperplasia are characterized by thickening and pseudostratification of the tracheal or bronchial mucosa by a population of tall, mucus secreting cells with abundant pale cytoplasm. In addition, goblet cells extend further down the airways than in normal animals and mucus may fill or distend the airways or impact in the alveoli. In less florid cases, a simple increase in the number of goblet cells may be found

without other structural change.[73] Goblet cell hyperplasia of the lining epithelium may be accompanied by an increase in size of the underlying submucosal glands. This has clearly been demonstrated in patients with chronic bronchitis and in rats where submucosal glands are normally quite prominent.[199,201] Species differences may exist because the airways of laboratory animals are variably endowed with goblet cells and submucosal mucous glands. The normal rat has more goblet cells lining the airways than either mouse or hamster.[199]

The factors controlling these alterations are uncertain but is has been long suggested that increased mitotic activity as well as cell conversion, probably by metaplasia of serous or Clara cells to mucous cells, is involved.[202] It has more recently been shown in mice sensitized to ovalbumin and subject to a single antigen challenge by aerosol that Clara cells in the proximal airways show great plasticity and become mucin-secreting cells.[86]

Pharmacological agents can induce goblet or mucous cell hyperplasia. Rats given six or 12 daily injections of isoprenaline, a non-selective β receptor agonist showed a dose- and time-dependent increase in the number and size of alcian blue-positive goblet (mucous) cells as well as serous cells in the tracheal and bronchial mucosa. This was associated with an increase in length, width and depth of submucosal glands.[203] Similar changes were produced by pilocarpine, although both alcian blue- and PAS-positive cells were increased in number following this agent, suggesting that pilocarpine induced both acid and neutral glycoprotein secretion. Comparison of the distribution of these changes in the rat following isoprenaline, with those of salbutamol, pilocarpine and tobacco smoke, showed that there were regional differences in the distribution of these changes in the airways.[204] Isoprenaline produced a greater increase in secretory cells in peripheral airways than tobacco smoke, which itself produces a greater increase in mitotic activity. Isoprenaline and pilocarpine produced a more diffuse change than the more selective β agonist, salbutamol. The changes induced by these therapeutic agents are presumably the result of their pharmacological activity.[204] Sturgess and Reid showed that the changes in the rat were accompanied by hypertrophy of the pancreas, submaxillary and parotid salivary glands[203] (see Digestive System, Chapter 8).

Unlike the rat and mouse, the hamster appears predisposed to develop minor multifocal epithelial hyperplasia of the tracheal and bronchial mucosa spontaneously with advancing age. These changes are flat or polypoid in nature and are composed of clear cells and goblet cells.[68,69]

Squamous hyperplasia, squamous metaplasia

The epithelium of the bronchi shows squamous metaplasia in response to chronic irritation or injury. It is characterized by three or more layers of epithelial cells with abundant eosinophilic cytoplasm with prominent cell boundaries. It may be associated with degenerative alterations to the mucosa or goblet cell hyperplasia. Squamous metaplasia can also develop in the alveolar parenchyma as a response to prolonged damage such as produced by large burden of

inhaled irritant or insoluble dusts. The metaplasia is also characterized by the presence of several layers of flattened epithelial cells showing squamous differentiation. The term *pulmonary keratinizing cyst* has been recommended for pulmonary cystic lesions lined by non-neoplastic squamous epithelium without excessive proliferative change.[205]

Hyperplasia, bronchiolo-alveolar (type II cell hyperplasia)

Hyperplasia may involve the lining epithelium of the alveoli or bronchioli. This form of hyperplasia has been termed *alveolar hyperplasia*, *adenomatosis*, *alveolar bronchiolization* or *epithelialization*. It occurs spontaneously but can be induced by infections and administration of irritant xenobiotics in rats,[47,206–208] mice[64,209] and hamsters.[210]

Histologically, the lesions consist of localized but unencapsulated foci of hyperchromatic regular, cuboidal or columnar cells investing airspaces without appreciable distortion of alveolar walls.

Neuroendocrine hyperplasia

Neuroendocrine hyperplasia is well described in hamsters. Although small aggregates of neuroendocrine cells (neuroepithelial bodies) are found at various levels of the bronchi and bronchioli in normal hamsters, administration of nitrosamines and 4-nitroquinoline 1-oxide produces neuroendocrine hyperplasia.[211–213] Hyperplastic lesions are recognizable as groups of non-ciliated cuboidal, oval or columnar cells located in the bronchial or bronchiolar epithelium. They contain argyrophilic granules that show immunoreactivity for corticotrophin (ACTH) and neurone-specific enolase. Ultrastructural examination reveals the presence of dense-core cytoplasmic granules of APUD type. Proliferative changes have also been reported in other species, including rats and humans in hypoxic conditions, although it has been suggested that these changes might be a result of increased peptide content rather than cell proliferation.[90,214]

Neoplasia

The most frequently diagnosed neoplasm world wide is lung cancer, where it is usually caused by smoking tobacco.[215] Bronchogenic squamous carcinoma is generally the most common subtype but in North America the incidence of adenocarcinoma now exceeds that of squamous cell tumours for reasons not fully understood. Some of the most aggressive subtypes are small and large cell neuroendocrine lung cancers, defined as small or large tumour cells with greater than ten mitoses per $2\,mm^2$.[216] These seem to be seen almost exclusively in heavy cigarette smokers.

In contrast to findings in people, squamous cell lung tumours are only occasionally seen arising spontaneously in laboratory animals. Even laboratory animals – rats, mice, hamster, monkeys and dogs – exposed to tobacco

smoke for long periods and at high doses fail to develop an increase in lung tumours.[217,218] Moreover, there appears to be no good experimental model for neuroendocrine lung cancer. Thus, particular caution is merited if using animal models for prediction of lung tumorigenic potential of inhaled substances.

By far the most common primary pulmonary neoplasms found in laboratory rats, mice and hamsters are adenomas and adenocarcinomas. These appear to develop from the bronchiolar or alveolar epithelium, although their precise histogenesis is somewhat disputed. Although spontaneous squamous neoplasms are uncommon in rodents cystic keratinizing lesions can be induced in rats by high burdens of particulate material in the lungs.[205] Pleural mesotheliomas and mesenchymal neoplasms also occur in these species but are uncommon. They can be induced in rodents by mineral fibres.[219] Mesenchymal tumours have similar histological features to those in soft tissues and mesotheliomas may show either epithelial or mesenchymal differentiation or both.

Rat

In most rat strains alveolar or bronchiolar neoplasms occur spontaneously in relatively small numbers, but morphologically identical neoplasia can be induced by administration of chemical carcinogens.[220] The most common are classified as bronchiolo-alveolar adenoma (pulmonary adenoma) and bronchiolo-alveolar carcinoma. The National Toxicology Program database on control Fischer 344 rats used in carcinogenicity studies indicates an overall percentage of less than 3% of animals with bronchiolo-alveolar adenomas and less than 1% with bronchiolo-alveolar carcinomas.[66] However, the range of bronchiolo-alveolar adenomas in different studies was between 0 and 14% in this series.

Histologically, bronchiolo-alveolar tumours are mostly small, discrete, rounded nodules located in the lung parenchyma and composed of fairly uniform cells with moderately hyperchromatic nuclei arranged in solid (alveolar), tubular, papillary or mixed growth patterns. They usually compress surrounding tissues without infiltration or metastatic spread (adenoma), although loss of differentiation, infiltration and spread to adjacent tissues can occur (adenocarcinoma). Ultrastructural study of bronchiolar-alveolar neoplasia in Fischer 344 rats has shown the presence of osmiophilic, lamellated inclusion bodies similar to those found in alveolar type II cells. Therefore it has been suggested that the neoplasms are derived from this cell type.[220]

Pulmonary squamous carcinoma occurs but is a very uncommon spontaneous neoplasm in the rat.[62] The large proliferative but benign cystic lesions found in the lungs of rats following accumulation of large amounts of particulate matter have been termed *pulmonary cystic keratinizing epitheliomas* for they have been regarded as benign neoplasms. When these lesions show evidence of tissue invasion they are regarded as *pulmonary squamous cell carcinomas*. Similar lesions are very occasionally reported as spontaneous lesions.[221]

Mice

Analogous neoplasms are found more commonly in most strains of laboratory mice used in carcinogenicity bioassays although considerable variation in incidence is reported. They are common in strain A mice where they are observed in low frequency at 3–4 months of age and incidences reach nearly 100% by 24 months of age.[222] Fewer, but significant numbers are found in $B6C3F_1$ mice, although there is considerable inter-laboratory variation.[223] The National Toxicology Program database on control $B6C3F_1$ mice used in carcinogenicity studies indicates an overall percentage of about 16% of males and 6% of females with bronchiolo-alveolar adenomas but only about 5% and 2.5% respectively with bronchiolo-alveolar carcinomas.[66] However, the range of bronchiolo-alveolar tumour varied considerably between studies in this series. Even in the same laboratory, mice housed under similar conditions show variation in incidence in these neoplasms with time. The incidence of lung adenomas and adenocarcinomas occurring in CD-1 mice used as controls in 18-month carcinogenicity bioassays in the same laboratory under similar conditions for a period of 3 years varied from between 19 and 36% in males and 6 to 16% in females.[224] By contrast, some strains of mice such as the C5781/10J strain show a very low predisposition to the development of lung adenomas.[225] Although these mouse pulmonary adenomas and adenocarcinomas do not resemble the common lung tumours in humans, strain differences have been exploited to study genetic susceptibility and resistance to pulmonary adenomas and carcinomas.[226,227]

Histologically, pulmonary tumours of this type in mice are generally small, sharply circumscribed nodules composed of fairly uniform, closely packed columns of cuboidal or columnar cells arranged in tubular or papillary structures with scanty fibrovascular stroma (Figure 6.5). They may be less well differentiated, with cellular pleomorphism, and show intrabronchial growth, invade lung parenchyma and produce metastatic spread.

The histogenesis of mouse pulmonary adenomas and adenocarcinomas is disputed. On the basis of sequential light and electron microscopic study of pulmonary adenomas induced in Bagg–Webster Swiss mice by transplacental exposure to ethylnitrosourea, it has been suggested that they develop from either alveolar type II cells or Clara cells.[228,229] Careful, stepwise analysis using light microscopic and electron microscopic examination has suggested that adenomas can be divided into three principal groups. Some are composed of solid growths of uniform cuboidal cells with expanding margins limited to alveolar septae (alveolar pattern). These cells contained concentrically arranged cytoplasmic lamellar bodies and abundant, large mitochondria similar to mitochondria found in alveolar type II cells. Tubular or papillary patterns are composed of cuboidal cells showing histological and ultrastructural features of Clara cell differentiation.[228]

However, immunocytochemical studies of chemically induced and spontaneous pulmonary neoplasia in B6C3F1, BALB/c or A strain mice have shown that the majority of adenocarcinomas, including those showing papillary patterns,

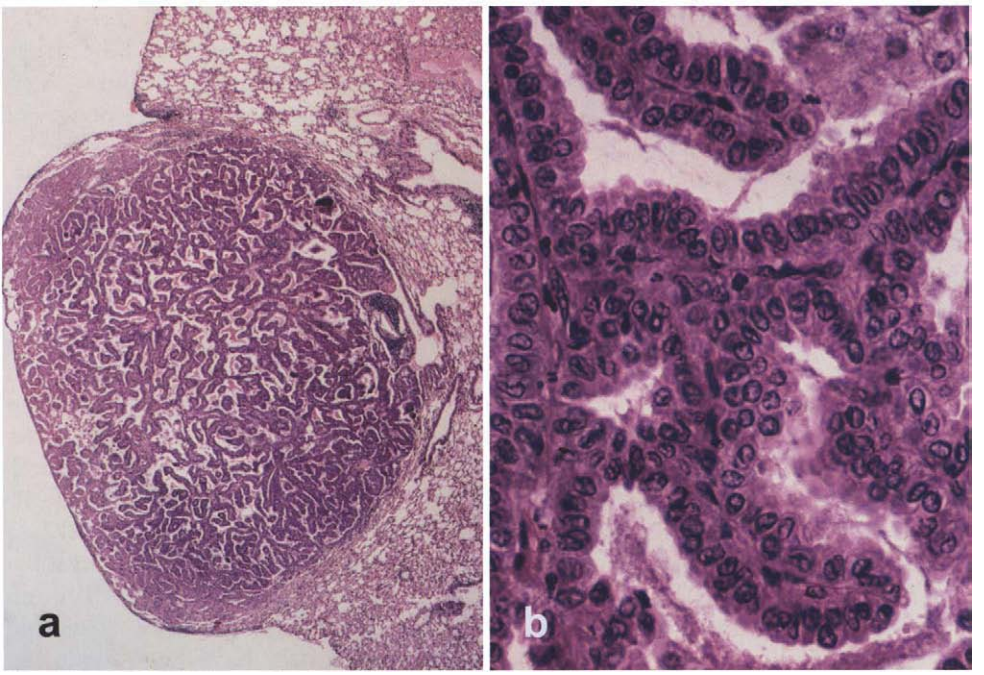

Figure 6.5 Lung from a 2 year old CD-1 mouse showing a typical rounded, well-circumscribed pulmonary adenoma. *Panel a:* Low power magnification showing uniform tubular and papillary structure (H&E ×45). *Panel b:* Higher power view shows the well-differentiated pattern of relatively uniform, low columnar or cuboidal cells (H&E ×190)

contain surfactant apoprotein, typical of type II antigens, suggesting that most neoplasms show alveolar type II differentiation.[230] However, in view of the plasticity of Clara cells, this does not exclude a Clara cell origin of the tumours. Immunocytochemistry of specific Clara cell secretory protein expression in a transgenic mouse model of lung carcinomas developing from Clara cells has shown that the protein is lost during tumour cell progression.[231] It has also been shown in strain A mice that the proportion of tumours with papillary and solid/alveolar growth patterns varies with the inducing agent.[232] This also suggests biological differences exist between histological subtypes.

Very few squamous carcinomas are reported in most series of mouse studies. A chemically induced mouse model of squamous cell carcinoma has been generated by administration of N-nitroso-tris-chloroethylurea. Strain differences in susceptibility to squamous cancer development have been demonstrated in this model, with NIH Swiss, A/J and SWR/J being highly susceptible, AKR/J and C57BL/6J being resistant and FVB/J and BALB/cJ mice showing an intermediate response to carcinogen.[233]

Safety assessment

The high incidence and the inherent variability of pulmonary adenomas and adenocarcinomas in conventional mouse carcinogenicity bioassays sometimes gives rise to statistically significant differences between control and treatment groups. There is considerable risk in over-interpretation of such group differences in conventional mouse bioassays. In the analysis of group differences, consideration needs to be given to tissue sampling procedure, age-standardization, historical control incidence, effects on food intake as well as the results of mutagenicity studies and carcinogenicity bioassays in other rodent species. Indeed, a considerable number of widely employed therapeutic agents of different classes have produced an increase in benign or malignant pulmonary tumours in carcinogenicity studies performed in mice without this proving of any significance to humans. Davies and Monro counted at least 17 drugs of this type in the 1994 Physicians' Desk Reference of the United States.[234]

For instance, in a carcinogenicity bioassay in which CF1 mice were treated for 80 weeks with the synthetic analgesic tilidine fumarate, a statistically significant difference ($p < 0.01$) was reported in the incidence of lung adenocarcinomas between the top dose female group (24%) and concurrent controls (10%).[235] It was argued that group differences did not indicate tumorigenic potential of tilidine fumarate on the basis that the incidence in the high dose group was within the historical control range (27%) and that there was no tumorigenic effect in a parallel 104 week rat carcinogenicity study.

A more difficult evaluation concerned metronidazole, a nitroimidazole which is an important therapeutic agent active against anaerobic organisms and trichomonas species. Administration of this compound led to an increased incidence of pulmonary adenomas and carcinomas in three separate mouse carcinogenicity bioassays.[236,237] The analysis of these findings was somewhat complicated by evidence that metronidazole shows mutagenic activity in bacterial assays using some strains of *Salmonella typhimurium*. It was argued that the risk to human patients was slight because the increase in prevalence in pulmonary tumours was likely to be a result of changes in nutritional status of the mice through the effect of metronidazole on gut flora, as similar differences could occur between *ad libitum* fed mice and those fed the same but restricted diet.[237] It was also postulated that the positive findings in bacterial mutagenesis assays were an inherent part of the antibacterial activity of metronidazole as a result of nitroreduction that does not occur in normal mammalian tissues. This conclusion was supported by negative effects in hamster carcinogenicity bioassays as well as lack of excess cancer risk in women followed up for 10 years or more.[237]

Strain A mouse pulmonary tumour bioassay

The common occurrence of lung adenomas in strain A mice has been utilized in the development of a quantitative bioassay for carcinogenic activity. This followed the demonstration that administration of carcinogens such as 3-methylcholanthrene to this strain could significantly increase the incidence

of pulmonary adenomas within periods of up to six months.[238] Over many years the strain A mouse pulmonary tumour assay has been used to test a large number of chemicals of different classes, including polycyclic hydrocarbons, nitrosamines, food additives, alkyl halides, metals and chemotherapeutic agents.[222,232] However, as with many test systems, correlation of results in the strain A test with 2 year carcinogenicity study data and genotoxicity results have been shown to be poor so prudence is needed in the use of this test.[239]

Hamsters

Hamsters develop lung adenomas spontaneously in small numbers with advancing age. They are composed of uniform cylindrical cells similar to those found in bronchial epithelium or goblet cells showing distinct mucus production.[68,69,240] An immunohistochemical study of similar pulmonary neoplasms induced in hamsters by N-nitrosodiethylamine showed the presence of Clara cell antigen in early phase of development, but as the tumours developed they became more squamous in type and showed immunoreactivity for cytokeratins.[210] A Clara cell origin was suggested for most of these neoplasms.

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