Experimental models: pleural diseases other than mesothelioma

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ANIMAL MODELS FOR PLEURAL DISEASES

Why we need animal models

The pleura is involved in many pulmonary and systemic disorders. As a result, pleural effusions are common clinical presentations. In vivo studies have played an invaluable part in enhancing our understanding of the etiology of various pleural diseases. While in vitro studies can provide information on isolated cell types, pleural pathologies are inevitably a result of complicated interactions between residental mesothelial cells and infiltrating (e.g., inflammatory, malignant) cells. The pleura is also under close influence of products from the systemic circulation (e.g., cytokines) that cross the vascular and mesothelial barriers. These interactions can only be adequately studied in vivo.

Animal studies are also important in evaluating the efficacy and safety of novel therapeutic modalities, information difficult to obtain from humans and in vitro experiments. For example, animal studies have assessed the efficacy and adverse effects of various pleurodesing and anti-cancer agents. Animal studies can also provide useful data on the pharmacokinetics of drug delivery into the pleural space, such as antibiotic penetration in empyema, and on physiological responses in health and disease.

The ideal animal model should accurately represent the human disease under investigation; be readily available, affordable, and easy to handle; yield reproducible results and provide adequate biological samples for analysis.

While most animal models cannot exactly emulate human disease, a good animal model has sufficient similarity to provide useful insights.

A large number of animal models have been employed in the investigation of pleural diseases. The design of meaningful in vivo experiments demands a good understanding of the advantages and limitations of the available models. This chapter outlines the species and methods used in pleural disease studies, as well as the pros and cons of specific models for different pleural diseases.

GENERAL RULES

There is no substitute for careful planning: the objectives and experimental endpoints should be clearly defined in advance. Investigators must attempt to minimize the number of animals sacrificed and the pain or distress to each animal.

Approval from local animal care committees must be obtained before experiments are performed. National and international guidelines on standards of animal use must be strictly adhered to. The Guide for the Care and Use of Laboratory Animals is one of the most commonly used reference guides. It is mandatory in many countries that researchers must attend training courses prior to using animals for experiments. The laws and regulations governing laboratory animal research can be found elsewhere.

Expert veterinarian advice is invaluable to ensure minimal animal discomfort, optimal surgical approach and efficient specimen collection.
Which animal model to use

CHOICE OF ANIMAL SPECIES

... it is proper to choose certain animals which offer favorable anatomical arrangements or special susceptibility to certain influences. ... the proper choice of animals is so important that the solution of a physiological or pathological problem often depends solely on the appropriate choice of the animal for the experiment so as to make the result clear and searching.

Claude Bernard (1813–1878) – for many the founder of experimental medicine – so stated in 1865.4

Many animal species are used in pleural disease investigations. Rabbits, mice, rats and sheep are the most common. Several factors govern the choice of species for experimentation, including size of animals, their anatomy, costs, availability of reagents suitable for the species, possibility of genetic manipulation, etc.

Smaller animals (e.g. mice) cost less and are easier to handle. Small size, however, makes intrapleural injections difficult and provides a limited amount of biological material for examination. Larger animals are costly, but provide larger sample quantities.

Investigators must realize two important anatomical characteristics of the species they use. First, many animals (e.g. mice, dogs), have incomplete mediastina and the two pleural cavities communicate freely, prohibiting the use of the contralateral pleura as a control. Second, larger animals (e.g. sheep) have a thick visceral pleura resembling that of humans, whereas smaller animals (e.g. rabbits, mice) have a thin visceral membrane. This difference bears implications on fluid and particulate transport across the pleura.

The choice of species may also be dictated by the availability of reagents to process the experimental samples. Commercial enzyme-linked immunosorbent assays (ELISA) are commonly used for measurement of cytokines, but are usually available only against humans and mice.

The choice of species for experimentation is often influenced by the knowledge of the genomic sequence of the species and by the availability of research tools to manipulate gene expression in animals. The mouse (Mus musculus) has emerged as the most popular choice and genetically engineered mice are increasingly used in pleural disease investigations, providing valuable insights into the molecular pathogenesis of pleural diseases. Murine models have several advantages: (i) The mouse genome shares sufficient homology with that of humans; (ii) a wide array of genetically engineered mice are available; (iii) the large litter size of Mus musculus makes breeding timelier and easier; (iv) high-throughput genotyping methods (e.g. using genomic DNA from tail fragments) have been developed for the mouse; (v) many inbred strains of Mus musculus have been isolated over the years. Inbred animals share a great degree of genetic identity, reducing experimental variability. Moreover, differences between inbred mouse strains can be exploited to discover mechanisms of disease.

The use of novel molecular biology technologies in the mouse has greatly enhanced research into the pathogenesis of pleural diseases. The pleura has been used for overexpression of gene products using various vectors.9,10 Several constitutive and conditional gene knockout and knockin mice have been used in pleural disease investigations,11–13 and the development of systems that facilitate conditional gene overexpression or silencing in the adult mouse, such as the tetracycline on-off models, is expected to greatly enhance research in this area.14,15 Recently, methods to silence gene expression in vivo using RNA interference were introduced, which are anticipated to further boost pleural disease investigations.16,17

ACCESS TO PLEURAL SPACE

The next question is how to deliver the experimental agent to the pleural space. Direct intrapleural injection with a fine needle is the least invasive; a small injection volume is adequate. Using Tc-99 labeled fluid, it has been shown that an injection volume as low as 0.5 mL is enough to allow the injectate to be distributed throughout the whole pleural surface of rabbits. Rotation of the animals is unnecessary for distribution of the injectate throughout the pleural space.18

Alternatively, small plastic 'chest tubes' can be inserted into the pleural space (see Appendix 14.1). This has the advantage of allowing repeat intrapleural administration of reagents, pleural fluid sampling or lavage of the pleural cavity. This provides longitudinal data on the biological changes within the pleural space, helping reduce the number of animals required in time-course studies. Chest tubes can induce mild inflammation,19 which in our experience is insignificant. In studies of pleurodesis, the insertion of chest tubes more closely resembles the procedure performed in clinical practice.

Thoracotomy20 and thoracoscopy21 have also been used to deliver material into the pleural space, but are more invasive. Systemic delivery of substances can be achieved by intravenous injections. For repeated blood sampling, central venous access can be established.

EXPERIMENTAL END-POINTS

Several parameters are commonly used as endpoints in animal pleural studies. In lethal models, survival is the definitive endpoint. Pleural tissues can be collected at autopsy for macroscopic and/or microscopic examination and semi-quantitative assessment for inflammatory or malignant changes. For pleural fibrosis studies, the pleura can be macroscopically graded for adhesions (see Table 14.1), which correlates well with histological measurements of collagen deposition and pleural thickening.22
The rabbit model is most commonly used in pleurodesis studies, though mice, sheep (see Appendix 14.2), rats, dogs and pigs have been employed. The results of common pleurodesing agents applied to different species appear similar; hence the choice of species depends mainly on experience and cost.

With New Zealand white rabbits, the pleurodesing agent is injected either directly or via a chest tube, the method of delivery having little effect on the outcome. Likewise, the pH of the pleurodesing agent does not affect its effectiveness. Conventional agents, e.g., talc, doxycycline and bleomycin, induce acute pleural inflammation and denudement of mesothelial cells. Inflammation may resolve (failed pleurodesis) or, if sufficiently intense, will persist and lead to collagen deposition, fibrosis and symphysis. Pleurodesis is usually evident 14 to 28 days after administration of the pleurodesing agent. However, with pro-fibrotic transforming growth factor beta (TGF-β), significant adhesions can be seen as soon as after 24 hours.

Pleural fibrosis can be graded macroscopically at autopsy (Table 14.1). In addition, pleural thickening and collagen deposition can be measured microscopically using multiple samples from different lung regions to avoid sampling bias, and the contralateral pleural space as the control. In our experience, chest tube insertion and saline or albumin injections do not result in significant adhesions. Hemothoraces, however, either from trauma or the experimental agent, can induce adhesions. Recently, a pleurodesis grading system based on transthoracic ultrasound findings, specifically the disappearance of the normal pleural gliding sign, was developed in rabbits and was validated against pleurodesis grading at autopsy (Table 14.2). Whether this system is applicable to other species, including humans, remains to be tested.

One concern of the published pleurodesis studies is that they were performed in animals with normal pleura, whereas human pleurodesis is usually applied to patients with abnormal pleura (especially malignant pleural metastases). However, pleurodesis results from animal models are similar to those from clinical investigations. For example, talc was effective in producing pleurodesis.

### Table 14.1 Pleurodesis grading scheme

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
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<tbody>
<tr>
<td>0</td>
<td>Definitely present</td>
</tr>
<tr>
<td>1</td>
<td>Questionable</td>
</tr>
<tr>
<td>2</td>
<td>Definitely absent</td>
</tr>
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</table>

Score 0–2 is determined at three sites as far away from the diaphragm as possible: midclavicular line (anterior chest wall); midaxillary line (lateral chest wall); midscapular line (posterior chest wall). Total score = sum of scores for each of the three sites. Range = 0–6 (Score 0 represents a normal appearing pleura, while score 6 represents greater likelihood of effective pleurodesis, e.g., definitely absent pleural gliding sign at all three sites tested).

### Specific pleural disease models

#### Models for Pleural Fibrosis/Pleurodesis

- The study of pleural fibrosis (pleurodesis), frequently employed to treat recurrent pleural effusions and pneumothoraces, represents one of the most common uses of animal studies in pleural disease research. There is active ongoing research for better pleurodesing agents, as currently available compounds either have suboptimal efficacy or carry significant adverse effects (see Chapter 46, Pleurodesis).
and was significantly more so than bleomycin in both rabbit\textsuperscript{18} and sheep pleuridosis studies\textsuperscript{59} – the same as in randomized clinical trials.\textsuperscript{40}

There are no available models for pleuridosis in malignant pleural effusions. Most models for malignant effusions are created in mice (see below), which are too small for adequate assessment of pleuridosis. On the other hand, attempts have been made to mimic the setting of pleuridosis for pneumothorax, where chemical pleuridosis remained effective despite the presence of air in the pleural space or active air leaks.\textsuperscript{38,41}

**MODELS FOR MALIGNANT PLEURAL EFFUSION**

Malignant pleural effusions, most commonly resulting from adenocarcinomas of the lung and breast, affect about 200,000 patients each year in the USA alone, causing significant morbidity and mortality.\textsuperscript{42-45} The pathogenesis of malignant effusions and their best management are still unclear. Animal studies can shed light on the mechanisms of effusion formation, as in recent studies on vascular endothelial growth factor (VEGF),\textsuperscript{46-48} and on the proinflammatory axes of interleukin (IL)-6/Stat3\textsuperscript{49} and of tumor necrosis factor (TNF)-α/nuclear factor (NF)-κB.\textsuperscript{50}

Most animal studies on malignant pleural effusions have been performed using mice. Athymic nude mice are commonly used as they allow the development of pleural metastases by xenogenic (e.g., human) tumor cells. Various human cancer cell lines have been successfully introduced into the pleural space of immunodeficient mice, which give rise to malignant effusions.\textsuperscript{47,48} Among the different tumor cell types used, adenocarcinomas produce the highest rate of effusions, similar to clinical presentations where adenocarcinomas tend to produce pleural metastases.\textsuperscript{51} The shortcoming of these models is the need for immunodeficient (e.g., severe combined immunodeficient [SCID] or recombination activation gene-RAG-2 null) mice, which have impaired immunogenic responses to malignancies.

Statopoulos et al.\textsuperscript{50} recently described a murine model of adenocarcinoma-induced malignant effusion in immunocompetent mice, which will allow better elucidation of the mechanism of malignant effusion formation and its treatment.

Malignant cells can be implanted into the pleural space directly (by surgery or intrapleural injection) or indirectly from metastases from tumors deposited in the lungs. Orthotopic implantation of freshly isolated human adenocarcinoma tissues into nude mice can produce a high takeup rate of the cancer\textsuperscript{52} but requires thoracotomy and tying of tumor tissues into the visceral and parietal pleura. This method is more invasive and does not provide additional information compared with intrapleural or intravenous injection of tumor cells. In the latter cases, commercially available cancer cell lines grown in cell culture conditions are titrated before injection to standardize the tumor load per mouse. Intravenous injection of human adenocarcinoma (e.g., A549) cells produces numerous lung lesions, pleural metastases and effusions, in contrast with human squamous or large cell carcinoma cell lines, where malignant effusions are uncommon.\textsuperscript{47,51} Transfection of antisense VEGF gene reduced VEGF expression, tumor vascularity, pleural metastases and effusions induced by adenocarcinoma cells.\textsuperscript{67}

Tumors can also be injected directly into the pleural space, which is best performed by a left lateral subdiaphragmatic approach aimed cephalically. Extra-fine needles (28G) should be used. Up to 1.0 mL of fluid can be injected into an adult mouse, but small volumes (e.g., 50–100 μL) usually suffice. Accurate intrathoracic delivery is confirmed by transient chest expansion and dyspnea. Alternatively, a small skin incision (~5 mm) can be made, preferably on the left hemithorax, and tumor cells can be injected into the pleural space under direct vision. This method requires minimal surgery and has the advantages of on-site confirmation of orthotopic tumor cell delivery to the pleura and is reliably reproducible.\textsuperscript{50}

Intrapleural injections of tumor cells (usually 10^6–10^8 per mouse) result in local implantations in the chest wall, mediastinum, lungs and diaphragm in 1–2 weeks (Figure 14.1a). Pleural effusions (usually bloody exudates) develop approximately 2–3 weeks after inoculation.\textsuperscript{50,51} At later stages, ascites may accumulate. Where immunocompetent mice are used with syngeneic tumor cells (e.g., C57BL/6 mice – Lewis lung adenocarcinoma cells), the effusions are rich in inflammatory cells (Figure 14.1b).\textsuperscript{50} Most mice eventually develop respiratory distress and weight loss, and die from local effects of the effusion, cachexia, and distant metastases.

Weight loss and survival are commonly used endpoints. The volume of pleural effusion, tumor load (number, size and weight), and the presence of distant metastases are other parameters useful for assessing therapeutic response. Pleural fluid and tissue collected allow the study of pathological mediators. Effusion-associated vascular permeability can be easily determined by intravenous injection of an albumin-binding dye (see 'Models for the study of pleural vascular permeability' and Figure 14.1c).\textsuperscript{50}

Using these models, the role of important mediators and biological pathways (e.g., VEGF/VEGFR, IL-6/Stat3 and TNF-α/NF-κB) in effusion formation have been uncovered.\textsuperscript{49,50} Other studies have assessed novel anticancer therapies, such as IL-12 and IL-15,\textsuperscript{53} and inhibitors of topoisomerase II\textsuperscript{41} and of VEGF receptor tyrosine kinase.\textsuperscript{48} The intrapleural injection model has also been successfully applied to transgenic mice, such as nitric oxide synthase knockouts.\textsuperscript{11}

**MODELS FOR PLEURAL INFLAMMATION**

The pleural cavity is regarded by some as 'the ideal site for the induction of inflammatory reactions'.\textsuperscript{54} In the clinical setting, inflammation is often assessed histologically which is subject to sampling error and can only provide...
qualitative rather than quantitative data. Also, if inflammatory mediators are to be investigated, they have to be extracted from histology tissues. In vitro studies of inflammatory cells fail to provide knowledge on the complex interactions between various cells and mediators. Therefore, investigators often employ animal models of pleural inflammation that allow the study of cells and fluids accumulated during inflammation.55

Pleural models of inflammation offer several advantages. The pleural cavity provides a confined compartment lined by mesothelial cells in close contact with the systemic circulation where inflammatory cells and mediators collect. These can be monitored in a dynamic fashion by assaying the pleural fluid. The histological changes of inflammation can be assessed in pleural tissues and effects of pro- or anti-inflammatory agents can be investigated following their intrapleural administration.

Various methods have been used to induce pleural inflammation (Table 14.3), and each yields an inflammatory reaction characterized by a distinct profile of cell and mediator accumulation. Depending on the predominant inflammatory cell type recruited to the pleural cavity, the various animal models have been coined models of neutrophilic, mononuclear or eosinophilic pleural inflammation. For example, intrapleural lipopolysaccharide (LPS) results in mainly neutrophilic, but also, to a lesser extent, mononuclear, and eosinophilic pleural inflammation.56-58

The most widely used pleural inflammation model is the carrageenan pleurisy model.55 Carrageenan can be administered intrapleurally by a needle injection, causing dose-dependent inflammation.55 The detailed onset, progression and resolution of carrageenan pleurisy have been extensively studied and validated.59 In brief, pleural exudation begins within an hour of injection, and is characterized by neutrophil followed by mononuclear influx, followed by vascular hyperpermeability and pleural exudation.55 Pleural fluids and serum can be serially collected for analysis, and pleural tissues can be obtained at necropsy. Numerous studies have utilized this model successfully in mice, rats and rabbits.60 For example, mice that do not express IL-61 or nitric oxide synthase61 exhibit significantly reduced pleural inflammation, confirming the
Table 14.3 A large variety of agents have been used to induce pleural inflammation. This table outlines the common and some of the uncommon agents used. Interested readers can refer to the references for individual agents for details.

<table>
<thead>
<tr>
<th>Agent to induce pleural inflammation</th>
<th>Representative reference</th>
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<tbody>
<tr>
<td>Most common</td>
<td>Bilven et al.55</td>
</tr>
<tr>
<td>Carrageenan</td>
<td>Vinegar et al.58</td>
</tr>
<tr>
<td>Common</td>
<td>Broaddus et al.55</td>
</tr>
<tr>
<td>Endotoxin or lipopolysaccharide</td>
<td>Fukumoto et al.66</td>
</tr>
<tr>
<td>Reverse Passive Arthur Reaction</td>
<td>Yamamoto et al.54</td>
</tr>
<tr>
<td>Zymosan</td>
<td>Berkenkopf et al.67</td>
</tr>
<tr>
<td>Uncommon</td>
<td>Utsunomiya et al.100</td>
</tr>
<tr>
<td>Azoics</td>
<td>Hanada et al.109</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>Saleh et al.110</td>
</tr>
<tr>
<td>Calcium pyrophosphate crystals</td>
<td>Periariini et al.111</td>
</tr>
<tr>
<td>Ionophore A23187</td>
<td>Wang et al.112</td>
</tr>
<tr>
<td>Kaolin</td>
<td>Kawamura et al.177</td>
</tr>
<tr>
<td>Phorbol myristate acetate</td>
<td>Oh-ishi et al.114</td>
</tr>
<tr>
<td>Platelet activating factor</td>
<td>Tarayre et al.175</td>
</tr>
<tr>
<td>Substance P</td>
<td>Frode-Saleh et al.178</td>
</tr>
</tbody>
</table>

essential roles of these compounds in the inflammatory process.

Using the carrageenan model in conditional macrophage-deficient mice, the important role of resident pleural macrophages in neutrophil recruitment to the inflamed pleura was elucidated.6 This process occurs via production of 15-deoxy-prostaglandin (PGI2) that induces transcription factor Nrf2, to facilitate recruitment of neutrophils to the pleura, to switch off neutrophilic and switch on monocytic inflammation.65

Several authors have injected LPS into the pleural cavity of rats and mice to generate inflammation; LPS induces influx of neutrophils within 4 hours, followed by monocytes, lymphocytes and eosinophils.56-58 These studies have yielded interesting results on the differential role of the various selectins (L-, P- and E-) and of IL-8 in inflammatory neutrophil and eosinophil recruitment to the pleura.64-66

The reverse passive Arthus reaction has also been studied, particularly in rats.54 Intravenous bovine serum albumin (BSA), followed by an intrapleural injection of purified anti-BSA 20 minutes later, induces pleural inflammation characterized by fluid extravasation (peak at 6 hours), neutrophil (peak at 6 hours) and mononuclear (peak at 12-24 hours) influx.

MODELS FOR EOSINOPHILIC (ALLERGIC) PLEURITIS

While allergic pleuritis is uncommon in humans, the pleural cavities of rats and mice have been used as surrogate systems for the study of the mechanisms of allergic responses. The pleural responses have been extrapolated to explain the pathophysiology of other allergic diseases, such as asthma and atopic dermatitis.

In these models, mice or rats are sensitized with ovalbumin adsorbed to Al(OH)3; gel injected subcutaneously 14 days prior to the experiment. This sensitization process elicits peripheral and pleural eosinophilia. The experiment is then performed with an intrapleural injection of ovalbumin (10 μg per pleural cavity), which induces mast cell degranulation, immunoglobulin E (IgE) accumulation and eosinophil chemotaxis.59,60 The role of mediators in eosinophilic recruitment and the efficacy of anti-allergy therapies can then be evaluated.59-70 Pleural lavage can be performed to quantify eosinophil influx and allergic mediators, such as leukotrienes and platelet-activating factor. Using ovalbumin allergic pleurisy in nuclear factor of activated T cells (NFAT1)-gene-deficient mice, the role of NFAT1 in the suppression of T-helper type 2 immune responses was elucidated.71 In addition to ovalbumin, direct intrapleural delivery of biological mediators has been used to provoke eosinophilic inflammation. Intrapleural injection of chemokine ligand (CCL) 22, macrophage inflammatory protein (MIP)-1α, RANTES (regulated upon activation, normally T-cell expressed and secreted) and eotaxin resulted in dose- and time-dependent recruitment of eosinophils.72,73

Another mouse model of eosinophilic pleural inflammation exploits the pleural eosinophilia observed in patients with pneumothoraces. In this model, large numbers of eosinophils, among other inflammatory cells, are retrieved from the pleural lavage of mice after transthoracic injection.
of air.\textsuperscript{74} Using IL-5- and IL-13-gene-deficient mice, investigators revealed that pleural recruitment of eosinophils is dependent on IL-5 but not IL-13.

As described above, intrapleural injection of LPS in rats and mice results in recruitment of eosinophils, along with other cell types.\textsuperscript{56-58,84,92} Eosinophil levels rise significantly after 24 hours and their recruitment appears to be mediated by T lymphocytes.\textsuperscript{58} LPS-induced pleural eosinophilia is mediated by P-selectin and IL-8 and is inhibited by corticosteroids.\textsuperscript{56,84,92} The clinical value of this model remains unclear.

**MODELS FOR PLEURAL INFECTION BY COMMON PATHOGENS (EMPYEMA)**

Thoracic empyema remains a common disease with significant morbidity and mortality.\textsuperscript{75,76} Animal experiments on empyema are most commonly performed using New Zealand white rabbits, though mice and sheep have been used.

Developing an adequate empyema model is difficult. Direct introduction of bacteria (such as *Streptococcus* or *Peptostreptococcus* species) into the pleural space usually results in their complete clearance.\textsuperscript{77} To successfully initiate pleural infection, prior injury to the pleura may be required. However, under such situations, overwhelming sepsis and death can occur if too large a pathogen load is administered.

Sahn et al.\textsuperscript{78} used an intrapleural injection of turpentine in rabbits, resulting in inflammation and an exudative neutrophilic pleural effusion by 72 hours.\textsuperscript{79} At that point, bacteria (e.g. *Streptococcus pneumonia*,\textsuperscript{76} *Escherichia coli*, *Peptostreptococcus anaerobius*, *Bacillus fragilis*\textsuperscript{79} or their combinations) were introduced by thoracotomy into the effusion to create an empyema. Turpentine, however, may impose artifacts, and the authors reported that a high percentage of animals did not develop an exudative effusion to allow bacterial inoculation.\textsuperscript{79}

Alternatively, Sasse et al.\textsuperscript{77} showed that intrapleural injection of a potent rabbit pathogen, *Pasteurella multocida*, in brain–heart infusion agar could produce empyema without prior administration of turpentine. In this model, rabbits required daily intramuscular penicillin injections, to prevent death from sepsis. One drawback of this model is that *P. multocida* is a rabbit pathogen but rarely infects humans.\textsuperscript{80} Hence, this model is not suitable for certain investigations, such as studies of antimicrobial treatment of empyema.

These models are well validated and exhibit pleural changes similar to that of human empyemas. In the models of Sahn et al.\textsuperscript{78} and Sasse et al.\textsuperscript{77} the induced pleural fluids showed significant increases in leukocytes and inflammatory indices, as well as markedly reduced pH and glucose. Pleural adhesions and macroscopic supplicative changes were evident at necropsy.

Another interesting model of pleural infection by *Staphylococcus aureus* was developed using a common mouse strain, C57BL/6.\textsuperscript{13} Although intrapleural inoculation of the microorganism did not result in frank empyema, inflammatory cell influx and local cytokine and chemokine production was observed. This model is interesting for two reasons. First, it can be applied to genetically engineered animals. In the aforementioned study, CD4 knockout mice showed reduced inflammatory response and retarded pathogen clearance compared with wild-type mice.\textsuperscript{13} Second, *S. aureus* is also pathogenic to humans, making these data more readily applicable to humans. Other methods to introduce empyema in guinea pigs and sheep have been published, but did not gain popularity.\textsuperscript{81-83}

There is one important limitation of all these models. In humans, empyema occurs usually as a complication of pneumonia, while isolated pleural infection is uncommon. The animal models used in the literature all involve direct introduction of microbes into the pleural cavity and development of isolated pleural infection without pneumonia. Hence, the results of these experiments may not be directly extrapolated to humans.

Animal models of empyema have facilitated the study of the pathogenesis of the disease. Studies on rabbits and mice have revealed the important role of CD4+ lymphocytes in empyema-associated inflammation and bacterial clearance\textsuperscript{13} and of TGF β1 in empyema-associated pleural fibrosis.\textsuperscript{84} Other studies have provided valuable information on the optimal treatment of empyema. Animal studies have lent support to repeated early thoracentesis,\textsuperscript{85} early chest tube insertion\textsuperscript{86} and intrapleural (single or combined) fibrinolytics for empyema.\textsuperscript{79,87} Finally, the penetration of antibiotics into the pleural space has been studied using rabbit models of empyema.\textsuperscript{88-90}

**MODELS FOR PLEURAL INFECTION BY MYCOBACTERIA (TUBERCULOUS PLEURISIS)**

Tuberculosis (TB) pleuritis continues to be a common clinical challenge in the new millennium. Our understanding of its pathogenesis and best management strategies remain limited. Animal models were used to study TB pleuritis as early as 1917 when Patterson investigated the disease using guinea pigs.\textsuperscript{91} Rabbits and mice have also been used subsequently.

In the guinea pig model modified by Widstrom et al.\textsuperscript{92,93} outbred guinea pigs are first vaccinated with intradermal 0.1–0.4 mg Bacille Calmette–Guerin (BCG). A higher dose of either BCG or heat killed *Mycobacterium tuberculosis* is injected intrapleurally 10–15 weeks later using a blunt needle connected to a manometer\textsuperscript{93} or a 20G needle directed subdiaphragmatically into the pleural space.\textsuperscript{94} With prior vaccination, most guinea pigs remain clinically well after intrapleural mycobacteria injection, despite the development of TB pleuritis.\textsuperscript{92} Vaccination is successful in >90 percent of cases and can be confirmed by purified protein derivative (PPD) testing 3 weeks later. Shorter time gaps between vaccination and intrapleural infection produced inconsistent results and higher
incidence of hemothoraces.\textsuperscript{52} Antony et al.\textsuperscript{95,96} applied similar strategies on New Zealand white rabbits to induce a TB pleuritis, rabbits being relatively resistant to \textit{M. tuberculosis}, but susceptible to BCG. They also induced neutropenia in the rabbits by pretreatment with nitrogen-mustard, a modification that can allow the study of TB pleuritis in immunocompromised hosts.\textsuperscript{96}

Several important points concerning these models deserve mention. It is believed that the dose of organisms, but not their virulence, is important for short-term TB pleuritis models. In the guinea pig model, both heat-inactivated \textit{M. tuberculosis}\textsuperscript{84} and BCG\textsuperscript{92} induce TB pleuritis. Unilateral injections of mycobacteria in guinea pigs often result in bilateral pleural reactions.\textsuperscript{32} Whether this is due to incomplete mediastinal separation of the pleural cavities or represents bacterial dissemination is unknown. Nonetheless, the contralateral pleural cavity should not be used as a control.

Tuberculosis pleuritis induced using these animal models closely represents the disease in humans. Exudative pleural effusions develop following intrapleural BCG injections, characterized by early neutrophilic, intermediate monocytic and late lymphocytic infiltrates.\textsuperscript{92,93,94} Similar to humans, lymphocytes in guinea pig TB pleuritis are mainly CD4+ T-lymphocytes.\textsuperscript{94} However, while TB pleural effusions in humans are sometimes characterized by low pH and glucose, this is not the case in animal models, presumably due to lower levels of infection and inflammation. Histologically, the pleura of infected animals shows casing granulomas, multi-nucleated giant cells and late pleural fibrosis.\textsuperscript{92,94,95} Regional lymph nodes can also be affected. If live mycobacteria are used, they can be recovered from cultures of the pleural fluid or lymph nodes.\textsuperscript{92}

Smaller animals such as mice (either Balb/c or C57BL/6 strains) have been used to investigate the early inflammation accompanying TB pleuritis.\textsuperscript{87,98} Without prior vaccination, intrathoracic injection of BCG can effectively induce pleuritis while inactivated \textit{Mycobacterium leprae} (isolated from livers of armadillo) cannot.\textsuperscript{97} While the pleural fluid cellular composition in mouse TB pleurisy appeared similar to that in humans, the histological changes with this model have not been described.

Investigators should be aware of certain limitations of existing models. In these models, isolated TB pleuritis is induced by transbronchial mycobacterial inoculation, while human TB pleuritis usually develops as a result of pleural spread from adjacent lung parenchymal infection. Also, only BCG or heat-killed \textit{M. tuberculosis}, rather than live \textit{M. tuberculosis} (the most common pathogen in humans), are used in these models. Thus, it is difficult to determine if results of therapeutic interventions using these models can be directly applied to humans.\textsuperscript{95}

Despite their limitations, the guinea pig and rabbit models of tuberculous pleuritis have provided valuable insights into the pathogenesis of the disease. Using rabbits, Antony et al.\textsuperscript{96} have shown the importance of neutrophils in recruiting mononuclear cells into the TB-infected pleural space. Using guinea pigs, Allen et al.\textsuperscript{99,100} have charted the time-course of pleural fluid accumulation, leukocyte influx and mediator expression after intrapleural injection of heat-inactivated \textit{M. tuberculosis}, and reported continual increase of TGF-β1, even during the resolution phase of the pleuritis.

**MODELS FOR BENIGN ASBESTOS-INDUCED PLEURAL DISEASES**

Asbestos is a recognized cause for various pleural diseases, such as circumscribed (plaque) and diffuse pleural thickening, benign asbestos pleural effusion (BAPE), rounded atelectasis and malignant mesothelioma.\textsuperscript{101-103} Mesothelial cells appear sensitive to the toxic effects of asbestos fibers, either by direct injury or via indirect effects from other asbestos-exposed cells.\textsuperscript{104} (see Chapter 10, Pleural reaction to mineral dusts). Extensive research has been conducted in the pathogenesis and treatment for malignant mesothelioma (See Chapter 15, Experimental models: Mesothelioma), but relatively little work has been invested in the study of benign asbestos-induced pleural diseases, despite their much higher prevalence. In addition to the pleura, asbestos damages the lung parenchyma causing fibrosis (asbestosis) and increasing lung cancer risk. Experimental models for the study of these conditions are outside the scope of this chapter.

**Route of delivery**

To study the effect of asbestos on the animal pleura, fibers can be introduced either via the respiratory tract or by direct intrapleural injection.\textsuperscript{104,105} The former resembles human exposure to asbestos and fibers can be delivered by intratracheal instillation,\textsuperscript{106-109} by direct delivery to a lobar bronchus\textsuperscript{110} or by inhalation of aerosolized fibers in a closed chamber.\textsuperscript{111,112} The resulting pleural and pulmonary inflammatory and fibroproliferative reactions have a dose-response relationship with the amount of fibers delivered.\textsuperscript{111,112}

Both the inhalation and the intrapleural injection methods have drawbacks. Administered by inhalation, fiber deposition in the peripheral lung, and hence their toxic effects to the pleura, varies. In addition, pleural changes in animals may take months to years to develop, similar to humans.\textsuperscript{115,116} By contrast, direct intrapleural injection can ensure immediate delivery of a known amount of fibers into the pleural cavity and accurate identification of time zero of pleural injury. It also facilitates the study of isolated fiber effects on the pleura without the influence by other lung parenchymal changes. However, this method of delivery differs significantly from how fibers reach the pleura in humans. Wagner et al. compared intrapleural and inhalation delivery of chrysotile to rats. Mesotheliomas developed more frequently with intrapleural injections, whereas malignant lung tumors were much more common than mesotheliomas if fibers were delivered by inhalation.
Choice of species

Rats, mice and hamsters are most commonly used. Larger animals, such as rabbits, guinea pigs and dogs, have also been used, especially if direct intrapleural injection is employed. It is important to note that intra- and interspecies differences in susceptibility to asbestos-induced damage exist. The propensity of mice to develop pulmonary fibrosis in response to asbestos varies significantly among strains, in accord with fibrotic susceptibility to radiation or bleomycin. Mice of the 129 strain respond to asbestos with lower TNF-α and TGF-β expression and minimal fibroproliferative lung lesions, compared with C57BL/6 mice. Balb/c mice, another commonly used strain, have also been found to develop lung changes similar to human asbestosis after exposure to aerosolized chrysotile. Although no studies have compared murine strain susceptibility with asbestos-induced pleural fibrosis, investigators should be aware that variation is likely to exist. Interspecies comparison is an important issue, particularly regarding extrapolation of animal study results to humans. Several reviews concurred that the rat model is most appropriate for extrapolation of toxicological data to humans. Maxim and McConnell found a significant difference in relative incidence of mesotheloma and lung cancer between rats and hamsters and concluded that the rat is a better model than hamsters for human risk evaluation. They also reported that cells of humans and rodents have comparable sensitivity to asbestos exposure, in terms of cytotoxicity and production of mediators. The deposition rate of respirable fibers is lower in humans than in rats, but so is the clearance rate. Hence, humans and rats develop fibrosis at comparable normalized fiber burdens.

Fiber types

The potency of different types of asbestos fibers to induce fibrosis and malignancies differs and appears to be related to their physical properties, especially fiber length and biopersistence. Long, but not short, crocidolite fibers were able to induce fibrotic reactions in the lung and pleura. However, no fiber type should be considered harmless. Contamination of the asbestos fiber preparation with other mineral dusts is common and can make the results of studies difficult to interpret. It is therefore crucial that investigators analyze and document the physical characteristics of the asbestos preparation used in their experiments. Chrysotile, crocidolite and amosite are the commonly used fibers in experimental models. In most studies, administration of the vehicle in which the fibers were suspended served as the control. Alternatively, wollastonite, a relatively non-pathogenic calcium silicate fiber, has been used as control and is known to induce significantly less mesothelial cell damage than crocidolite. Recently, models for asbestos-induced pleural disease have been extended to investigate the effects of man-made fibers, especially fibreglass.

Endpoints

Ideally, development of the asbestos-induced pleural diseases, such as pleural fibrosis, BAPE or mesothelioma, should be the experimental endpoint. However, since the lag time for development of such disease is long, many studies focused on the more immediate/early effects of fibers upon the lung and pleura. Pleural lavage can be analyzed for mediators induced after fiber exposure of the pleura. Mesothelial cells can be harvested for assessment of proliferative responses, apoptosis or other immunohistochemical analyses.

Animal studies of asbestos exposure have been invaluable in revealing the mechanisms of asbestos-induced lung and pleural injury. After asbestos inhalation, the pleura can be assaulted via direct or indirect mechanisms. Using scanning electron microscopy, inhaled asbestos has been shown to produce cystic degradation of the pleural surface, allowing penetration of single fibers through the visceral pleura. Inhaled chrysotile fibers have been detected in pleural cells of rats by electron microscopy within varying time intervals, ranging from 1 week to 3 years after exposure.

Rodent studies have shown morphological changes in mesothelial cells within 2 hours of intratracheal amosite instillation, followed by early mesothelial proliferation and macrophage influx within 24 hours. In addition, asbestos has been shown to stimulate intracellular signaling cascades such as mitogen-activated protein kinases (MAPK) and extracellular signal-regulated kinases (ERK) in mesothelial cells. These acute changes occur in the absence of direct penetration of amosite fibers into the pleural space, supporting the view that pleural reactions result from pleural migration of mediators induced by asbestos in the Airways and lung parenchyma.

In fact, antibodies to keratinocyte growth factor (but not to platelet-derived growth factor) reduce crocidolite-induced mesothelial proliferation.

The pleural inflammatory reaction to asbestos is multifactorial. Pleural macrophages produce large quantities of proinflammatory nitric oxide and TNF-α after inhaled chrysotile. Reactive nitrogen species and nitrosyrosine were also found in both visceral and parietal pleurae and are likely to play a role in pleural injury. Intrapleural crocidolite in rabbits also resulted in significant elevation of IL-8 synthesis and neutrophil influx. These inflammatory changes are usually accompanied by mesothelial cell proliferation. Knockout mice deficient in both the 55 and 75 kDa TNF-α receptors are protected from the pulmonary fibroproliferative changes induced by chrysotile, further confirmation of the essential role of TNF-α.

Although BAPE is a common asbestos-induced pleural condition that precedes diffuse pleural thickening (fibrosis), it has only occasionally been studied in experimental settings. Shore et al. injected crocidolite intrapleurally into rabbits and showed the development of a neutrophilic exudative effusion within 4 hours of injection. When the
fluid was reinjected into another rabbit, a polymorphonuclear neutrophil (PMN) response was also elicited.122

Asbestos-related lung and pleural diseases are likely to continue to increase. While legislation has been implemented in most developed countries to minimize occupational and environmental asbestos exposure since the early 1970s, the long lag time between exposure and clinical presentation means there is still a large population at risk of developing disease. Also, chrysotile now constitutes 99 percent of current global asbestos production and sales remain strong in developing nations despite the recent conclusion of the International Program on Chemical Safety of the World Health Organization that ‘exposure to chrysotile poses increased risks for asbestosis, lung cancer and mesothelioma in a dose-dependent manner’.104 Hence, it is anticipated that animal studies will remain important in the ongoing effort to understand the pathogenesis of asbestos pleural damage and to design new treatment strategies.

MODELS FOR CHYLOTHORAX

Chylothorax, the accumulation of chyle in the pleural space, results from impaired lymphatic drainage due to various causes, such as surgery or trauma to the thoracic duct and malignancy (see Chapter 29, Effusions from lymphatic disruptions). Surgical ligation or interruption of the thoracic duct has been performed in a canine model. Mongrel dogs were fed milk fat prior to surgery to increase lymphatic drainage and allow easy identification and transection of the thoracic duct, leaving chest tubes in place for drainage. Using this model, octreotide has been shown to enhance closure of the fistula and reduce chyle leak.133

Congenital defects of the lymphatic duct and lymphangiectasia are uncommon causes of chylothoraces. Recently, mice homozygous for a null mutation of the gene encoding the α9 subunit of the α9β1 integrin were bred to examine the roles of the α9 integrins. Unexpectedly, these mice had genetic defects in their lymphatic system resulting in bilateral chylothoraces by 3–6 days after birth, and died eventually of respiratory failure.134 The role of the α9β1 integrin in lymphatic development appears to be mediated via binding to lymphangiogenic VEGF-C and -D.135 This model may be useful for further investigation of the mechanism of chylothorax development.

MODELS FOR PLEURAL EFFUSIONS FROM ESOPHAGEAL RUPTURE

Esophageal perforation is an uncommon cause of pleural effusion. Only one model has been developed using insertion of a 16F Foley catheter into the esophagus of anesthetized New Zealand white rabbits.136,137 Pleural effusions developed 2 hours later and were bilateral in half of the rabbits. Serial thoracenteses revealed exudative effusions with progressively increasing acidity and leukocytosis, rising levels of protein, very high amylase and low glucose, and positive bacterial cultures, consistent with the classical pleural fluid findings in patients with ruptured esophagus.138 Interestingly, when the animals were rendered neutropenic by pretreatment with nitrogen mustard, no reduction in pleural fluid pH was observed, suggesting that the reduced pH results from neutrophil metabolism rather than anaerobic bacterial infection.137

MODELS FOR PLEURAL EFFUSIONS FROM FLUID OVERLOADS

Human transudative pleural effusions, commonly caused by congestive cardiac failure, renal failure or hepatic cirrhosis, are not easily recapitulated in animal models. A sheep model of intravenous oleic acid infusion has been used to induce pulmonary edema and bilateral pleural effusions.139 Oleic acid resulted in reduced cardiac contractility, raised pulmonary arterial pressures, alveolar edema and pleural transudation. The biochemical composition of the pleural fluid was similar to that of alveolar fluid. While this model is useful, it is not entirely similar to pleural effusion from cardiac failure. First, oleic acid infusion causes alveolar damage. Second, the pleural fluid to plasma protein ratio was 0.6–0.7, classifying the pleural fluid as an exudate.

A murine model of renal failure and fluid overload has been described by Song et al.139 Renal failure is generated by bilateral renal vessel ligation and fluid overload by intraperitoneal delivery of isotonic saline of 40 percent body weight. After 3 hours, the mice develop bilateral pleural effusions (approximately 100 μL).139 This model was developed to study the role of aquaporin water channels in pleural fluid homeostasis, but can be used to study other aspects of pleural effusion secondary to renal failure.

MODELS FOR PLEURAL PHYSIOLOGY STUDIES

Numerous animal models have been employed for the investigation of pleural physiology in health and disease. The main areas of interest are mechanisms of pleural fluid formation and absorption and pleural pressure changes in the presence of effusion or pneumothorax.

To study the transfer of lung water into the pleural space through the visceral pleura, Broaddus et al.140 subjected anesthetized ventilated sheep to volume overload. The chest was open and a bag was wrapped around the exposed lung to collect the fluid leak from lung parenchyma into the pleural space. Their experiments confirmed that the pleural space provided an important route of clearance of pulmonary edema. Similar methods have been used in mongrel dogs to study the factors that alter the permeability of the visceral pleura.141,142

Different animal models have been used to study the removal of pleural fluid and proteins from the pleural space. Many studies have been performed in sheep and rabbits,143 and occasionally dogs.143 Labeled particles, such
as 125I-albumin or fluorescent isothiocyanate-labeled dextran can be used as a tracer to follow the efflux and reabsorption of protein in the pleural space.

Readers should beware of potential drawbacks when extrapolating the results of these animal studies to human physiology. First, fluid exchange mechanisms in animals may not always parallel those of humans, as humans and sheep have thick visceral pleura in contrast to the thin visceral pleural membranes of rabbits; thus, the results of rabbit fluid absorption studies may not represent human conditions. Second, in humans, pleural fluid often accumulates in pathologic conditions with abnormal (e.g. inflamed or malignant) pleura, and the pathophysiology of fluid formation and regression may well differ from studies performed in animals with normal pleurae. Third, respiratory patterns may affect pleural fluid absorption, making sedated animals not ideal representatives of real life situations. In rabbits, the rate of pleural absorption of particles can be influenced by their molecular weight.

Whether this applies to other animals remains to be tested.

Animal studies have also been used to assess cardiorespiratory impairment induced by pleural effusion or pneumothorax. Fluid or air can be introduced intrapleurally and physiologic changes (e.g. electrocardiogram, hemodynamic changes, arterial oxygenation, lung function) measured. Large pleural effusions affect the dynamic elastance and resistance of the respiratory system, and produce hypoxemia in a dose-dependent manner. It is noteworthy that in animal experiments effusions are produced acutely, whereas most human effusions (e.g. malignant) accumulate over time. Chronic measurement of the effects of artificially induced effusion is difficult, but has been reported by Murphy et al., who surgically inserted catheters in the serosal layer of the rat esophagus, allowing measurement of pleural pressures for up to 14 weeks.

MODELS FOR THE STUDY OF PLEURAL VASCULAR PERMEABILITY

Leakage of protein-rich exudate from blood capillaries in the lung interstitium, and beneath the mesothelium into the pleural space, has been implicated in the pathogenesis of various pleural effusions and has been demonstrated in human disease and animal models. Albumin is the most abundant protein contained in exudative pleural effusions and is, as a result, most commonly used to determine vascular permeability. Studies of pleural vascular permeability are feasible in any species, but mice have been used preferentially.

Pleural vascular permeability can be assessed using two mutually complementary methods. Permeability can be determined using an albumin tracer (e.g. Evans' blue, fluoro-isothiocyanate or radioisotope-labeled albumin) introduced intravenously into animals bearing pleural effusion or inflammation. Shortly thereafter (e.g. 5-30 minutes), the levels of the tracer in the pleural fluid or lavage can be determined (e.g. measuring absorbance, fluorescence or scintillation), and reflects the rate of albumin leakage into the pleural space.

Another experimental approach, the Miles vascular permeability assay, determines the effects of mediators contained in pleural fluid on vascular permeability in the mouse or rat skin (Figure 14.1c). Cell-free pleural fluid supernatants are injected intradermally and Evans' blue is administered intravenously. After a predetermined time interval, extravasation of the dye into the mouse dermis is determined by measuring Evans' blue levels in tissue extracts or by morphometry. Vascular permeability induced by the fluid under examination can be compared with saline (negative control) or VEGF solutions (positive control), and the contribution of individual mediators to overall vascular permeability can be assessed after their neutralization in the pleural fluid prior to injection into the skin.

Using these methods, the contribution of VEGF, IL-6 and TNF-α (Statopoulos et al., unpublished observations) to the induction of vascular hyperpermeability in malignant pleural effusions has been established.

OTHER MODELS

Various other models have been published over the years. For example, the turpentine model (see Models for pleural infection) has been used to mimic effusions from rheumatoid arthritis.

Intrapleural gene therapy for replacement therapy or for mesothelioma has been studied in animal models. In our experience, mesothelial cells can be easily transfected in vitro and in vivo. Plasmids delivered intrapleurally can transfect the mesothelial cells and the protein product can be recovered from pleural fluids and from plasma.

Rabbits have also been used to study the pharmacokinetics of intrapleurally administered drugs. This may be relevant to intrapleural chemotherapy, which has been increasingly used in clinical trials for control of malignant mesothelioma.

IN VITRO STUDIES OF MESOTHELIAL CELLS AND IN SITU STUDIES OF MESOTHELIAL MONOLAYERS

While in vitro studies have limitations, the study of cultured mesothelial cells in isolation can provide information supplemental to animal studies. Mesothelial cell lines are commercially available but are transformed by viral infection or transfection. Mesothelial cells from the pleura or the peritoneum can be harvested for primary culture. While their biological behaviors are likely to be similar, this has seldom been compared or confirmed. However, when in vitro studies of mesothelial cells are undertaken to address the pathophysiology of pleural diseases, it would be ideal to use pleural and not peritoneal mesothelial cells.
Normal human pleural mesothelial cells are difficult to obtain, and have most commonly been isolated from pleural effusions caused by heart failure. Culture methods of human and animal mesothelial cells are similar.\(^{94,165}\) However, mesothelial cells from human effusions, even from transudative ones, have likely been exposed to mediators and may not truly represent 'normal' mesothelial cells. For that reason, studies often employ primary culture of pleural mesothelial cells from animals, e.g. rabbits or mice. Principles of harvesting the cells and points of caution are summarized in Appendix 14.3. In our experience, primary rabbit mesothelial cells grow rapidly and maintain their biological activities up to seven or eight passages. In contrast, murine mesothelial cells divide very slowly (the initial growth rate increases with density of seeded cells), and rarely survive a third passage.

To overcome the limitations of human mesothelial cell culture outlined above, Kim et al. devised a new in vitro system for culture of mesothelioma tissues in the form of spheroids, based on a technique previously developed for culture of intact bronchial mucosa.\(^{162,163}\) Using this method, mesothelioma tissue retained many of its in vivo characteristics. This model closely emulates intrapleural mesothelioma growth and is expected to greatly facilitate studies on mesothelioma apoptotic resistance to novel therapies. Such techniques can be adopted for experiments on non-malignant pleural tissue cultured ex vivo.

Other studies have been performed on isolated mesothelial barriers in situ, most commonly obtained from sheep.\(^{184-186}\) Using chambers, special devices that function as voltage clamps, have been used to measure the transpleural resistance in pleura stripped from animals, before and after an intervention. The resistance measured supposedly reflects the permeability of the pleural barrier. This model has its limitations: pleural fluid formation is governed largely by vascular (rather than mesothelial) permeability, and the findings of these studies of normal pleura may not be applicable to pleural structures in disease states.

CONCLUSIONS

'The animal model is the key to the understanding of disease.'

Leader (1969) at the Federation of American Societies for Experimental Biology

Animal experimentation represents one of the fundamental approaches in the long arduous path towards the understanding of the pathogenesis of various pleural diseases. In vivo studies are essential in the evaluation of novel therapeutic approaches in pleural diseases, in the study of pharmacokinetics of drug delivery in the pleural space and in the investigation of pleural physiological changes in both normal and disease states.

No animal model is ideal. Investigators should understand the advantages and limitations of the use of different animal species and models. Only through doing so would they be able to choose or design the most suitable in vivo model or in vitro experiment that provides the best chance of answering the scientific question(s) raised. Animal studies should be planned, conducted and supervised with a similar degree of scrutiny as that applied to clinical trials. The ultimate aim should always be to provide better care to patients with pleural diseases.

Advances in other areas of biomedical sciences, especially animal imaging techniques, should allow the design of more sophisticated animal models that will improve our understanding and clinical management of pleural diseases.

KEY POINTS

- Animal models have been invaluable in the study of the pathogenesis of pleural diseases. In vivo studies are essential in evaluating new therapeutic options for pleural diseases, in assessing the pharmacokinetics of drug delivery to the pleura and in examining physiological changes in the pleural space in health and disease.
- It is critical for researchers to understand the characteristics and limitations of experimental models available in order to use the most appropriate method that can best answer the scientific question asked.
- Researchers must adhere to standard guidelines for animal care and gain approval from local ethics committees. Every effort should be made to minimize animal discomfort and the number of animals required.
- In vitro studies allow the study of mesothelial and other cells that may engage in the pathogenesis of pleural diseases in isolation. In vitro experiments can help explain pathologies observed in animal or human studies. Conversely, novel information derived from cell culture experiments can be tested in vivo using appropriate animal models.
- Animal models exist for common types of pleural diseases (e.g. pleural effusion due to malignancy, infection and inflammation), as well as for pleural pathologies that are uncommonly encountered in clinical practice (e.g. chylothorax, esophageal rupture).
- Advancement in biomedical technology, e.g. novel methods for gene transfer and further development of genetically engineered mice, will allow the design of increasingly sophisticated models to provide further significant insights into pleural diseases.
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APPENDIX 14.1: RABBIT CHEST TUBE INSERTION

1. New Zealand white rabbits (>1.5 kg) are anesthetized with an intramuscular injection of ketamine hydrochloride (35 mg/kg) and xylazine hydrochloride (5 mg/kg). The chest is shaved and the skin sterilized with 10 percent povidone iodine.*

2. The rabbit is placed in the lateral decubitus position and a small (<3 cm) skin incision is made midway between the tip of the scapula and the sternum approximately 2 cm above the costal margin. Chest tubes are made from intravenous solution set tubes with three extra openings near the distal end of the tube to enhance drainage.

3. The chest tube is inserted by blunt dissection into the right pleural cavity and secured at the muscle layers with purse-string sutures (3.0 ethilon). The proximal end of the chest tube is then tunneled underneath the skin and drawn out through the skin posteriorly and superiorly between the two scapulae. The exterior end of the chest tube is sealed with a one-way valve with cap via an adapter and sutured to the skin using a 2.0 silk suture.

A three-way stopcock is attached to the end of the chest tube through which any aspirated air is immediately evacuated from the pleural space.

5. Reagents can be administered intrapleurally via the chest tube, followed by the instillation of 1.0 mL of 0.9 percent sodium chloride solution or sterile phosphate-buffered saline (PBS) to clear the dead space.

6. The chest tubes can be aspirated for pleural fluid. Alternatively, pleural lavage can be performed by the administration of 5–10 mL of sterile PBS via the chest tube. The rabbit is then rotated and the PBS aspirated back from the chest tube.

7. The chest tube should be removed under light sedation as soon as it is no longer required in order to minimize risks of infection and discomfort.

8. At time of sacrifice, the rabbits are sedated and killed with carbon dioxide. The thorax is removed en bloc. The lungs are expanded by the injection of 50 mL of 10 percent neutral-buffered formalin into the exposed trachea via a plastic catheter. The trachea is then ligated and the entire thorax submerged in 10 percent neutral-buffered formalin solution for at least 48 hours.

APPENDIX 14.2: SHEEP PLEURODESIS MODEL

1. Yearling sheep of mixed breeds can be anesthetized with an intravenous injection of 2.5 percent sodium thiopental at 20 mg/kg.

2. The chest should be shaved and the skin sterilized with 2 percent chlorhexidine and then with 10 percent povidone iodine.

3. Using a laryngoscope, an endotracheal tube (8.5 mm internal diameter) is inserted with an attached plastic 'bite block' and secured with tape. Anesthesia is maintained with a gaseous mixture of room air, oxygen, and 1.5–2.5 percent halothane at a ventilation rate of 10 breath cycles/minute with a volume of 15 mL/kg per breath cycle. The sheep is placed on its side on a surgical table and the feet secured to the table.

4. A 5 cm incision is made in the lateral chest wall at the 7th intercostal space. By blunt dissection, an 18G French Foley balloon-catheter with 30 mL balloon volume is inserted into the pleural space under aseptic conditions, tunneled underneath the skin and brought to the surface just lateral to the vertebral. The tube is secured at the skin with purse-string sutures.

5. The sheep is then ventilated with a positive end expiratory pressure of 15 cmH₂O. A three-way stopcock is attached to the end of the Foley catheter through which all air is evacuated from the pleural space immediately after the chest tube insertion.

6. Intrapleural injection of agents can be made via the chest tube. The buffer or vehicle can be injected to the contralateral side and serve as the control.
The chest tube is aspirated (with the Foley catheter balloon inflated) regularly for any pleural fluid produced. To minimize discomfort and risk of infection, we recommend that the chest tubes be removed as soon as no further intrapleural injections or pleural sample collections is needed.

For pleural fibrosis/pleurodesis studies, the sheep are killed 14 days after the chest tube insertion with an intravenous injection of sodium phenobarbital solution.

**APPENDIX 14.3: METHODS FOR HARVESTING RABBIT AND MICE MESOTHELIAL CELLS**

1. Pleural mesothelial cells are obtained from mice of adult size or from New Zealand white rabbits (commonly 2 kg).

2. After the animals are killed, the abdomen is opened to expose the diaphragm. Hank’s Balanced Salt Solution (HBSS) is injected into the pleural cavity from beneath the hemi-diaphragms under direct vision and then aspirated out after 2 minutes. This is to remove surface proteolytic enzymes to allow greater efficacy of trypsin (see below). In mice, there is no mediastinal separation and a single injection of 1 mL of HBSS is sufficient to rinse the pleural surface in the left and right chest.* In rabbits, 10 mL is injected into each pleural cavity.

3. Trypsin-EDTA (ethylenediamine tetraacetic acid) (0.25 percent) is injected into the pleural cavity and left *in situ* for 10 minutes during which the animal should be rotated. The solution, with the mesothelial cells, is then aspirated and put into fetal calf serum (FCS) or culture media (e.g. DMEM [Dulbecco’s modified Eagle medium] with 10 percent FCS) on ice. The serum contains trypsin that will terminate the action of, and any potential damage from, the injected trypsin. An injectate volume of 1 mL is used in mice, and 10 mL (for each side) in rabbits.

4. The FCS is centrifuged at 1000 rpm for 5 minutes. The cell pellet is washed and resuspended in DMEM with 1 percent (v/v) L-glutamine, 1 percent (v/v) penicillin–streptomycin and 10 percent (v/v) FCS.

5. The cells can be plated in standard cell culture flasks, and incubated at 37°C with 95 percent of air and 5 percent of CO₂. Initial cell population will consist of a large number of erythrocytes and leukocytes as well as the mesothelial cells.

6. The media should be changed after overnight incubation. Mesothelial cells should adhere to the culture flasks, and contaminating cells can usually be removed with the media.

7. The cells can be stained for mesothelial markers to confirm the epithelial origin of the cells. Fibroblasts that may have adhered to the culture flasks are cytokeratin negative. Most investigators are able to achieve a 95 percent purity of mesothelial cells using this technique.

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* In our experience, very fine needles (28G) should be used in mice pleural injections.