SYMPOSIUM III:
Animal Models

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DEMYSTIFYING YOUR EXPECTATIONS OF RESPIRATORY PHYSIOLOGIC ASSESSMENT OF VARIOUS ANIMAL MODELS

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Transgenic technology has greatly enhanced the insight derived from mice in respiratory research. While the genotype is explicitly defined in these animals, it is the phenotype that often leads to discovery. Yet there is no universally accepted or easy method to obtain 'respiratory phenotype' in the mouse. Defining features include physiology, structure (imaged), and histomorphometry of the respiratory tract. Of these features, the *physiologic function of the respiratory system* most closely predicts clinical outcome, hence there is renewed enthusiasm for the inclusion of *in vivo* diagnostics in translational research protocols. Despite the enthusiasm, there is yet no consensus on *how* to evaluate pulmonary function in the mouse, in particular airway function in the conscious mouse[1]. The measurement of elastance of the lung is generally not disputed and requires intubation and anesthesia. Newer automated systems make implementation of pressure-volume measurements easy and accurate.

For measurement of airway function unlike pressure-volume measures, it is feasible to measure pulmonary function in the conscious state and this is very tempting but not without controversy. Indeed the gap between accurate/precise invasive yet obtrusive methods, and imprecise yet non-invasive techniques has not been bridged by more technologic advances[2]. Recently, concern has been expressed over the widespread use of unrestrained plethysmography to characterize 'airway function' per se[1-5]. Since unrestrained plethysmography is a commonplace application, this has left many users searching for alternatives[6]. Indeed, veterinary pulmonologists have joined the ranks of the perplexed in this regard. For use in laboratory animals, several alternative methods have been proposed, which provide more direct measure of airway mechanics. For example, mid-tidal expiratory flow (EF50) has been used extensively to characterize bronchoconstriction in conscious mice[7-10]. While EF50 tracks invasive measures of pulmonary resistance and dynamic compliance during allergen, cholinergic agonist, and hyperoxia challenges[7,9,11], this is only a measure of flow and therefore lacks the power derived from the classic comparison of driving pressure (alveolar, transpulmonary) with flow. Hence, one can only infer resistance and it must be assumed that all flow limitation results from bronchoconstriction (vs altered breathing pattern, air trapping). Specific airway resistance (sRaw), the product of airway resistance (Raw) and functional residual capacity (FRC) should improve the insight into airway caliber since both components are affected during bronchoconstriction[12,13]. The techniques available to measure sRaw include double chamber plethysmography (DCP)[14] and restrained whole body plethysmography (RWBP)[15]. The current problem with DCP is the use an uncomfortable and potentially restrictive neck seal and complex restrainer[13]. The reproducibility of airway reactivity derived from double chamber plethysmography (DCP) has also been challenged[16], and strain-specific responses to methacholine were discordant with more invasive methods[17]. For these reasons, the current method to measure sRaw using DCP is not widely cited. In contrast RWBP provides values of sRaw and measures of airway responsiveness which are highly comparable to invasive measures (e.g. forced oscillation technique) but does not

utilize a neck seal. The RWBP technique employs the principle that rodents are *thigmotaxic*. Rodents in general seek surfaces, holes, cubbies, etc, in part due to their poor eyesight so feel comfortable in a standard restrainer; furthermore the restraining system pre-conditions inspired air improving the signal. While promising for longitudinal studies in allergen models and pharm-tox studies, RWBP is a very new adaptation of the original and time-honored method of measuring sRaw[18] and the general acceptance of this method awaits future trials. Transfer impedance (Ztr) is yet another conscious method, which is used to discriminate central (airway) vs. peripheral (tissue) contributions to bronchoconstriction, and permit longitudinal measurements[19]. One limitation of Z_{tr} is the investment in time to acclimate the animals for at least one day to the instrument in order to obtain acceptable reproducibility. Invasive techniques that describe airway phenotype include the forced oscillation technique (FOT), respiratory resistance and compliance, and forced (fast-flow) maneuvers, all of which will be granted some perspective with regard to their application to transgenic phenotyping, and in models of asthma (ovalbumin allergy), emphysema (elastase exposure), acute lung injury (saline lavage), and pulmonary fibrosis (bleomycin exposure). It is noteworthy that the European Union will greatly expand its evaluation of chemical exposures by toxicologic screening in rodents as well as non-animal cell culture based methods over the next 5 years. Veterinarians must be at the forefront of understanding pulmonary function testing in laboratory animals, in order to improve animal welfare *and* optimize the phenotypic certainty derived from *in vivo* tests.

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MURINE MODELS OF ASTHMA AND LUNG INFECTION: DETERMINATION OF PHYSIOLOGICAL, IMMUNOLOGICAL AND HISTOLOGICAL PARAMETERS

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Human asthma is characterized by variable airflow obstruction in response to allergen (early- and late phase response) and airway hyperresponsiveness. Structurally, the airways of asthmatics are characterized by the presence of chronic allergic inflammation with intense infiltration of the bronchial mucosa by lymphocytes (Th2) and eosinophils, accompanied by epithelial desquamation, goblet cell hyperplasia and thickening of the submucosa (airway remodeling). An animal model of asthma must recapitulate these features of the human disease to be reliable.

Murine models that reproduce certain features of asthma have been developed. In standard protocols inbred mice are first sensitized systemically to allergen and then challenged by aerosol. Critical for the induction of an asthma-like phenotype with allergic airway inflammation and airway hyperresponsiveness (AHR) are: 1. Selection of the antigen: frequently used allergens are proteins (e.g. ovalbumin and Aspergillus fumigatus extract) or microorganisms (e.g. Aspergillus fumigatus, Schistosoma mansoni egg); 2. Selection of the mouse strain: the choice of the mouse strain is very dependent on the allergen used. Balb/c mice are often used for ovalbumin protocols; 3. Selection of the protocol: the protocol should ascertain a reproducible sensitisation against the allergen (e.g. repeated systemic injection of the allergen associated with an adjuvant like alum) and the induction of a long lasting allergic airway inflammation (e.g. repeated aerosol challenges over weeks). Classical read out parameters are: allergen specific IgE and IgG1 in serum, eosinophil numbers and Th2 cytokines in broncho-alveolar lavage, quantification of inflammation and remodelling in lung sections by (immuno-) histology and determination of early phase response and airway hyperreactivity by lung function measurement.

An excellent overview over the most common used protocols is given in Lloyd et al (Lloyd, et al. 2001). The acute models are very effective in inducing acute airway inflammation - mainly consisting of Th2 cells and eosinophils - and airway hyperresponsiveness. In order to induce a chronic inflammation, the protocols have been further developed. Multiple aerosol challenges and/or live aspergillus fumigatus connidia were used to induce a long lasting (chronic) inflammation in the airways that were associated with airway remodeling and AHR. Recent progress in developing these models and improvement of lung function measurements made it possible to acquire both early- and late phase response due to allergen provocation in mice (Glaab et al. 2005). Most of those new therapeutic strategies for asthma that are designed to neutralize central mediators in asthma pathology have been developed and tested in murine asthma models. Prominent examples are anti-IgE, anti-IL-5, anti-IL-13 and anti-eotaxin (for review (Lloyd et al. 2001).

These models are used to test efficacy of drugs as well as to elucidate the mechanisms of allergic airway inflammation. Therefore often mechanistical studies were performed in which certain components of the immune response were deleted by genetic or

pharmacological strategies (Braun et al. 2006, Epstein 2006). In addition, cell transfer models gave new insights in the participation of cells like T-cells and dendritic cells in the pathogenesis of allergic asthma (van Rijt. et al. 2005).

In the mouse, lung infection model for bacterial, viral and fungal pathogens were described (Stark et al. 2006, Liebmann et al 2004). In these models the primary read out parameter is often the survival of the animals. In addition, systemic parameters like body temperature and weight loss give important information regarding the health status of the animals. The determination of pathogen numbers in lung and other organs like spleen or in serum is often performed but has its methological limitations. For characterisation of the immune response inflammatory parameters like neutrophil numbers or Th1 cytokines are determined in the lung or bronchoalveolar lavage fluid. In addition, a histological analysis of the lung and other organs is helpful to characterize the localization of the infection and the degree inflammation.

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IN VIVO ASSESSMENT OF AIRWAY REMODELLING

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Among the pathophysiological changes occurring in chronic inflammatory lung diseases such as asthma, chronic obstructive pulmonary disease, chronic bronchitis etc. airway remodelling is particularly challenging and is gaining increasing interest in respiratory research (Bousquet et al., 2000; Vignola et al., 2000). Remodelling occurs in response to airway inflammation and accounts for functional as well as for histological pulmonary changes, e.g. airway hyperresponsiveness, mucus hypersecretion, epithelial hyperplasia, basal membrane thickening, bronchial smooth muscle hyperplasia, vascular changes etc. (Bousquet et al., 2000; Rutgers et al., 2001). Progressive decline in lung function, decreased responses to bronchodilator or anti-inflammatory therapies as well as some acute exacerbations of airway disease have been shown to be associated with airway remodelling (James and Carroll, 1998).

Given the close relationship between airway inflammation and remodelling, the assessment of remodelling takes into account inflammatory processes of the respiratory tract. Different approaches might be used for investigating inflammation-associated remodelling: lung function testing, lung or airway imaging and the determination of biomarkers.

1. Functional approach of inflammation-associated remodelling: hyperresponsiveness

Bronchial hyperresponsiveness is defined as an abnormal sensitivity and exaggerated response of the airways to pharmacological, chemical, physical or physiological stimuli. Airway luminal diameter, smooth muscle mass, elastic recoil, airway inflammation, epithelial injury and neural activity may all play a role in hyperresponsiveness (Rutgers et al., 2001). Remodelling-associated hyperresponsiveness can be suspected in case of treatment-resistant and irreversible hyperresponsiveness. Numerous lung function tests are available and might be adapted to different animal species, allowing assessment of bronchial reactivity in clinical situations as well as in respiratory research.

2. Diagnostic imagin: radiography, computed tomography, bronchoscopy, thoracoscopy, biopsies

Diagnostic imaging can be performed using different techniques and is complementary to functional or biochemical evaluation of remodelling. A classical but not very sensitive method is thoracic radiography. This technique is mainly used for clinical purposes and bears the advantage of being applicable to large and small animal species. Pulmonary computed tomography (CT) provides qualitative information about large lung fields and offers the possibility of quantifying structural changes. CT is widely used in human respiratory medicine and research, whereas its application in veterinary medicine or animal respiratory research is still limited to some animal species.

The evaluation of the large airways' aspect by use of bronchoscopy provides valuable information about mucosal aspect and mucus production. However, this technique can only be used in animals of a certain size. Bronchoalveolar lavage fluid, bronchial biopsies or brushings might be sampled using a bronchoscope, which provides insights about cytological and histological changes of the airways. In large animals, histological changes

of peripheral airways and lung tissue might be evaluated using thoracoscopy-guided biopsies.

3. Assessment of biomarkers

Inflammation- and remodelling-associated biomarkers can be determined in bronchoalveolar lavage fluid, tracheal washes, biopsy samples, induced sputum (in humans), exhaled air and exhaled breath condensate. In some particular cases, blood markers associated with a pulmonary process might also be used.

The choice of the matrix used for biomarker determination depends on the animal species and the marker selected. All sampling techniques are potentially applicable in large animals, whereas the reduced size might become a limiting factor in small animals. The need for anaesthesia in small animals is a further limiting factor, especially with regard to repeated investigations. The sampling technique used further depends on the chemical properties (molecular weight, stability, solubility,…) and the synthesis and secretion of the biomarker.

Among the biomarkers that are frequently assessed figure cytokines, prostaglandins, leukotriens, growth factors, transcription factors, volatile molecules (NO, ethane), H_2O_2, pH, metalloproteinases,…. (Rutgers et al., 2001).

The equilibrium between matrix metalloproteinases (MMP) and their endogenous inhibitors (TIMP) seems to play a particularly important role for the development of airway inflammation and remodelling (Lagente et al., 2005) and is therefore investigated in human and animal respiratory research.

Remodelling of the respiratory tract covers many physiological and pathophysiological processes which are often correlated. Assessment of remodelling remains challenging for the future, especially *in vivo*.

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ANIMAL MODELS OF EMPHYSEMA

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Background. Chronic obstructive pulmonary disease (COPD) is predominantly a disease of the sixth decade of life and later. It is characterized by an irreversible airflow limitation measured during forced expiration, which is caused by either an increase in the resistance of small airways due to chronic bronchitis, or an increase in lung compliance due to emphysema, or both [1]. Emphysema is defined as the "*abnormal permanent enlargement of the airspaces distal to the terminal bronchioles, accompanied by destruction of their walls*" [2]. Several types of emphysema are distinguished in humans as e.g., centriacinar emphysema, which is associated with tobacco smoking, and panacinar emphysema, which is most frequently seen in α_1 anti-trypsin deficiency. The pathogenetic pathways leading to emphysema are still a matter of debate and, even more importantly, curative therapies are still lacking. Various lines of research have been followed to establish animal models of emphysema including the development of mutant or transgenic animals, the induction of emphysema by inhalation of cigarette smoke or noxious gases, by instillation of elastase or LPS, or by calorie restriction [3, 4].

General aspects. An important question before choosing an animal model of emphysema is to ask whether the pathogenesis of the disease or a therapeutic approach shall be investigated. Studying aspects of emphysema formation requires that the model closely mimics the pathogenetic pathways of the human disease, whereas for the study of a therapeutic approach a model that is characterised by a well defined end-stage of the disease may be more attractive. As pulmonary emphysema is anatomically defined, the validity of a potential animal model of emphysema has to be tested by quantitative histopathologic methods measuring both airspace enlargement **and** destruction of the alveolar walls. Unfortunately, most studies rely on the quantification of airspace enlargement alone assessing mean linear intercept length or mean chord length as indices of airspace size. These parameters are highly sensitive to inflation during fixation and tissue shrinkage during embedding [5, 6]. As airspace size increases with age [7, 8], "*abnormal enlargement*" can only be demonstrated compared to age-matched control lungs, "*permanent enlargement*" only when additional groups are implemented to demonstrate persistence of airspace enlargement. However, to conclude that emphysema is present, it is not sufficient to reveal abnormal permanent airspace enlargement alone. Additionally, destruction of alveolar walls has to be demonstrated [9], e.g. as a decrease in total alveolar wall volume, total alveolar surface area, total capillary length and/or total number of alveoli [10-13]. Although even in patients with severe emphysema an amplified inflammatory response is observed [1], the mere presence of inflammatory cells characteristic of human COPD as e.g., neutrophilic granulocytes, activated alveolar macrophages and $CD8^+$ T-lymphocytes, can not be considered to be a conclusive indicator of emphysema alone.

Genetic models of emphysema. Studying mutant, gene-targeted or transgenic animals, the analysis of the time course of the formation of an emphysema-like phenotype is of major importance to distinguish developmental defects resulting in an impairment of

alveolarisation from the loss of existing alveoli, i.e. pulmonary emphysema. Alveolarisation, the formation of alveoli, is achieved by secondary septa sprouting into the saccular airspaces thus subdividing a saccule into several alveoli [14]. In humans, rats, and mice, a sudden and extensive formation of alveoli termed "bulk alveolarisation" is observed during early postnatal life. In humans, it starts at about foetal week 36 and continues until a postnatal age of about 1-2 years. In rats and mice, bulk alveolarisation begins at about day 4 after birth and is completed by about postnatal day 14. Therefore, only those genetically modified animals that exhibit normal postnatal alveolarisation followed by a secondary loss of mature alveoli can be considered as models of human lung emphysema. Distinction of developmental and post-alveolarisation effects is not possible using conventional transgenic animals, in which the gene of interest is active or deficient during all stages of lung development, but can be achieved by using conditional transgenic animals [15].

Inhalation models of emphysema. After completion of bulk alveolarisation, the lung continues to grow until adulthood [7, 14]. This is accompanied by a considerable increase e.g., in total alveolar surface area and total alveolar wall volume [7]. In rats and mice, a steep increase in lung volume, total alveolar surface area and total alveolar wall volume is seen until an age of about 5-6 months [7]. Recent data indicate that alveoli, too, are continuously formed until the lung reaches its final volume [16, 17]. Most frequently, animals of only a few weeks of age are used in inhalation experiments, which may require a total exposure time of many months as e.g., in cigarette smoke inhalation. Unless appropriate age-matched and follow-up groups are implemented in such studies, it is difficult or even impossible to distinguish a true loss of alveolar walls and accompanying airspace enlargement from any effect caused by the inhibition or acceleration of lung growth and aging. In addition, long-term inhalation exposure to noxious gases is frequently associated with a loss of body weight [18], which alone may result in emphysematous changes of the lung parenchyma [12]. In this case, appropriate calorie-restricted control groups are necessary to distinguish effects caused by inhalation alone from those effects that may ensue as a result of reduced food consumption.

Elastase instillation models of emphysema. The elastase model of emphysema was established more than forty years ago by Gross and co-workers [19], and had great impact on the development of the proteinase-antiproteinase concept of emphysema formation. Since then, it has been adapted by many others. The attractiveness of this model is that a single hit, the instillation of a bolus of porcine pancreatic or human neutrophil elastase, results in the loss of alveolar walls [10], the defining feature of emphysema. Animals can be used for the study of therapeutic concepts about 3 weeks after the initial injury. However, the desired effect of elastase is frequently limited to a narrow window of dosage, below which no significant loss of alveolar surface area may be observed, whereas a higher dose may result in death due to severe haemorrhage. Using a rat model of emphysema induction by elastase, Massaro and Massaro presented first evidence of a potential therapeutic effect of all-trans-retinoic acid (ATRA) for the treatment of pulmonary emphysema [20]. However, some groups failed to reproduce these findings in rats [21], and no evidence for a beneficial effect of ATRA was observed in mice [22].

Conclusion. Today, numerous animal models are available to study various aspects of pulmonary emphysema. Each one has its merits and limitations. The latter have to be carefully taken into account. In any case, however, the validity of each animal model of emphysema has to be tested by quantitative histopathologic methods measuring both airspace enlargement and destruction of alveolar walls.

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VARIOUS ANIMAL MODELS OF ACUTE LUNG INJURY: GENERAL ASPECTS AND PARAMETERS OF OUTCOME

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The lung is a critical organ in terms of dysfunction to maintain oxygen demands of critically dependent and vital organs like the brain or the heart. Therefore, Acute Lung Injury (ALI) or Acute Respiratory Distress Syndrome (ARDS) (1) and infant respiratory failure (2) are important conditions of rather high morbidity and mortality in the human situation So far, only supportive interventions, i.e. a low tidal volume ventilation of 6 ml per kg and the use of a rather low plateau pressure of 30 cm of water could decrease mortality and morbidity in this severe condition (3).

Depending on the specific addressed scientific question, animal models can be used e.g. to reveal pathophysiologic processes or to study interventions on many aspects of outcome. Whereas large animal models allow assessing many physiological parameters similar to clinical situations, they also tend to lead to a laborious intensive care-like handling (4-6).

On the other hand, many aspects of ALI and ARDS are studied in small rodents like rats or mice. Whereas rats can be a bit easier instrumented and operated on due to their larger size, mice bear the advantage that many tools like antibodies and genetic variations including transgenic mice are available, and smaller amounts of substances are needed for treatment compared to larger animals. However, e.g. the assessment of arterial blood gases is rather difficult due to the limited amount of blood in the mouse. This is of particular importance as the extent of gas exchange disturbance is one key parameter in acute lung injury in the human clinical situation. Furthermore, pulse oxymetry is usually not working well in the mouse, and end tidal CO_2 measurements, a good parameter of arterial carbon dioxide pressure and thus of ventilation, are virtually lacking in ventilator settings.

Many models are more fulfilling a “one hit” acute lung injury. E.g. gram negative bacterial lipopolysaccharide (LPS) -induced lung injury (4), given i.v., intraperitoneally, or intratracheally, causes a rather defined lung injury and/ or sepsis- like syndrome, and also acid instillation (5), oleic acid lung injury (6), thoracic trauma (7), repetitive *in vivo* lung lavage leading especially to surfactant depletion (8), lung ischemia-reperfusion injury, i.v. application of cobra venom factor (9), or some immune complex lung injuries behave more as suggested by such a schedule.

Other models behave more like a “continuous hit or stress” and are closer to clinical situations like sepsis. Examples include pulmonary bacterial, fungal or viral infection models, or abdominal infection models like caecal ligation and puncture peritonitis (10).

By a similar, but probably less variable model like fecal peritonitis defined quantities of stool/bacteria are injected intraperitoneally (11). Oxidant-induced lung injury by high fractions of inspiratory oxygen or ozone, or toxicant-induced lung injury by paraquat or phorbol myristate acetate, or radiation-induced lung injuries also behave like that. Also models like bleomycin induced lung injury bear within a first phase of about two weeks aspects of acute lung injury and turn later to a fibrotic phase or stage. A model of progressive organ failure that covers more aspects than bleomycin is that of zymosan induced lung injury (12). Novel alternatives to bleomycin lung injury may be a fluoroisothiocyanate (FITC) mediated lung injury model (13,14) and adenovirus-mediated lung injury model by transient interleukin-1β overexpression in fibrosis prone mice (15).
"Overventilation" or ventilator induced lung injury models much depend on their design whether they are rather classified in "one hit" or are more continuous injury models.

As a very general simplification, in many models the act of aggression results in inflammation, apoptosis or necrosis, and later repair mechanisms may lead to fibrosis. The majority of investigations focus on very early points of time and thus pathophysiologically mainly at the act of aggression involved in acute lung injury. From the very first beginning of any injury there is a repair program starting, so that usually there may be in early stages of acute lung injury a mixture of programmed inflammation, cell demise, and repair. The lung injury may be as severe that artificial support may be necessary; alternatively the animal may die. Such a situation is therefore either modelled by ventilator support to the animal, or the injury is very gentle – which is frequently the case in mouse lung injury models in order to have surviving mice and not an intensive care situation.

Specific aspects of models can further be reduced to models of isolated lungs, where e.g. physiologic aspects like vascular or pulmonary mechanical function, corresponding mediators or signalling cascades can often be studied more easily (16,17) than directly *in vivo*.

Unilateral acute lung injury models may be of preference in situations where bilateral lung injury may be too severe to keep the animal alive (18). This approach is also preferred when in the same animal a compatible control is ideal, e.g. when biological variation may be important. This may be the case e.g. in settings of high biological variation, as e.g. in stem cell therapy, as cells could vary between animals. With that approach every animal serves as its own control, which is usually also a statistically powerful design. Two points should be kept in mind on models of unilateral lung injury: First, acute lung injury and ARDS are defined as bilateral lung injury, so that this definition is not fulfilled. Second, the pathophysiological situation concerning the circulatory situation of the injured lung seems quite dissimilar to real bilateral injury: in bilateral lung injury the whole blood flow has to pass the injured lung, whereas in a unilateral model the blood flow may be directed more to the non-injured lung which may protect the injured organ in terms of oedema genesis and inflammation.

Outcome of acute lung injury models can base on individual data like survival. Surrogate parameters of lung injury are usually taken; they should relate as much as possible to the target of intervention and the question to be answered by the study. Frequently there are redundant read-outs to corroborate the findings. The assessment of surrogates of the lung injury like gas exchange by arterial blood gas analysis (18) or the pulmonary function/lung mechanics (19) can be determined. Histopathological alterations including morphology and grading (20-23), and associated techniques like immunohistochemistry can be used. They

allow e.g. to assess cell proliferation or cell death, altered metabolism, specific mediators or occurrence of specific cell types, or *in situ*-hybridisation techniques can be used. *In vivo* microscopical approaches of the lung, be it on the open chest or with a microscopy window, or using ectopic lung tissue, can reveal aspects like inflammatory cell migration. As acute lung injury is a permeability oedema, microvascular permeability e.g. with protein leakage or specific tracer leakage, or gravimetric methods (19,24) (wet to dry weight ratio of lung tissue) can be assessed besides the morphological quantification of oedema in histological slices. Bronchoalveolar lavage (BAL) is of high interest to investigate the alveolar space where acute lung injury, i.e. breakdown of both the endothelial-interstitial and the interstitial-alveolar barrier takes place (25); it reflects much less mainly interstitial changes. It allows determining the alveolar cell count and differential. Protein content is a rough marker of lung injury, and mediators of inflammation and repair can be determined. The BAL composition of phospholipids and surface activity may be used assess aspects of surfactant quantity and activity. Lung homogenate can also be used to assess mediators, and molecular biological methods can be performed.

There are “common avenues” of injury due to a certain stereotypic reaction of lung tissue to a certain stimulus and some specific pathways, as e.g. between ventilator induced lung injury and LPS injury of the lung (16), but only a few aspects have been determined in detail. Knowledge of such similarities and differences may allow better understanding pathogenic mechanisms and defining therapeutic targets. Acute lung injury bears a rather severe outcome, and selected outcome parameters should reflect such biological relevance if possible. The model should be a close picture of the clinical situation we wish to study.

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PARTICULATE MATTER-INDUCED LUNG INJURY – ANIMAL MODELS

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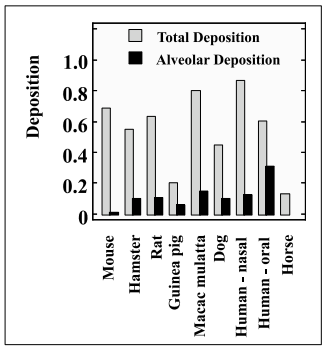
Introduction There is wide agreement that certain individuals are more susceptible to the hazardous effects of air pollution than others. This is supported by a strong body of epidemiological evidence showing that children and the elderly, in particular those with cardiopulmonary diseases, are more frequently affected by the adverse outcomes associated with inhaled ambient particulate matter (PM). The current working hypothesis states that the individual risk is determined by "gene-environment interactions", i.e., an individual's final outcome is determined by a combination of his immutable genes and the quality and intensity of the environmental burden throughout his life span. It has also become clear that the complex interactions of multigenetic traits and a wide array of environmental burdens result in a riddle hardly to be solved by a single approach. Although epidemiological studies and clinical trials have made substantial progress, most of our current understanding still results from experimental studies under controlled conditions. These studies, however, always require the extrapolation of toxicological data as obtained in a certain animal species to the human scenario. An ideal animal model should therefore mimic human conditions as perfectly as possible, a demand which even in terms of respiratory system anatomy can hardly be fulfilled. In addition, differences in physiological and immunological features have to be considered. The purpose of this brief review is therefore to show the prospects and limitations of animal experiments, focusing on asthma models in the evaluation of particulate matter health effects.

Dosimetric aspects If the assumption is made that comparable PM doses at comparable target sites cause comparable effects across species, extrapolation "only" requires dose and site to be adequately defined for the respective experimental conditions. Commonly, equivalent dosage is normalized to body weight or respiratory region surface area, which differs substantially between species: mice, 0.1 m^2 (0.067 m^2/ml lung volume (V_L)); rats, 0.5 m^2 (0.033 m^2/ml V_L); beagle dogs, 60 m^2 (0.04 m^2/ml V_L), adult humans, 100-140 m^2 (0.02 m^2/ml V_L). Inhalations and instillations are common exposure routes in animal experiments. While the latter approach guarantees exact dosage, it is hampered by an unequal particle distribution in the lungs, with regions receiving no particle load at all and others getting a substantial overload. Moreover, the entire dose is given instantaneously, which obviously does not reflect the situation in humans who are usually being continuously exposed to low levels of PM via the inhalatory route. Inhalation studies require consideration of species-specific deposition curves. The Figure exemplarily illustrates total and alveolar deposition of 3 µm particles in various laboratory animal species. Note the substantial variation in total deposition from 20% to 80%, and also the fact that total deposition is not related to the dose delivered to the alveolar region. Beside anatomical differences, breathing pattern (see Table), e.g. shallow and fast vs. slow and deep breathing, and the route, i.e. nasal vs. oral breathing, have a substantial impact on the amount and site of deposition. In the mouse, for example, most 3 µm particles are



delivered to the nose and only very few of them reach the alveolar region due to the fact that mice are obligate nose breathers with high nasal filtration efficiencies for this particle size range. Deposited insoluble particles are mainly cleared via two mechanisms: (i) the mucociliary escalator in the conducting airways, and (ii) the alveolar macrophages in the lung periphery. Particle clearance efficiencies differ substantially between species, which is of specific importance for adequate dosing in subacute and chronic exposure studies. Rats, for example, which are commonly used in chronic exposure studies, may clear up to 80% of insoluble particles from the alveolar region within 100 days while humans only clear 10%.

Animal models of asthma There is epidemiological evidence that PM can cause exacerbation of asthma, i.e. increase frequency and severity of asthma attacks in individuals pre-sensitized to allergens. The role of pollution in the development of asthma is less clear, but diesel exhaust exposure or high levels of metal-containing PM have been inflicted to act as adjuvants and support the induction of a Th2 immune response, thus increasing the potential for a genetically susceptible individual to become sensitized. The latter hypothesis has been studied in different experimental animal models, with measuring endpoints focusing on inflammatory responses in the lungs and, in terms of lung function, increased airway resistance and bronchial responsiveness to specific or unspecific stimuli.

Physiological and anatomical parameters of different species

	Mouse (sleep)	Hamster (rest)	Rat (rest)	Guinea pig	Macacus mulatta	Beagle dog	Human	Horse
BW (g)	25	70	250	600	7.000	15.000	75.000	500.000
V_T (ml)	0.2	0.9	1.2	1.75	20	275	500	6000
f (min^{-1})	200	75	100	90	25	15	14	12
MV ($ml\ min^{-1}$)	40	67.5	120	157.5	500	4.125	7.000	72.000
TLC (ml)	1.5	7	10	15	500	2.000	6.500	45.000
S_A-nose (cm^2)	-	-	10.4	27.4	61.6	220	181	
S_A-alveolar (m^2)	0.1	0.25	0.5	1	13.3	65	143	1171

BW: Body weight, V_T : tidal volume, f: respiratory rate, MV: minute ventilation, TLC: total lung capacity, : S_A-nose: surface area of nasal airways, S_A-alveolar: surface area of alveolar space

The first model of allergic airway disease, introduced almost 90 years ago, were ***guinea pigs*** (GP) followed by rabbits. Anatomically, GP have well-developed airway smooth muscles, a favourable feature in comparison to other small animals like rats and mice. GP can easily be sensitized to antigens. An antigen challenge of allergic GP produces an acute, histamine-mediated bronchoconstriction, a transient agonist-dependent airway hyperreactivity and substantial lung eosinophilia, and GP may also develop a late phase response to the antigen. A major drawback is that the immune response is usually mediated by a subclass of IgG (IgG1) rather than IgE so that the response may not reflect atopic asthma in humans. With respect to lung physiology, spontaneous breathing is characterized by a relatively high respiratory rate (see Table). There are several options to assess airway resistance and responsiveness to agonists in GP. Classical measurements of resistance and compliance as well as ventilatory parameters can be obtained in anesthetized GP by single chamber bodyplethysmography. In conscious animals, a double chamber plethysmograph - consisting of a head and a body chamber - allows measurement of ventilatory parameters, but only specific airway resistance and conductance can be determined. Finally, there is the option of whole body plethysmography, where unrestrained animals can move freely within the chamber and ventilatory parameters are derived from pressure swings in the chamber induced by gas conditioning in the body (i.e. heating and humidification) and alveolar gas compression. Airway responsiveness is derived from Penh, an index inferred from the pattern of inspiratory and expiratory timing and flow rates. Because of its easy applicability, whole body plethysmography has become

a very common approach, but it has to be emphasized that the parameter Penh depends on many factors, i.e., it is sensitive but not specific, and it is not a direct measure of airway mechanics. A cautious interpretation of results is therefore mandatory.

Among larger species, sheep and dogs have often been used as allergic models. ***Sheep*** can develop sensitivity to *Ascaris suum* and exhibit early and late phase allergic responses. The sheep model does resemble human disease in terms of pulmonary inflammation, increased levels of IgE and a nonspecific airway hyperreactivity, but there is little evidence for spontaneous bronchoconstriction or persistent obstructive airway disease. Sheep are unique in that restrained but conscious animals can be instrumented through the nasal passages with an esophageal balloon and a cuffed endotracheal tube so that repeated measurements are possible. Monitoring of flows and pressures allows assessment of compliance and airway resistance changes in response to mediators of airway constriction.

Sensitivity and responsiveness to allergens seem to have a genetic component in ***dogs***. Basenji-Greyhounds have constitutional airway hyperreactivity, and distinct breeding colonies have been established of Beagle dogs which are prone to develop allergic sensitivity. Ragweed immunized dogs have increased mucus production, and the number of eosinophils in their lungs correlates with serum IgE levels. Dogs exhibit airway hyperreactivity and obstruction, but they do not wheeze. The large size of their conducting airways - 175 ml in a Beagle dog with 1750 ml lung volume - may reasonably account for this finding. Dogs have been used as experimental animals for a long time, and a whole array of methods has been customized for the characterization of canine lung function. Beside various techniques for the measurement of respiratory mechanics, more sophisticated parameters like airway volume and intrapulmonary gas mixing can be derived from foreign-gas wash-out measurements which are well established and compare to these techniques as applied in clinical studies. Usually, the experimental animals are anesthetized, but respiratory impedance measurements by impulse oscillometry have been reported in trained, conscious animals.

Rats and mice have most extensively been used as models for allergic airway disease. Among ***rats***, the Brown Norway rat model is widely established. Their inflammatory response to antigens shows several features of the human allergic response, in particular elevations of Th2 cytokines like IL-4 and IL-5 and a reduction of the Th1 cytokine IFN-γ. Airway hyperreactivity and early and late-phase responses after antigen challenge can be observed. Allergic bronchoconstriction seems, however, to be primarily mediated by serotonin rather than histamin. Moreover, the rat is a weak bronchoconstrictor to unspecific stimuli, and high levels of mediators are required for bronchial challenge. With respect to respiratory physiology, rats have much higher breathing rates than humans: about 100 breaths/min at rest but 300 to 400 breaths/min when active and stressed. The implications for intrapulmonary gas transport are obvious, but high breathing rates also affect particle transport and deposition in the lungs which is of importance for inhalative challenges. Several options exist for the assessment of respiratory mechanics in rats: the techniques introduced for GP are available for rats, and forced expirations as well as the more sophisticated forced oscillation technique are also well established.

In the past 10 years, the ***mouse*** has experienced a remarkable “career” as an experimental animal, and more is probably known about its immune responses and genetics than of any other species. There are significant differences among inbreed mouse strains in their intrinsic responsiveness to bronchoconstrive agents and to the ability to produce allergic responses. A/J and BALB/c mice are most often used as susceptible strains. Their allergic airway inflammation resembles in many important ways that of humans, namely with respect to airway hyperresponsiveness, Th1/Th2 cytokine profile, eosinophilic airway inflammation, and airway remodeling in terms of mucus-cell hyperplasia and metaplasia

and thickening of basement membranes. Moreover, the broad spectrum of genetically engineered mice allows targeted studies on the role of specific cytokines, cells or pathways in the pathogenesis of allergic airway disease. One of the major disadvantages of the mouse model is that mice seem to develop tolerance upon repeated allergen exposures, and the fading of obstructive and pulmonary inflammatory responses precludes the study of chronic effects. Besides, there are substantial differences in respiratory physiology in comparison to humans. Respiratory rates in mice are extremely high. Even in sleeping animals frequencies of 150 to 200 breaths/min are common. Consistent with their breathing pattern, conducting airway volume is relatively large and may exceed 20% of lung volume. Techniques for assessing murine respiratory mechanics and airway reactivity have substantially been refined in the last years, and all techniques applicable to rats are also available for mice. Yet, reliable measurements are still difficult to obtain due to the small animal size, with lung volumes ranging between 1 ml and 2 ml. As in rats, high levels of agonists are required to induce bronchoconstriction. Agonists are often injected intravascularly or intraperitoneally. This approach bears the risk of considerable cardiovascular side effects and the potential for adrenergic responses which affect bronchoconstriction. Inhalative challenges via the nasal airways are, however, hampered by the high nasal filtration efficacy for particle sizes as produced by common nebulizers (1.5 to 3 μm).

Conclusion: It is evident, that no single animal model will be suitable to reproduce the full spectrum of human physiology, anatomy and disease. Exposure studies must be performed with an awareness of the limitations and strengths of currently available animal models. Besides, the assessment of lung function and airway responsiveness in animals frequently involves anesthesia which affects lung and cardiovascular performance and also autonomic reflexes. Finally, the species of first choice can often not be used for ethical reasons, economic aspects or the capabilities of the available animal care facility.

THE MOUSE MODEL AT THE INTERFACE BETWEEN PATHOLOGY AND GENETICS: FROM THEORY TO PRACTICE USING PNEUMOTROPIC *PARAMYXOVIRIDAE*

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The *Paramyxoviridae* family includes some of the most important and ubiquitous disease-causing viruses, most of which cause significant infections of the respiratory tract. Evidence is accumulating in humans as well as in animals that genetic factors are involved in the severity of clinical presentation. As a first step toward the identification of the genes involved, a set of studies was undertaken to establish whether laboratory mouse strains differ in susceptibility to Sendai virus, the murine counterpart of human type-1 parainfluenza virus, and to the pneumonia virus of mice, which is the murine counterpart of bovine and human respiratory syncytial viruses.

With this purpose in mind, double–chamber plethysmography data were collected daily for 7 days after inoculation of the viruses in six inbred strains of mice. In parallel, histologic examinations and lung viral titration were carried out from day 5 to day 7 after inoculation.

Pulmonary structure/function values closely reflected the success of viral replication in the lungs and revealed a pattern of continuous variation, with resistant, intermediate and susceptible strains. The results unambiguously suggest that (1) BALB/c (resistant) and 129Sv (susceptible) strains should be used in crossing experiments aimed at identifying the genes involved in resistance to *paramyxoviruses* and (2) SJL (resistant) and DBA/2 or 129/Sv (susceptible) should be used in crossing experiments aimed at identifying the genes involved in resistance to *pneumoviruses.*

MOUSE MODEL OF PNEUMOCOCCAL PNEUMONIA

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Community-acquired pneumonia (CAP) is a significant cause of morbidity worldwide. In severe cases, CAP may result in acute respiratory failure requiring mechanical ventilation, and sepsis [1]. Despite the availability of potent antibiotics, mortality of CAP remains high. In most cases, lung failure due to pneumonia is caused by inadequate host-pathogen responses [2]. Therefore, a better understanding of host-pathogen interactions is needed.

Streptococcus pneumoniae is the most important causal pathogen identified in CAP [3, 4]. When pneumococci enter the lung, they are recognized by diverse receptors. Cell wall phosphorylcholine of the pathogen binds to the receptor for platelet activating factor (PAF) [5], which is an essential step in cell invasion. Further, recognition of intact pneumococci by toll-like receptor (TLR) 2 was recently demonstrated by our group [6], and in the same study pneumolysin, an exotoxin and important pathogenic factor of *S. pneumoniae*, was recognized by TLR 4 [7]. Following invasion into epithelial cells, pneumococci were recognized by the cytosolic receptor nucleotide-binding oligomerization domain (NOD) 2 [8]. Activation of TLRs and NODs by pneumococci or pneumolysin evoked NFκB translocation into the nucleus, and generation of different cytokines.

To investigate the host response to pneumococcal invasion *in vivo*, we employed a mouse model of pneumococcal pneumonia. C57/Bl6 mice, transnasally infected with serotype 3 pneumococci, developed clinical signs of severe illness within 24 hrs, including loss of body weight, reduced activity, decreased body temperature, and dyspnea. Lung function was impaired, as assessed by measuring lung compliance and oxygenation index. Bacteremia and sepsis developed in the later course of disease. Survival-time and -rate depended on the number of pneumococci used for the infection. In sum, the clinical course of murine pneumonia in this model was partly comparable to severe human pneumonia.

NFκB translocation into the nucleus results in the production of different cytokines. Well in line with the *in vitro* observations on pathogen recognition and NFκB activation, lung levels of diverse proinflammatory mediators were increased in murine pneumonia, including Interleukin (IL-)15. The expression of IL-15 was upregulated in bronchial and alveolar epithelium (unpublished data). IL-15 has critical impact on the homeostasis and activation of NK, NKT, γδT and CD8+T cells, and contributes to antimicrobial defense particularly at mucosal sites. Notably, IL-15 neutralization with soluble IL-15 receptor α dramatically increased mortality, which was independent of NK or NKT cell function, because depletion of these cells did not alter survival (unpublished data). Further studies may clarify the mechanism of IL-15 contribution to survival and evaluate the therapeutic perspective for IL-15 treatment in pneumococcal pneumonia.

The activation of the immune system by proinflammatory cytokines evokes neutrophil recruitment to the site of inflammation, which is crucial in host defense. However, the mechanism of neutrophil trafficking in the lungs is not unequivocally clear. In a recent study, we defined a role for angiopoietin 2 in neutrophil recruitment in several models of inflammation [9]. Angiopoietin 2 is an autocrine ligand of the receptor tyrosine kinase tie2, which is rapidly released from endothelial stores upon endothelial cell activation. Though being important for neutrophil recruitment in many organs, angiopoietin 2 was not involved in neutrophil trafficking in pneumococcal pneumonia [9].

Lung neutrophil activation may have beneficial effects in host defence, but may also evoke pulmonary hyperpermeability, contributing to edema formation and acute respiratory failure. Interestingly, in *S. pneumoniae* infected mice, early-onset lung microvascular leakage was observed before neutrophil influx started. We hypothesized that a pneumococcal pathogenic factor may serve as the direct causative agent, and focused on pneumolysin. By studying the impact of aerosolized or intravascular recombinant pneumolysin in a model of isolated perfused and ventilated mouse lungs, we found that pneumolysin may play a central role for early-onset acute lung injury due to severe pneumococcal pneumonia by causing impairment of pulmonary microvascular barrier function, and severe pulmonary hypertension [10]. Further studies suggested an important role for platelet-activating factor in both hyperpermeability and pulmonary arterial hypertension, caused by pneumolysin (Witzenrath et al., submitted).

Although potent antibiotics against *S. pneumoniae* are still available, treatment of pneumococcal pneumonia has become more difficult in recent years, due to increasing resistance of the pathogen against multiple antibiotics [3]. Therefore, alternative treatment strategies need to be investigated. We employed a lytic enzyme (Cpl-1) of a pneumococci-specific bacteriophage [11] to treat pneumococcal pneumonia (Witzenrath et al., submitted). According to clinical appearance, functional parameters and morphologic changes in the lungs, mice suffered from severe pneumonia at the onset of therapy. All mice treated with Cpl-1 survived otherwise fatal pneumonia. Cpl-1 distinctively reduced pulmonary bacterial counts, prevented bacteraemia and systemic hypotension, and evoked rapid convalescence. Bacterial lysis by Cpl-1 did not cause significant inflammatory response *in vivo*, as determined by multiplex cytokine assay of lung and blood samples. In vitro experiments provided evidence for the specificity of Cpl-1 for *S. pneumoniae*, as neither other pathogens nor host cells were affected by the enzyme. Thus, Cpl-1 may provide a therapeutic perspective in pneumococcal pneumonia.

In summary, mouse models of pneumococcal pneumonia allow investigations of pathogen-host interactions, which aim at the development of new therapeutic perspectives.

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MODELS OF PULMONARY INFECTION - MORPHOLOGY AND EVALUATION

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Models of pulmonary infection are useful tools in basic as well as in applied research. They are used in human and veterinary medicine to study the pathogenesis of bacterial and viral infections. Furthermore, they are applied to test the effects of drugs against the pathogens of these infections [5].
Depending on the problems to be investigated, the experiments are carried out in rodents (rat, mouse) or non-rodents (dog, pig, cattle). The handling of rodent studies is somewhat easier in terms of application, animal maintenance, necropsy etc. . Additionally, from rodent studies many data are available for the comparison with own results. Concerning the morphology of the lungs, rodents have the advantage that whole lung specimens can be evaluated, whereas from non-rodent lungs only small parts can be investigated. In general, the infection of the animals is carried out via intranasal or intra-tracheal application, in single cases intra-bronchially [7]. Following an intranasal application, the microbiological spread in the respiratory tract and other organs/tissues can be studied with close relation to the field situation.

Study design
Prior to study begin, all scientific disciplines involved should decide about the study design including details of pathomorphological evaluation. The following questions have to be answered :

1. Which route of application will be applied?
2. How often are pathogens applied and which interim sacrifices and/or recovery periods have to be taken into account?
3. Which control groups are included (negative, positive, vehicle)?
4. Where (in which organs/tissues) are induced effects to be expected?
5. Which organs/tissues should be fixed, trimmed and evaluated?
   - Lungs (how detailed)?
   - Other organs/tissues of the respiratory organs?
   - Extra-respiratory organs?
6. Is a blinded evaluation preferred?
7. Which endpoints/methods are scheduled (clinical findings, lung function testing, BAL, radiology, hematology etc.) and are there mutual interferences concerning specimens, results etc.?

Methods
Following a detailed gross examination during necropsy including organ weight determination, trimming, paraffin embedding and light microscopical evaluation are carried out. Apart from “normal” H&E-stained slides, several special staining and immuno-histochemical methods can be applied to demonstrate morphological structures in the respiratory tract. In the case that special questions are addressed, other methods might be included, e.g. electron microscopy (TEM, SEM), proliferation markers (PCNA, BrdU), morphometry. With these additional methods the effects of pulmonary infection models can be clarified up to subcellular findings and secondary effects, like localized increasing of cell proliferation and inflammation associated mediators [4].

Evaluation

The pathomorphological evaluation on the one hand can be done qualitatively. This approach can generally answer the question whether or not inflammatory findings occur. Furthermore, detailed findings such as hemorrhages, necroses, thromboses, interstitial alterations or involvement of the pleura can be recorded [2, 6]. Additional relevant findings occurring in the organs/tissues after respiratory/pulmonary infection are certain cell populations (e.g. PMNs, eosinophils, mast cells) or fibrin and collagenous fibers. The qualitative evaluation gives also information on the location of the lesions and the affected lung lobes.

Evaluating bacteria induced lesions, findings in the airways, in the alveolar region and, species-depending, in the interstitium can predominantly be expected. In virus-induced models the bronchus-associated lymphoid tissue (BALT) is frequently altered [1, 5, 6].

However, an optimal application of histopathology should include semiquantitative evaluation of the lesions, possibly with a computer program such as for example PathData®, and a grading of the findings from „0“ to „3“ or „0“ to „5“ [2, 7]. This has the advantage that morphological results without detailed morphometry can easily be compared with the results from other methods which primarily give quantitative data (lung function testing, BAL).

If several specimens from various locations are evaluated, the semiquantitative histopathology provides the possibility statistical evaluation (mean and standard deviation) for certain locations, animals and groups [3]. This so-called „Lung Inflammation Index“ (L I I) is an indication for the degree of pulmonary inflammation. In studies testing antibiotics, this index – mostly in comparison with a positive control group – is a clear indication for an anti-inflammatory effect of the test substance.

Additional (“real”) morphometry might be necessary if semiquantitative methods are not sufficient to detect differences between the groups, if a no-effect level can not be established, or if a comparison of different models and substances has to be done.

However, a comparison of results from different models/studies is only possible, if morphological methods in the experiments are the same concerning necropsy technique, collection of specimens, fixation, trimming and histopathological diagnosis, including terminology.

Assessment

In general, the assessment of morphological findings differentiates between artifacts, spontaneous findings, handling-induced lesions and “real” effects of the infection, the substance or the treatment. Control groups (positive and negative) are indispensable for this assessment.

In relation to the quality and the grading of morphological effects, the assessment includes the differentiation of for example degenerative or proliferative lesions, and if the alterations adaptive and/or reversible.

The results of morphological evaluation in models of pulmonary infection have an important impact on the validity of the model. In many cases, they give relevant information to clarify the pathogenesis of a disease model or the efficacy of the test compound. During drug development, morphological results, together with other methods, are necessary to decide further steps of the developmental process.

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EXHALED MARKERS OF AIRWAY INFLAMMATION IN ANIMALS AND ANIMAL MODELS

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Because exhalate is the product of alveolar gas exchange and airway water loss, it most likely contains metabolites from the lungs or substances originating from biochemical reactions in the airway mucosa. With respect to respiratory physiology and the metabolic functions of the lung, various gaseous and non-gaseous components have been detected in the exhaled breath of humans and animals.

Measurements of volatile substances in exhaled breath have a long history. In traditional Chinese medicine, strong body scent, bad breath and strong smelling stools or urine were attributed to different indications. Hippocrates also recognised that abnormal odours were associated with certain conditions. For example, in the text Aphorisms, there is an entry which reads "In persons affected with phthisis [an ancient Greek term for Tuberculosis], if the sputa which they cough up have a heavy smell when poured upon coals….". Measurement of ammonia in exhaled breath was first reported approximately one hundred years ago and in last 100 of so years a number of odours have been associated to different medical conditions.

Odour	**Site/Source**	**Disease**
Baked brown bread	Skin	Typhoid
Stale beer	Skin	Tuberculosis
Butchers shop	Skin	Yellow fever
Grape	Skin/sweat	*Pseudomonas* infection
Ammoniacal	Urine	Bladder infection
Rancid	Stool	Shigellosis
Freshly plucked feathers	Sweat	Rubella
Sweet	Sweat	Diphtheria
Foul	Infant stool	Cystic fibrosis
Full	Sputum	Bacterial infection
Putrid	Breath	Lung abscess
Acetone-like	Breath	Diabetes mellitus
Sweet/fruity or boiled cabbage	Breath (infant)	Hypermethionemia

In modern human medicine, exhaled nitric oxide (NO), but also carbon monoxide (CO) and other gases, including breath alkanes (e.g. ethane, pentane) are measured in exhaled breath and viewed as markers of airway inflammation. In animals, measurements of NO in exhaled breath have been reported in elephants, horses, cattle, pigs, sheep, dogs and cats, as well as in anaesthetised and ventilated laboratory animals, including mice, rats, guinea pigs and rabbits.

From the current knowledge concerning exhaled NO analysis in human medicine, a number of limitations arise in applying exhaled breath analysis to spontaneously breathing animals:

- The concentration of NO in exhaled breath is dependent on the expired flow rate, which cannot easily be regulated in conscious animals.
- Due to the very high NO production in nose and paranasal sinuses, nasal NO should be eliminated from NO measurements in exhaled breath in order to assess the peripheral respiratory system and airway inflammation. However, nasal breathing is the predominant route of ventilation in most animals and is obligatory in the horse.
- Larger animals such as the horse and cow also have a significantly greater proportion of tidal volume that is dead space (somewhere between 50% and 75%), which is primarily due to a larger anatomical dead space.
- Inspired air must not contain any NO.
- The N_2-balance of the total organism may influence exhaled NO levels (carnivores are different from herbivores; the protein content of the diet could affect exhaled NO).
- NO analysers are mainly based on chemiluminescence (and are still relatively expensive).

Despite these biological and technical limitations the concentration of NO in exhaled breath has been successfully determined in both cats and horses.

There are far fewer studies of the concentration of CO in exhaled breath compared to NO in either human subjects or animals. However, a number of studies have demonstrated that exhaled CO concentration is greater in human patients with asthma, chronic obstructive pulmonary disease or cystic fibrosis compared to healthy controls. CO has been demonstrated to be undetectable in the exhaled breath of both healthy horses and cats (<0.1 parts-per-million, ppm). There are a wide range of volatiles that can be measured in exhaled breath and therefore there is the potential for application of techniques such as GCMS, SIFT-MS and electronic nose as opposed to analysis of single, specific volatiles which may afford greater potential to diagnose specific conditions rather than generalised airway inflammatory responses.

As an alternative to the measurement of volatile markers in exhaled breath, a variety of compounds can be measured in condensed exhalate. There is increasing interest in this technique, because the method of condensate collection is simple, completely non-invasive, repeatable and does not necessarily require patient cooperation. The rationale for making measurements in EBC is based on the hypothesis that water and aerosols (which are present in the exhaled breath) contain a range of compounds which reflect the concentrations within the extracellular epithelial lining fluid in the peripheral airways. To date, EBC samples have been collected in horses, calves, pigs, dogs and cats using different collection methods. A variety of custom made systems have been described to collect EBC from conscious, non-sedated animals The only commercially available system that has been used in animals is the ECoScreen (Viasys Healthcare, Hoechberg, Germany) and its use has been limited to calves and pigs in combination with a facemask using a non-rebreathing valve.

Substances measured in the EBC of animals include H_2O_2, LTB_4, urea, ammonia and hydrogen ion activity (pH). The collection system configuration, materials used in construction and level or pattern of ventilation can have a marked effect on the composition of EBC from animals.

This presentation will review evidence for the various markers that have been measured in exhaled breath of animals or animal models as indicators of non-infectious airway inflammation.

INFLUENCE OF RESPIRATORY INFECTIONS ON ACTIVE ION TRANSPORT IN CALVES

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Objectives: Mucus clearance is an important component of the lungs innate defence against diseases. The ability of the airways to clear mucus is strongly dependent on the rate of ciliary beating and the hydration state of the airway surface liquid (ASL). The aims of this study were (i) to determine physiological mechanisms and rates of ion transport and (ii) to show the influence of respiratory infections on active ion transport mechanisms in calves.

Methods: The airway mucosa of 4 adult cows, 8 healthy calves, 18 calves experimentally infected with *Mycoplasma (M.) bovis,* and 1 calf with chronic pneumonia was dissected and mounted as flat sheets in Ussing chambers. Under short circuit conditions, resistance (R_t), current (I_{sc}), voltage (PD) were measured. Both the unidirectional mucosa(m)-to-serosa(s) and s-to-m fluxes (J_{ms}, J_{sm}) of Sodium (Na), Chloride (Cl) and Mannitol (J_{ms}) were determined.

Results: Under short circuit conditions, there was a net flow of Na in the absorptive (m→s) direction and a net movement of Cl in the opposite (s→m) direction in both cows and calves. In calves, the s-to-m Na transport was significantly higher and therefore the net absorption was significantly lower compared to cows. The Cl secretion was nearly equal in both, calves and cows, but the Cl transport in calves was significantly higher. Calves showed also a significantly higher paracellular (Mannitol) movement. The sum of both net fluxes, Cl and Na, was calculated from matched experiments and was found to be slightly, but significantly different from the measures $I_{sc.}$ Probably there is a transport of HCO_3^-. In *M. bovis*-infected calves (subclinical model) ion fluxes were determined 3, 7, 10, 14, 21 and 35 days post infection (p.i.). It was shown that neither Na- and Cl- nor Mannitol-fluxes changed during the first 35 days p.i.. Addition of amiloride, a Na blocker (Na channel ENAC and Na/H-exchanger NHE), caused continuously decreased reactivity in I_{sc} over 35 days from 14,37 % to 5,46 % and had nearly no effect in some tissues. Only 2 animals showed mild cough and nasal discharge at the day of section (day 7-10). Compared to calves without cough and nasal discharge, the Na transport (J_{ms}, J_{sm}) in calves with cough and nasal discharge was lower but the net Na absorption was nearly equal in both. There was no difference in Cl transport but a significant lower transport of paracellular mannitol in calves with cough and nasal discharge. The chronic purulent bronchopneumonia caused by parainfluenza virus type 3 and *Pasteurella multocida* of a calf caused under short-circuit conditions a significant increase in $J_{sm}Cl$, indicating changes in net secretion of Cl, and the paracellular transport of 3-H-Mannitol decreased significantly. The Na transport was higher but the net absorption of Na was not affected.

Conclusions:
1. The active ion transport differs age-dependent.
2. Respiratory infections cause a change in active ion transport.
3. The change of active ion transport and clinical symptoms act simultaneously.
4. Tight-junctions are tighter in calves with clinical signs.
5. Probably *M. bovis* influences the apical Na-channel.