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## Experimental approaches to evaluate respiratory allergy in animal models

Jürgen Pauluhn<sup>a,\*</sup>, Ulrich Mohr<sup>b</sup>

<sup>a</sup>*Institute of Toxicology, BAYER HealthCare, Building No. 514, Wuppertal 42096, Germany*

<sup>b</sup>*Fraunhofer Institute of Toxicology and Experimental Medicine (FhG ITEM), Hanover, Germany*

Received 6 June 2004; accepted 5 October 2004

### Abstract

Asthma is defined as a chronic disease of the entire lung and asthma attacks may either be immediate, delayed or dual in onset. Allergic asthma is a complex chronic inflammatory disease of the airways and its etiology is multifactorial. It involves the recruitment and activation of many inflammatory and structural cells, all of which release mediators that result in typical pathological changes of asthma. A wealth of clinical and experimental data suggests that allergic asthma is due to an aberrant lung immune response mediated through T-helper type 2 (Th2) cells and associated cytokine-signaling pathways. The pathology of asthma is associated with reversible narrowing of airways, associated with prominent features that involve structural changes in the airway walls and extracellular matrix remodeling including abnormalities of bronchial smooth muscle, eosinophilic inflammation of the bronchial wall, hyperplasia and hypertrophy of mucous glands. The primary objective of respiratory allergy tests is to determine whether a low-molecular-weight chemical (hapten) or high-molecular-weight compound (antigen) exhibits sensitizing properties to the respiratory tract. This may range from reactions occurring in the nose (allergic rhinitis), in the bronchial airways (i.e., allergic bronchitis, asthma) or alveoli (e.g., hypersensitivity pneumonitis). Current assays utilize several phases, viz. an induction phase, which includes multiple exposures to the test compound (sensitization) via the respiratory tract (e.g., by intranasal or intratracheal instillations), by inhalation exposures or by dermal contact, and a single or multiple challenge or elicitation phase. The challenge can either be with the chemical (hapten), the homologous protein conjugate of the hapten or the antigen. The choice depends both on the irritant potency and the physical form (vapor, aerosol) of the hapten. The appropriate selection of concentrations (dosages) both for the induction and elicitation of respiratory allergy appears to be paramount for the outcome of test. Endpoints to characterize positive response range from the induction of immunoglobulins, cytokine or lymphokine patterns in serum (or the lung) to (patho-)physiological reactions typifying asthma. None of the currently applied animal models duplicate all features of human asthma. Accordingly, the specific pros and cons of the selected animal model, including protocol variables, animal species and strain selected, must be interpreted cautiously in order to arrive at a meaningful extrapolation for humans.

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**Keywords:** Animal models; Respiratory allergy; Respiratory function; Lung remodeling; Bronchoconstriction

\*Corresponding author. Tel.: +49 202 368909; fax: +49 202 364589.

E-mail address: [juergen.pauluhn@bayerhealthcare.com](mailto:juergen.pauluhn@bayerhealthcare.com) (J. Pauluhn).

## Introduction

Allergic asthma is a complex chronic inflammatory disease of the airways and its etiology is multifactorial. It involves the recruitment and activation of many inflammatory and structural cells, all of which release inflammatory mediators that result in typical pathological changes of asthma (Barnes et al., 1998; O'Byrne and Postma, 1999). Several features of asthma can suitably be investigated in animal models. These features include cellular infiltrations in the lung, antigen-specific IgE production, and a predominant T-helper type 2 (Th2) immune response characterized by elevations in the levels of typical cytokines seen upon allergen (haptens) sensitization and challenge. The mechanisms that control CD4<sup>+</sup> T lymphocyte polarization to allergenic Th2 phenotypes are incompletely understood but seem to involve genetic predispositions, local factors such as pre-existing cytokine concentrations and inflammation, and antigenic factors that include potency, dose, frequency and duration of exposure. The number of mediators involved in the sensitization process to an allergen and/or the development of a chronic inflammatory process in the mucosa of the lower airways, including airway remodeling, tends to confer an image of overwhelming complexity (Henderson et al., 2002; Leigh et al., 2004; Pauwels et al., 1997). Airway hyperreactivity is defined as an exaggerated acute obstructive response of the airways to one or more non-specific stimuli, often associated with airway epithelial damage and disruption, a common feature of even mild asthma (Laitinen et al., 1985). Increased hyperresponsiveness may also be associated with direct exposure of sensory nerve endings (Barnes, 1986) or by the loss of enzymes which metabolize sensory neuropeptides (Frossard et al., 1989; Barnes et al., 1991). Neurotrophins are important mediators between the (systemic) immune system and the local nervous system (Carr et al., 2001). Rather than having unique actions on immune cells, the neurotrophins often act in concert with known immune-regulating factors. They are produced locally during the allergic reaction and serve as amplifiers for Th2 effector functions and thus play an important role in the development of inflammation and airway hyperresponsiveness (Braun et al., 2000). This demonstrates that the microenvironment adjacent to the site of injury and/or sensitization and challenge may be important for the progression of disease. Commonly, animals are sensitized by two successive occasions, followed by challenge with the antigen (or haptens). This primary allergen challenge results in an asthmatic phenotype. However, to more closely resemble the human disease, secondary allergen challenges, after prolonged gaps, are often used. The route, method and dose of allergen exposure also determine the phenotype of the allergen response (Kannan and

Deshpande, 2003). The response to short-term, high-level exposures cause different airway lesions when compared low-level, chronic antigen challenge. Also the methods used to assess immediate or delayed bronchoconstriction, the kind and extent of airway inflammation and tissue remodeling have an impact on the outcome of studies involving allergen (haptens)-sensitized animal models of asthma.

The emphasis of this paper is to review methodologies suitable to identify respiratory allergens for the purpose of hazard identification in animal models currently used in toxicology. This includes an analysis how the test design, i.e., route, dose, frequency of dosing, timing of challenge exposures with the haptens or antigens and the endpoint selected, can affect the outcome of study. The validity and the limitations of the various approaches are discussed.

## Mechanisms leading to an asthmatic phenotype

The agents causing respiratory allergy may be present as a gas, aerosol or represent mixture of a volatile haptens partitioned with the aerosol phase. For irritant chemicals it is as yet not clear, for instance, whether induced airway hyperresponsiveness is a dose-dependent phenomenon or whether a brief high-level exposure plays a more important role. High-level exposure to irritant agents may cause airway hyperreactivity considered to be different from typical occupational asthma because of its rapid onset, specific relationship to a single environmental exposure, and no apparent pre-existing period of sensitization to occur with the apparent lack of an allergic or immunologic etiology. Hence, this illness is termed reactive airways dysfunction syndrome, or RADS. Mechanisms to explain the development of RADS focus on the toxic effects of the irritant exposure on the airways and may be attributed to neurogenic effect (Brooks et al., 1985).

The asthma phenotype is characterized by a chronic airway inflammation and inflammatory mediator cell products that orchestrate the disease. Mediator interaction may also occur by "priming" of inflammatory cells, leading then to augmented release of secondary mediators. This implies some uncertainty as to whether exogenously administered agents exert similar effects when the exposure is direct (by inhalation) or via systemic routes. To date, more than 50 different mediators have been identified in asthma, although, at the same time, current gene technology identifies an ever increasing range of molecules that could be involved in the sensitization process to an allergen and/or the development of a chronic inflammatory process in the mucosa of the lower airways. This evolution tends to confer an image of overwhelming complexity. The intricate interaction of structural and mobile cell

populations and the mediators that orchestrate their influx and activation, including their predilection to induce a somewhat self-perpetuating and self-amplifying type of inflammation, is not yet fully understood. Mediators may act synergistically to enhance each other's effect, or one mediator may modify the release or action of another mediator.

This disease is clinically characterized by recruitment and activation of specific inflammatory cells, chemotaxis, bronchoconstriction, increased airway secretion (and mucus cell hyperplasia), plasma exudation, neural effects, hyperplasia and hypertrophy of airway smooth muscle cells, and increased airway hyperresponsiveness which is defined as an exaggerated acute obstructive response of the airways to one or more non-specific stimuli, often associated with airway epithelial damage and disruption, a common feature of even mild asthma (Laitinen et al., 1985). How this increased bronchial responsiveness is precisely triggered, amplified, sustained and how it relates to inflammatory events remains, to a certain extent, incompletely elucidated (Kimber and Dearman, 1997). Many inflammatory cells are recruited to asthmatic airways or are activated *in situ*. These include mast cells, macrophages, eosinophilic and neutrophilic granulocytes, T lymphocytes, dendritic cells, basophils, and platelets. Although in the past much attention has been paid to acute inflammatory responses, such as bronchoconstriction, plasma exudation, and mucus hypersecretion, in asthma, it is increasingly recognized that chronic inflammation and airway remodeling constitute the most important aspects (Roche et al., 1989). Some of these changes may be irreversible, leading to fixed narrowing of the airways. The activation and coordination of allergic inflammatory/immune responses depend upon a complex interaction of multiple cell populations and diverse mechanisms. Numerous experimental and clinical studies have established that CD4<sup>+</sup> T-cell-mediated inflammation of airways is central to the pathogenesis of asthma (Foster et al., 2002). According to dogma, allergic airway responses are dependent upon Th2-type cytokines, including local production of IL-4, IL-5, and IL-13, including signal transduction proteins (Wilk-Karp, 1999). Several studies have suggested that Th1 cytokines actually contribute to the overall allergic microenvironment and play a significant role of the detrimental responses. Thus, the Th2-type cytokines during asthmatic/allergic responses may not be the only cytokines needed for inflammatory responses in the development of severe asthma (Raman et al., 2003) and may explain IgE-independent mechanisms of allergic inflammation (Wilder et al., 1999; Scheerens et al., 1999). Recent evidence support the notion that the 'exposure intensity' at the site of contact, including the additional factors that may enhance/attenuate the penetration/persistence of the hapten/antigen might play

a role. Kinetic factors might be involved that include the proliferation and maturation of dendritic cells at the site of contact and their migration to the draining lymph nodes. Additional components may cause Th1/Th2 imbalance by virtue of their abilities to enhance antigen-stimulated T-cell activation and by affecting the production of cytokines and chemokines in the local draining lymph nodes (Gu et al., 2000). Also the components of the airway wall that are critical in determining the response to inhaled substances are organized in the epithelial-mesenchymal trophic unit (Evans et al., 1999). The basic assumption is that the epithelial, interstitial, nervous (neural), and immunological components have an active interaction with each other.

The distinction between neurogenic and immunogenic asthma is limited to the catalysts because the two types cross over after the release of mediators. The two mechanisms might be differentiated by testing guinea pigs with challenging doses of specific antigens and non-allergic, non-specific pharmacological stimuli while monitoring the respiratory pattern or through the assessment of specific mediators. This situation is complicated further since chemical haptens may evoke non-specific irritation as well as specific allergic hypersensitivity responses and the responses ultimately occurring may be clinically indistinguishable. How this increased bronchial responsiveness is precisely triggered, amplified, sustained and how it relates to inflammatory events remains, to a certain extent, incompletely elucidated (Dearman and Kimber, 1999). Neural control of airways, including axon reflexes, is complex and the extent of contribution of neurogenic mechanisms to the pathogenesis of airway disease is still uncertain. Perturbation of one compartment creates imbalance in all parts of the airway wall, or rather, or a metabolic response in one compartment will produce alterations in the other compartments. This demonstrates that the microenvironment adjacent to the site of injury and/or sensitization and challenge may be important for the progression of disease.

In regard to high-molecular-weight substances, enzymes used in the pharmaceutical, food-manufacturing and detergent industry, and protein antigens from plant and animal sources have been characterized as workplace allergens. The mechanisms of sensitization by which the incriminated agents cause asthma are believed to be IgE-mediated for high-molecular-weight agents whereas for low-molecular-weight agents the immunologic and non-immunologic factors that incite and sustain the inflammatory cascade, which ultimately results in clinical asthma, are still not well understood. The reason(s) for the induction of immediate hypersensitivity to the antigen mediated by a predominant IgE and IgG<sub>1</sub> response may reside in properties of the antigen or allergen itself, the time frame investigated, or the dose and route of sensitization.

### Agents that cause respiratory allergy

Agents used in animal models have broadly been categorized into low-molecular-weight chemical haptens (molecular weight <1000 Da) and high-molecular-weight agents that include proteins from diverse sources, i.e., plants, bacteria, arthropods or animals. The low-molecular-weight agents are reactive chemicals and act as allergen when bound to appropriate carrier molecules, such as autologous proteins; a complete hapten-carrier antigen is then formed. The antigenic determinant may be the hapten or it may be a new antigenic determinant formed by chemical reaction between the hapten and the carrier protein. The incriminated etiologic low-molecular-weight agents all share a common toxicological characteristic of being irritant in nature. In some cases, these agents are present as a gas, in others the inciting agent is an aerosol or represent mixtures of a volatile hapten partitioned with the aerosol phase. The most prevalent class of low-molecular-weight asthmagens include platinum salts, acid anhydrides (e.g., trimellitic anhydride (TMA), phthalic anhydride), diisocyanates, such as diphenylmethane diisocyanate (MDI), toluene diisocyanate (TDI) and hexamethylene diisocyanate (HDI), reactive dyes, derivatives of cyanuric chloride to mention but a few. For a more comprehensive discussion of agents see Selgrade et al. (1994).

The influence of coexposure of particles (e.g., alum, airborne particles) and/or irritants (e.g., ozone) and allergens has provided direct evidence of adjuvant activity or that allergen-induced atopy exacerbates airway dysfunction upon exposures to irritants or particles (Steenberg et al., 2003). Coexposures of particles or irritants with allergens have shown a profound increase in immunoglobulins (IgE, IgG), cytokines typifying a predominant Th2 response, and an influx of eosinophils, however, the timing of coexposures (concurrent vs. sequential) appears to be critical for the outcome of test. The adjuvant effect of particles on the IgE antibody response in ovalbumin sensitized mice was associated with an early increased primary cellular response in the draining lymph node (Nygaard et al., 2005). Last et al. (2004) showed that ozone appears to antagonize the specific inflammatory effects of ovalbumin exposure, especially when given before or during exposure to ovalbumin.

### Advantages and disadvantages of current animal models

Studies of allergic sensitization employed single and multiple sensitization dosage regimens to non-irritant (low dose) to irritant (high dose) concentrations. Routes

range from injection (intra-/subcutaneous, intraperitoneal with or without adjuvant), epicutaneous exposure, bolus instillation (intratracheal/intranasal) or steady-state inhalation exposures over short or long periods of time (Sarlo and Karol, 1994). Commonly little attention has been directed towards the identification and quantification of induction-related local irritant/inflammatory responses and the ensuing systemic effects. Thus, with regard to common denominators amongst the variable protocols employed, it is difficult to judge the impact of a specific protocol design on the specific findings observed. With regard to antibody production (e.g., specific IgG<sub>1</sub>, total IgE), a concentration – number of booster or challenge exposure – relationship has been noted in many studies. For example, Kumar et al. (2000) found that whereas IgG-synthesizing cells were undetectable in normal airways, the overwhelming majority of immunoglobulin-producing cells in mice chronically challenged by inhalation with aerosolized antigen exhibited cytoplasmic immunoreactivity for IgG. The local immunoglobulin production does not contribute significantly to the induction of an IgE-mediated response. Animal models can be tailored for the production of IgE, even in guinea pigs where large doses of antigen tend to support earlier and more intense bronchopulmonary hypersensitivity and favor IgG, whereas smaller doses favor mixed IgE and IgG production (Pretolani and Vargaftig, 1993). The exposure concentration of the free chemical to elicit pulmonary responses in short-term animal models is commonly much higher than that recognized to elicit responses in humans. The available experimental evidence appears to suggest that a threshold concentration needs to be exceeded for responses to occur. The high-level exposure commonly used in animal models triggers acute inflammation in the lung parenchyma, especially perivascular and peribronchiolar inflammation, which is markedly different from the acute-on-chronic inflammation of the airway wall observed in human asthma (Cohn, 2001; Kumar and Foster, 2002). Moreover, the pattern of acute inflammation differs in several respects from that observed in individuals with asthma. Perhaps the most significant deficiency of the commonly used models of allergic bronchopulmonary inflammation is that, by their very nature, they involve short-term experiments. Thus they do not exhibit many of the lesions that typify chronic human asthma with regard to the intra-epithelial accumulation of eosinophils in the intrapulmonary airways, the chronic inflammation of the airway wall, and its remodeling that may have important consequences to both airway hyperreactivity and development of fixed airflow obstruction (Kumar and Foster, 2002). As to whether these divergent and conflicting results amongst animal models and humans are related to the extreme variety of sensitization and challenge protocols employed,

including the different readouts to define disease or response to challenge, remains obscure. Differences in susceptibilities related to the dosing of critical structures within the respiratory tract in obligate nasal breathing laboratory animals and oronasally breathing humans inflicted with airway disease contributes to the difficulty to define any “most appropriate” animal model. Moreover, for highly reactive low-molecular-weight chemicals there is need for synthesis of a chemical-carrier protein conjugate for the elicitation of pulmonary hypersensitivity when the challenge by inhalation in order to adequately dose the critical target within the lung. However, when instilled intranasally positive response were shown to occur also with the non-conjugated hapten (Vanoirbeek et al., 2003, 2004; Scheerens et al., 1996).

Despite these variables, animal models are indispensable for studies requiring an intact immune system, especially for studying the pathogenetic mechanism(s) in atopic diseases, modulator regulation, and related biologic effects. When designing an *in vivo* model of respiratory allergy, it is important to clearly decide which aspects of this complex disease is the focus. In addition to expedience and practicability, the species and strain selection, the sensitization and challenge protocols as well as the endpoint(s) chosen are paramount for the outcome of test. The relative abundance of biomarkers to probe disease may change from one compartment to another and, for example, it may be different in serum, lung parenchyma and draining lymph nodes or bronchoalveolar lavage fluid (BALF) (Schuster et al., 2000). In terms of endpoints, those that integrate independently a series of complex events might be most practical to probe positive response in animal models in order to detect lung-sensitizing agents. Suffice it to say, the limitations of most of the currently applied bioassays are that they model the acute rather than the chronic manifestation of asthma. Apart from the difficulties in transposing rodent to human data, it has to be borne in mind that each animal model is a trait associated with asthma, rather than for modeling the entire asthma phenotype (Kips et al., 2003).

### Species selection

Several rodent and non-rodent species have been used for respiratory allergy studies. The choice of species is made, primarily, with a view to extrapolating the experimental results to man. However, when selecting a particular species for a study, additional factors must be considered, such as the comparative morphology of the respiratory tract, the presence or absence of lung disease or susceptible states, and the similarity of biochemical and physiological responses to those in

man. The choice of animal models may also be based on practical considerations rather than validity for use in human beings. An animal species must be small enough to allow handling and exposure in sufficient numbers in relatively small inhalation chambers and large enough to allow measurement of all endpoints relevant to identifying the inherent toxicity of the substance under investigation. Most of the animal models are associated with an allergic alveolitis or hypersensitivity pneumonitis, which may overshadow the inflammatory lesions of the airways. However, using controlled exposure to low mass concentrations of aerosolized antigen succeeded in eliciting acute-on-chronic allergic inflammation of the airways without an accompanying alveolitis. Diisocyanate-induced extrinsic alveolitis has been reported to occur also in humans (Mapp et al., 1985; Vandenplas et al., 1993; Baur, 1995). Notwithstanding the wealth of valuable data that have been obtained in these models, their outcome per se is highly dependent on protocol design variables.

### Mouse models

An obvious advantage of the current mouse models is the availability of genetically characterized inbred strains and genetically manipulated strains at relatively low cost. This species allows for the application *in vivo* of an extremely wide diversity of immunological tools, including gene deletion technology. However, due to the poorly developed bronchial musculature, bronchoconstriction is more difficult to detect in this species. Using mice as models of human disease, in particular asthma, has certain shortcomings (Irvin and Bates, 2003; Tu et al., 1995; Gelfand, 2002; Persson, 2002) only some of which will be addressed. With regard to lung anatomy, the parenchyma of the mouse lung occupies a smaller fraction of the total lung than that of the rat but more than of the human (mouse: 18%, rat: 24%, human: 12% lung volume). The airways constitute a large percentage of the lung in mice (11%) compared to rats (5.7%). Two other significant features of the mouse lung are the thinness of the respiratory epithelium and the relatively large airway lumen. Exactly what significance these anatomical features of the mouse lung have for lung function is speculative, but several authors demonstrated that the baseline airway resistance of mice that have been sensitized and challenged with antigen differs imperceptibly from that of control animals (Cieslewicz et al., 1999; Tomioka et al., 2002). The majority of protocols used involves relatively short-term exposure to aerosolized antigen (usually no more than 1–2 weeks) and is thus devoid of the chronic inflammatory and epithelial changes that typify human asthma. Most of the models are associated with an allergic alveolitis or hypersensitivity pneumonitis, which may overshadow

the inflammatory lesions of the airways. However, using controlled exposure to low mass concentrations of aerosolized antigen succeeded in eliciting acute-on-chronic allergic inflammation of the airways without an accompanying alveolitis. Mice subjected to repeated challenge protocols exhibit abnormalities of airway epithelium similar to those observed in human asthma, as well as evidence of airway hyperreactivity to methacholine (MCh). Excellent papers on the advantages and disadvantages of murine systems to study asthma pathogenesis are available (Tu et al., 1995; Dearman et al., 1992, 2003a, b).

Currently, two approaches to the identification of respiratory chemical allergens in mice have been described. The first focuses on the induction of total serum IgE, the second is cytokine fingerprinting. Both have as their theoretical foundation, the fact that chemical allergens of different types induce in BALB/c mice divergent immune responses characteristic of the selective activation of discrete T lymphocyte subpopulations. Respiratory allergens provoke Th2 cell-type responses. Contact allergens, such as 2,4-dinitrochlorobenzene (DNCB), that are considered not to cause sensitization of the respiratory tract, stimulate in mice immune responses consistent with the preferential activation of T helper 1 (Th1) cells. Such responses are associated with the production by draining lymph node cells (LNC) of interferon- $\gamma$  (IFN- $\gamma$ ). The converse picture is seen with chemicals that have been shown to cause allergic respiratory hypersensitivity and occupational asthma in humans, although, when animals were administered high concentrations of DNCB a low but significant increase in IgE antibodies was noted in BALB/c mice (Potter and Wederbrand, 1995). Chemical respiratory allergens such as TMA elicit in mice Th2-type immune responses, associated with the production by draining LNC of high levels of interleukin (IL) IL-4, IL-5, IL-10 and IL-13 and of other cytokine products of Th2 cells. Type 2 T-helper lymphocytes are presently considered to be the main orchestrator of the allergic airway inflammation underlying asthma. It would appear that cytokine production, rather than the influx of eosinophils or production of immunoglobulin-E, is the cause of bronchial hyperresponsiveness (Kips, 2001). As a consequence it has been found that exposure of mice to TMA, but not to DNCB, results in the appearance of specific IgE antibody. In practice, assays are performed using three concentrations of the test material together with TMA and DNCB which serve, respectively, as positive and negative controls. To carry out the BALB/c mouse IgE test, chemical in vehicle is applied to the shaved flank of the mouse; 7 days later the chemical is applied to the dorsum of both ears; 14–21 days later serum is drawn and total IgE assessed using an ELISA assay.

Remarkable strain specific differences in mice have been found (Shinagawa and Kojima, 2003; Whitehead et al., 2003; Takeda et al., 2001). After repeated intranasal ovalbumin instillations over a period of 3 months a sustained eosinophilic inflammation and features typical of airway remodeling, such as airway wall thickening and increased collagen deposition, was observed in A/J mice whilst in the other strains used (BALB/c, C57BL/6 and C3H/HeJ) these changes were not observed at all. On the other hand, in A/J mice inhalation challenges after ovalbumin/alum sensitization led only to a transient increase in eosinophils and to less airway wall thickening, indicating the importance of the protocol used (Shinagawa and Kojima, 2003). However, it could be speculated that at least some of the differences amongst different strains reflect the differences in the method and especially the associated dose of airway antigen exposure.

### Rat models

As demonstrated for mice models of airway allergy rats are considered to demonstrate many features of allergic human asthma. In many instances, the rat appears to be more suitable than mice with regard to the experimental procedures, the amount of body fluids available for analyses, and the extent of already existing data on specific chemicals. In contrast to guinea pigs, which exhibit a mast-cell-dependent bronchoconstriction to histamine, allergic bronchoconstriction in rats seems to be primarily mediated by serotonin. In this species, pulmonary mast cell activation generates a relative meager inflammatory response. Similarities between responses in high-IgE responding Brown Norway (BN) rat and humans include the production of IgE, a reasonable percentage of rats that have both immediate- and delayed-phase responses following aeroallergen challenge of sensitized animals, airway hyperreactivity to MCh, acetylcholine (ACh) or serotonin, and the accumulation of neutrophils, lymphocytes, and particularly activated eosinophils in lung tissue and BALF (Waserman et al., 1992; Eidelman et al., 1988). In the BN rat, although airway smooth muscle contraction accounts for much of the changes in pulmonary resistance following allergen inhalation, microvascular leakage into the airway lumen may be an important contributing factor to the airway narrowing and the increased airway resistance that is observed during the early and late responses (Olivenstein et al., 1997).

Elevations of the Th2 cytokines IL-4 and IL-5 and a reduction in the Th1 cytokine IFN- $\gamma$  are also observed. However, some questions have been raised regarding the correlation between airway inflammation and airway hyperreactivity. However, the rat is a weak bronchoconstrictor, and higher levels of agonist are required to

induce the same level of response as compared to guinea pigs. Thus, this animal model focuses on the induction of airway inflammation, which comprise most of the characteristic features of asthma, depending on the protocol employed. Airway responses in spontaneous breathing rats with regard to bronchoconstriction are relative weak and somewhat inconsistent especially when challenge concentrations are limited due to the irritant potency of the hapten. Many features of respiratory allergy can aptly be modeled in the high-IgE responding BN rat (Arts et al., 1997, 1998; Schuster et al., 2000; Cui et al., 1997; Pauluhn et al., 2002a, b; Pauluhn, 2003).

### Guinea pig models

The guinea pig model for assessment of pulmonary allergenicity of low-molecular-weight chemicals and proteins, including pharmacological intervention studies, has extensively been utilized (Briatico-Vangosa et al., 1994; Blaikie et al., 1995; Sun and Chung, 1997; Griffiths-Johnson and Karol, 1991; Hayes and Newman-Taylor, 1995; Hayes et al., 1992a, b; Karol and Thorne, 1988; Karol, 1983, 1994; Pauluhn and Eben, 1991; Pauluhn, 1994a; Pauluhn and Mohr, 1998; Sarlo and Clark, 1992; Sarlo and Karol, 1994; Sugawara et al., 1993; Welinder et al., 1995). The particular advantages and disadvantages of this animal model have been reviewed extensively (Briatico-Vangosa et al., 1994; Sarlo and Karol, 1994; Karol, 1994; Pretolani and Vargaftig, 1993).

Conventionally, the guinea pig has been the species of choice for the toxicological evaluation of chemical-related respiratory allergy, primarily because it is possible in this species to elicit and measure with relative ease challenged-induced pulmonary reactions that resemble in some ways the acute clinical manifestations of human allergic asthma. The guinea pig is known to respond vigorously to inhaled irritants by developing an asthmatic-like bronchial spasm. This species possesses a developed bronchial smooth muscle, which contracts intensively and rapidly in response to *in vivo* or *in vitro* exposure to antigen. This anatomical prerequisite is required for both the expression of bronchoconstriction of the immediate hypersensitivity reaction, which evolves in minutes, and for its late component, which evolves in hours. This species has been used for decades for the study of protein-evoked anaphylactic shock and pulmonary hypersensitivity and can experience both immediate- and late-onset responses. Guinea pigs are quite prone to respiratory tract infections (Chengelis, 1990), which also causes increased breeder-to-breeder variability of pulmonary baseline inflammation.

The guinea-pig is considered by some to be unsuitable for the study of the effects of inhaled irritants because of its potential to respond to respiratory irritation by developing an asthmatic-like bronchial spasm. The measurement of pulmonary challenge reactions in sensitized guinea pigs permits considerations of dose-response relationships in the elicitation of respiratory hypersensitivity, which may be of use in the risk assessment process. However, as true for all animal models, it has been noted that extensive manipulation of the exposure regimen used for sensitization, repeated challenges and multiple concentrations are sometimes required to elicit a positive bronchoconstrictive response, raising concerns about the robustness of these methods (Dearman and Kimber, 1999). Guinea-pig anaphylactic responses usually involve IgG<sub>1</sub> antibodies, even though the model can be tailored for the production of IgE. Large doses of antigen tend to support earlier and more intense bronchopulmonary hypersensitivity and favor IgG, whereas smaller doses favor mixed IgE and IgG production (Pretolani and Vargaftig, 1993). In this species no clear association of pulmonary hypersensitivity responses and elevated specific IgG<sub>1</sub>-titres could be established. Thus, measurement of specific antibody formation provides ancillary evidence of an immunologically mediated response. As with other animal models, clearly, the inflammatory response varies according to the protocol, and antigen persistence and repetitive stimulation, in particular, may lead to appreciable differences.

### Manifestation of respiratory allergy

Bronchial hyperreactivity and symptoms of airway obstruction clinically characterize respiratory allergy (Temelkovski et al., 1998). Underlying the clinical manifestations is acute-on-chronic inflammation of the airway mucosa, with degranulation of mast cells, recruitment of eosinophils and neutrophils, as well as accumulation of activated T lymphocytes and other chronic inflammatory cells. Pulmonary parenchymal inflammation is not a significant feature of asthma, although limited numbers of eosinophils may be present in the alveoli immediately adjacent to involved airways. Abnormalities of the airway epithelium are prominent, including shedding and regeneration of ciliated cells, goblet cell hyperplasia and/or metaplasia, as well as a distinctive pattern of subepithelial fibrosis which, in humans, reversed upon pharmacological interventions (Fabbri et al., 1998). Subepithelial fibrosis is a distinctive lesion of asthma that involves accumulation of extracellular matrix proteins such as type III and type V collagen, fibronectin, tenascin and  $\beta_2$ -laminin, as well as various proteoglycans, in the lamina reticularis beneath



the true basement membrane Airway epithelial cells express a variety of growth factors with the potential to stimulate replication of and collagen synthesis by myofibroblasts of the mesenchymal trophic unit (Kumar et al., 2004; Zhang et al., 1999).

To elicit changes comparative to those of human asthma, it is necessary to allow for repeated inhalation exposure to the agent under consideration. Of considerable interest is the evidence that airway hyperreactivity did not correlate with IgE titers or with the magnitude of the inflammatory response in the airway wall (Temelkovski et al., 1998). Indeed, this model is relatively labor intensive and requires specialized equipment but does succeed in achieving a new level of fidelity of asthmatic lesions. For highly reactive low-molecular-weight volatile chemicals, acute alveolitis/hypersensitivity pneumonitis often confound the inflammatory response localized in the airways. While the inflammatory response within the airways has an atopic/allergic basis in at least two thirds of asthmatic patients (McFadden and Gilbert, 1992), the mechanisms involved in the development of airway obstruction remain poorly understood. Accumulation of inflammatory cells and exudate, increased airway smooth muscle mass, deposition of connective tissue, and epithelial hyperplasia may all contribute to thickening of the airway wall, which appears to be the basis for the excessive diminution of airflow that accompanies bronchoconstriction in asthmatics. The respiratory epithelium has long been recognized to be the primary barrier protecting the underlying tissue from inhaled irritants and noxious stimuli. The loss of this physical barrier function could be envisaged to play a role in hyperreactivity by permitting greater access of bronchoconstrictor stimuli to the underlying smooth muscle. Also the metabolic activity of this barrier is increasingly appreciated.

Investigation of the pathophysiological mechanisms of chronic asthma has been limited by the lack of a satisfactory animal experimental model. Attempts have been made to model the disease in a range of species (Karol, 1994; Fügner, 1985; Bice et al., 2000). The relevance of experimental systems which employ antigens that bear little relationship to triggers of human asthma or which use pathophysiologically inappropriate methods for delivery of these antigens is debatable. However, for highly reactive, i.e., irritant agents that are preferentially deposited in the upper airways of obligate nose-breathing laboratory rodents, adequate doses of antigenic determinants can only be delivered to the lower airways when the irritant properties of the chemical are masked. As illustrated in guinea pigs sensitized to and challenged with HDI, the inhalation challenge using the hapten was negative, whilst following the hapten-conjugate challenge immediate-onset respiratory responses were observed (Figs. 13 and 14;

duplicated from Pauluhn et al., 2002a, b). Accordingly, elicitation of respiratory allergy of potent respiratory tract irritants in their non-conjugated form are biased to be (false) negative. Despite this common outcome of testing, in many instances, sensitized guinea pigs and BN rats did fully display surrogate immediate-onset type asthmatic responses from challenge with the free TMA. It remains puzzling that the physiological effects (change in early and late breathing patterns upon challenge with the hapten, eosinophilic airway inflammation) of this respiratory tract sensitizer could be satisfactorily modeled in the absence of conjugation, whilst other known human asthmagens, such as the diisocyanates, produced only a mild allergic bronchial inflammation, if any, using similar protocols (Pauluhn et al., 1999).

### Endpoints of respiratory allergy

As detailed above, a common pathologic accompaniment or cause of increased airway hyperresponsiveness is a prolonged airway inflammation and remodeling after inhalation of specific haptens or antigens. It is suggested that the inflammation and/or remodeling are responsible for the change in increased agonist responsiveness. In contrast with the assays relying upon an induction of a specific set of characteristic immunologic biomarkers, some models do not depend upon a preconceived mechanism of sensitization and response. Rather, they function by reproducing the characteristics, which typify the hypersensitivity reactions, i.e., the immediate-onset physiologic response of the airways which is bronchoconstriction. The clinical manifestation of respiratory allergy is not characterized by one single, unique endpoint, except anaphylaxis that occurs in guinea pigs. Depending on the type of plethysmography applied, an analysis of additional characteristics typifying a change in breathing patterns is useful. In spontaneously breathing animals, in addition to measurement of respiratory rate, flow-derived endpoints, such as peak inspiratory and expiratory flows and respiratory-pattern related endpoints, e.g., inspiratory, expiratory, and relaxation times, enhanced pause (Penh) have been shown to improve the sensitivity of analysis (Pauluhn, 2003; Pauluhn, 2004). As detailed above, in mice and rats the magnitude of respiratory changes is markedly less pronounced as compared to guinea pigs.

The delayed-onset physiologic response is more difficult to quantify by physiological methods due to the spontaneous activities in unrestrained animals in whole-body plethysmographs (see below), however, this more sustained inflammatory component can suitably be probed by bronchoalveolar lavage (BAL) and histopathology. However, also the characteristic features of the acute and chronic types of allergic

inflammation of airways need to be considered. These endpoints appear to depend on the intensity of the priming response at the site of sensitization. For a better appreciation of dose-/concentration related effects observed following challenge, the specific responses occurring at the site of induction, including their draining lymph nodes, should be observed.

### Antibody determinations and biomarkers of exposure

Recent studies have focused on the initial reactivity of diisocyanates with target airway macromolecules and peptides, including glutathione and lung epithelial proteins. Specific airway epithelial cell proteins that are selectively adducted by diisocyanates have been identified (Redlich and Karol, 2002). Moreover, HDI bound either to keratin-18 or keratin-10, was identified in human endobronchial and skin biopsy samples as the major protein adducted with HDI following inhalation and dermal exposure, respectively. In BALF albumin was found to be the major HDI-adducted protein (Wisniewski et al., 2000).

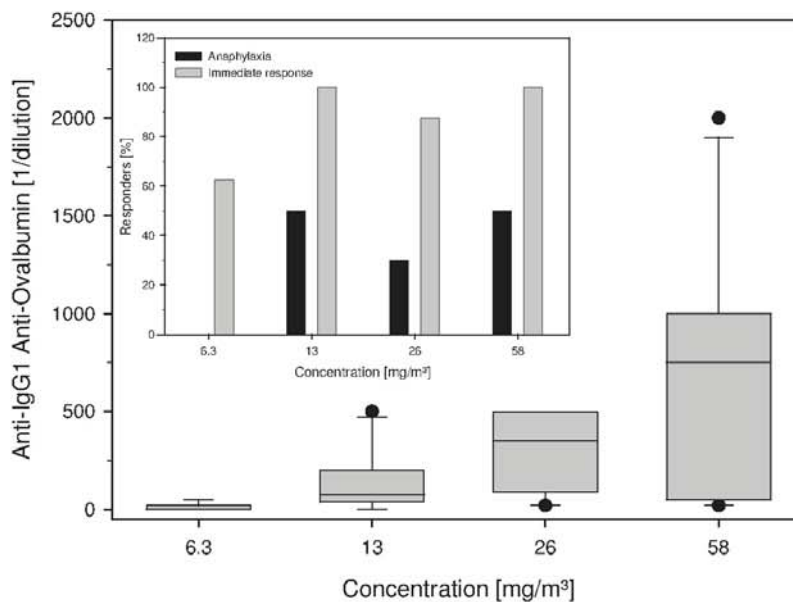
Common serological measurements assume that the endpoints addressed in the systemic circulation are reflective of changes at the sites of allergic inflammation. Before testing for specific immunologic responses to chemicals, appropriate antigens must be prepared and fully characterized to determine the number and kind of chemical ligands bound to each protein carrier molecule. Especially for multifunctional chemicals the selection of the most “antigenic” hapten–protein conjugate is critical because different conjugates have to be tested against *in vivo* antisera assuming that the synthesized conjugate that is recognized best by the antiserum is likely to be most similar to the conjugate formed *in vivo* (Pauluhn et al., 2002a, b). Accordingly, the quality and degree of conjugation affects the recognition in the ELISA assay. Subtle differences in laboratory procedures may result in conjugates of different antigenic activity, which renders the comparison amongst different laboratories difficult.

Measurements of total serum IgE in the mouse and BN rat are considered as endpoint for the identification and assessment of chemicals to induce respiratory allergy. Especially in mice, this test is extended further using a cytokine profiling (Th1 vs. Th2), in which cytokine levels produced by local draining LNCs are measured after topical induction (Dearman et al., 2003a, b; Kimber and Dearman, 2002). In guinea pigs successful sensitization to a low-molecular-weight chemical (hapten) or allergen has been demonstrated by the assessment of anti-IgG<sub>1</sub> antibody. Andersson (1980) demonstrated that low-dose antigen caused production

of both IgE and IgG in the guinea pig, while only IgG<sub>1</sub> was produced when guinea pigs are sensitized with larger amounts of antigen. Repeated exposure to ovalbumin has been reported to enhance IgE production in the BN rat (Siegel et al., 1997). Only few animal models have addressed the clinically relevant patterns of repeated daily exposures and intermittent/discontinuous antigen exposure. A comparison of acutely and ‘continuously’ ovalbumin exposed mice (6-week-‘continuous’ exposure for 1-h/day) revealed minimal airway eosinophilia, diminished airway B-cell lymphocytosis, less pulmonary inflammation, no airway hyperreactivity, and altered cytokine profiles; however, their systemic levels of allergen-specific IgE and IgG<sub>1</sub> were unabated. The maintenance of elevated serum IgE levels in the absence of airway inflammatory responses using the ‘continuous’ exposure protocol suggests this apparent tolerance was due to local mechanisms rather than systemic lymphocyte clonal deletion or anergy. Notwithstanding, the refractoriness to airway inflammation that developed in continuously exposed mice was clearly different from the inhalational tolerance demonstrated in other models (Schramm et al., 2004). A shift in T-cell phenotypes from a CD4+ to a CD8+ predominance, nor a systematic switch from a Th2 to a Th1 cytokine profile in BALF was observed.

Dose–response relationships are observed between the induction dose, this means in terms of exposure concentration and number of exposures, antibody titers, respiratory hyperresponsiveness, and morphological changes (Siegel et al., 2000; Pauluhn and Eben, 1991; Potter and Wederbrand, 1995). However, the animals experiencing marked respiratory reactions upon challenge were not always associated with the magnitude of IgG or IgG<sub>1</sub> antibody response mounted. This means, dose–response curves based on specific IgG<sub>1</sub> and severity of pulmonary response (anaphylaxis) do not necessarily parallel as shown in guinea pigs sensitized to and challenged with ovalbumin (Fig. 1).

In studies using various concentrations and fractional doses to obtain an overall similar cumulative dose it was shown that the apparent threshold for antibody production significantly depends on the number of applications of TDI (Potter and Wederbrand, 1995). A synopsis of diverse repeated inhalation exposure studies with HDI-homopolymers in Wistar rats and guinea pigs demonstrated a relationship of acute lung irritation and (sub)chronic lung inflammation in rats as well as the induction of IgG<sub>1</sub> antibodies in guinea pigs (Pauluhn, 2000a). Hence, it appears that increased titers of IgG<sub>1</sub> antibodies could only be detected at exposure concentrations causing membranolytic/cytotoxic effects of airway epithelial cells. In guinea pigs sensitized to HDI-monomer by three intradermal injections (3 × 0.3%; one injection/day of 100 µl of 0.3% on days 0, 2, 4) or to the HDI homopolymer by six intradermal



**Fig. 1.** Guinea pigs were sensitized to ovalbumin aerosol on four consecutive days (days 0–3, duration: 15-min/day, concentrations:  $6 \pm 1.9$ ,  $13 \pm 2.2$ ,  $27 \pm 5.9$ ,  $58 \pm 14.4$  mg/m<sup>3</sup>; mass median aerodynamic diameter, MMAD,  $\approx 1.5$   $\mu$ m). Tukey Box plot of IgG<sub>1</sub> antibodies (ELISA) from blood collected prior to challenge with aerosolized ovalbumin. Boundaries of the box represent the 10th and 90th percentiles, the medians are displayed as solid lines. Inset: percentage of guinea pigs (eight/group) displaying either a change in respiratory rate or anaphylaxis.

injections ( $6 \times 30\%$ ; two injections/day of 100  $\mu$ l of 30% HDI-BT on days 0, 2, 4), dissolved in the vehicle corn oil, almost equal IgG<sub>1</sub> anti-body titers were observed, showing that the monomer is a more potent inducer of specific IgG<sub>1</sub> antibody than the respective HDI homopolymer (Pauluhn et al., 2002a, b). Thus, for complex mixtures containing chemically related monomers and homopolymers antibody cross-reactivity might preclude a robust analysis of the causal relationship of mixed monomer/homopolymer exposure and antibody response. Moreover, the comparison of antibody titers in ELISA assays is known to be highly contingent upon the ‘antigenicity’ of the respective conjugate synthesized. Thus, monomeric HDI appeared to be a much more potent inducer of specific IgG<sub>1</sub> antibodies than the related homopolymers, although the role of specific IgG<sub>1</sub> antibodies in context with pulmonary allergy remains unresolved (Cullinan, 1998). This issue is complicated further due to the partition of the volatile monomeric HDI in the non-volatile homopolymer aerosol (Rando and Poovey, 1999) which makes a clear distinction of effects caused by highly potent monomers and low potent polymers difficult.

Collectively, it appears to be generally accepted that a specific IgG<sub>1</sub> antibody response provides the potential for the elicitation of a pulmonary response to occur; however, there is no clear relationship between antibody titer and pulmonary responsiveness (Blaikie et al., 1995;

Pauluhn and Eben, 1991; Lushniak et al., 1998). However, the exact role of locally synthesized IgG in the pathogenesis of asthma is unknown. In mice, IgG may play a role in type I hypersensitivity responses (Kumar et al., 2000). Recently, an alternative to the guinea pig anaphylactic antibody model has been proposed which assess the formation of specific IgG<sub>1</sub> antibody in a mouse intranasal test (MINT), the assumption being that specific IgG<sub>1</sub> antibody is a surrogate for anaphylactic antibody in this species. However, it was concluded, that this model requires substantial further validation before it can be adopted as an alternative model (Blaikie and Basketter, 1999).

### Immediate-/delayed-onset physiological response

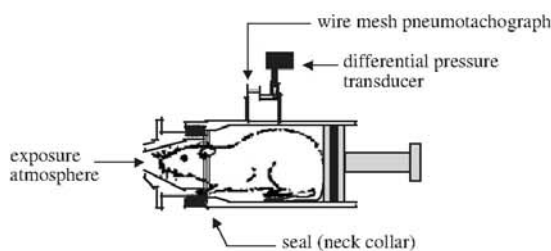
The analysis of responses associated with the clinical manifestation of respiratory allergy is based on the analysis of immediate- and/or delayed-onset pulmonary sensitivity by pulmonary function tests. They are integrative tools available to inhalation toxicologists to assess the function of the lung. Measurements can be made during or after exposure in spontaneously breathing conscious animals using various types of plethysmographs (e.g., whole-body, bias-flow or barometric and

nose-only, volume displacement). Investigators also used more in-depths type of measurements in anesthetized, cannulated animals for the determination of lung resistance ( $R_L$ ) and dynamic compliance ( $C_{dyn}$ ) (Hamelmann et al., 1997) or for forced maneuvers (Pauluhn et al., 1995). The definition of positive responses as well as their categorization varies from one method and laboratory to another (Karol, 1983; Sarlo and Clark, 1992; Botham et al., 1998, 1989; Blaikie et al., 1995; Pauluhn, 1997; Pauluhn et al., 2002a, b). Changes in respiratory rates and patterns are commonly recorded by either optical, plethysmographic fluctuations of pressure or by signs of respiratory distress. Most protocols employ single and/or repeated challenge periods of 10–30 min to elicit respiratory hyperresponsiveness. Sometimes measurements are followed by extended post-challenge measurements for the detection of delayed-onset reactions (Alarie et al., 1990; Karol et al., 1985; Pauluhn and Eben, 1991; Zhang et al., 2004; Thorne and Karol, 1988).

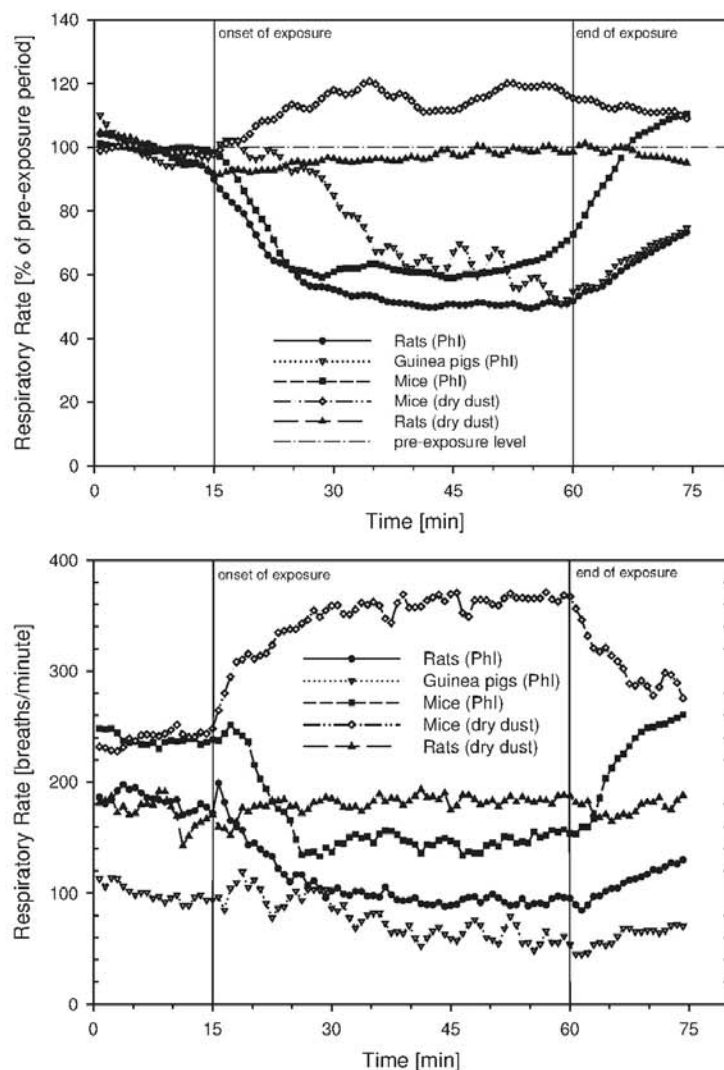
Ideally, respiratory patterns are measured during the challenge exposure in volume displacement plethysmographs (Fig. 2). For this purpose nose-only animal restrainers are equipped with a wire mesh pneumotachograph and a differential pressure transducer fitted directly onto the exposure restrainer (plethysmograph). The head and body compartments are separated using a double-layer latex neck seal separated by a stabilizer. Precautions have to be taken to avoid artifacts due to restraint and tight fitting seals around the neck. This type of plethysmograph provides a means to challenge animals to well characterized atmospheres with regard to actual breathing zone concentrations and particle size (Pauluhn and Eben, 1991; Pauluhn, 1994b; Pauluhn and Mohr, 2000) as well as an analysis of reflexively induced changes in breathing patterns. The onset and extent of the initial irritant-related response in guinea pigs, rats, and mice differs from one species to another, depending on the baseline respiratory rate (Fig. 3). Due to the relative abundance of irritant receptors at different locations within the respiratory tract, respirable dry, innocuous dust (kaolin) may evoke different changes in

breathing patterns in mice and rats. Depending on the agent under study, concentrations high enough to cause irritation-related stimulation of sensory nerve endings in the upper or lower respiratory tract may be associated with a decrease or increase in respiratory rate respectively (Arts et al., 2001; Pauluhn, 1994a; Vijayaraghavan et al., 1993). The advantages of volume displacement plethysmography include that confounding effects due to habituation and grooming as observed in whole-body plethysmographs do not occur, that the flow-derived breath structure can be better appreciated and that the tidal volume calculated is more precise than that based on box pressure changes in barometric plethysmographs as detailed by Epstein and Epstein (1978). However, a restraint-related increased basal respiratory rate is often observed in the volume displacement plethysmograph (Fig. 3) when compared to whole-body barometric plethysmography (Fig. 4). In the latter, no restraint is required and, therefore, this method is suitable for analysis of post-challenge respiratory reactions delayed in onset as suggested by Zhang et al. (2004). These responses may not be misconstrued with an elicitation-dependent inflammatory response causing sustained change in breathing patterns (Pauluhn et al., 2002a, b).

Common parameters derived from the breathing pattern and the breath structure include the breathing frequency (respiratory rate, RR), tidal volume (TV), the inspiratory and expiratory times (IT and ET) and the respective maximal peak flow rates during inspiration and expiration (PIF and PEF). Analysis of additional parameters may be useful to better understand the quality of changes observed, i.e., whether they originate from the stimulation of receptors in the upper or lower respiratory tract or are related to lung resistance. These include the relaxation time (RT, which is commonly defined as the time from start of expiration to the return to 35% of TV), apneic pause ( $\text{Pause} = T_e/RT - 1$ ), end inspiratory pause (EIP), a measure of the pause between inspiration and expiration defined, peak inspiratory and expiratory plethysmographic pressures (PIF and PEF), and enhanced pause ( $\text{Penh} = \text{Pause} \times \text{PEF}/\text{PIF}$ ). More details are described elsewhere (Chong et al., 1998; Hamelmann et al., 1997; Kimmel et al., 2002; Epstein and Epstein, 1978; Drorbough and Fenn, 1955). Thus, for the calculation of Penh a series of flow-derived breathing parameters, especially those dominated by the shape of the expiratory phase, are utilized. The pattern of this phase appears to be particularly dependent on the 'control of respiration', including pulmonary sensory or inflation/deflation reflexes and those involved in the mechanical control of the breathing apparatus (Jacquez, 1979). There is increasing evidence showing that Penh appears to be primarily related to ventilatory timing, unrelated to airway resistance ( $R_{aw}$ ). Therefore, this method should not be used to invoke terms such as "airway(hyper)reactivity" or "airway(hyper)



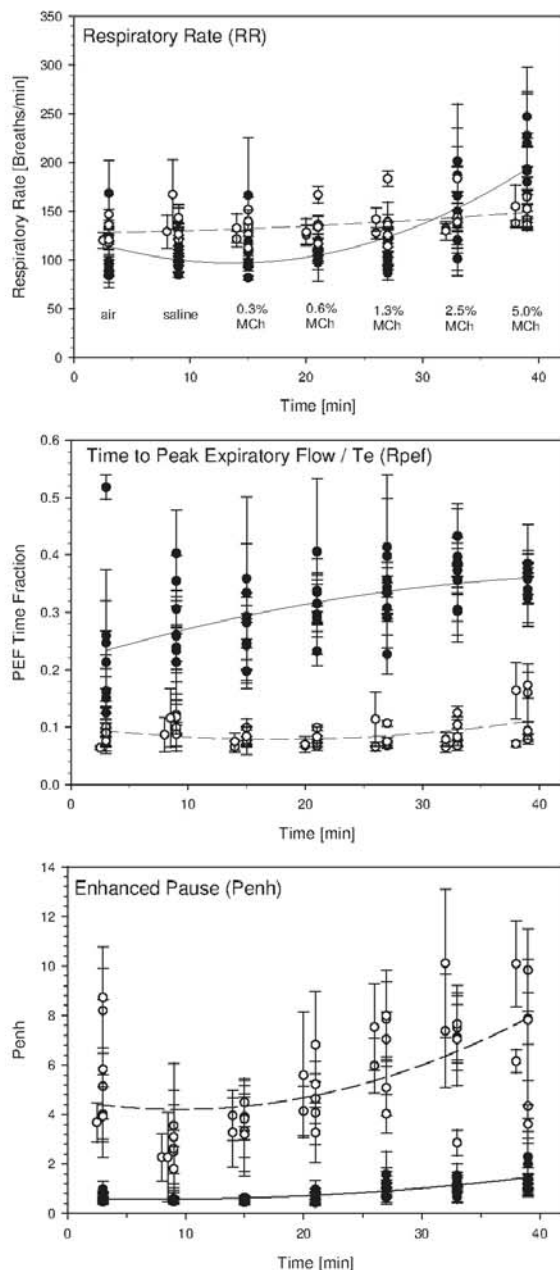
**Fig. 2.** Rat in a nose-only volume displacement plethysmograph for measurement of respiratory patterns during challenge.



**Fig. 3.** Change of respiratory rate, absolute (upper panel) and relative (lower panel). Baseline data were collected during a 15-min pre-exposure period followed by a 45-min exposure period to an upper respiratory tract irritant vapor (phenyl isocyanate, PhI, at approximately  $13 \text{ mg/m}^3$ ) or respirable dry dust particles (kaolin, approximately  $11 \text{ mg/m}^3$ ). Measurements were made in guinea pigs, rats, and mice in volume-displacement nose-only plethysmographs (see Fig. 2) and represent means of 4 animals.

responsiveness". The increasing uncritical use of unrestrained plethysmography imparts a danger of mass-producing false experimental evidence in the search for mechanisms of respiratory disease (Bates and Irvin, 2003; Bates et al., 2004; Hantos and Brusasco, 2002; Mitzner et al., 1998, 2003; Peták et al., 2001; Pauluhn, 2004). Nonetheless, despite its theoretical weakness, in some circumstances Penh may be suitable as a preliminary, simple technique to show substance-induced changes in breathing patterns (*vide infra*), where it is inappropriate scientifically to use Penh to reflect airway function, without an independent assessment of Raw.

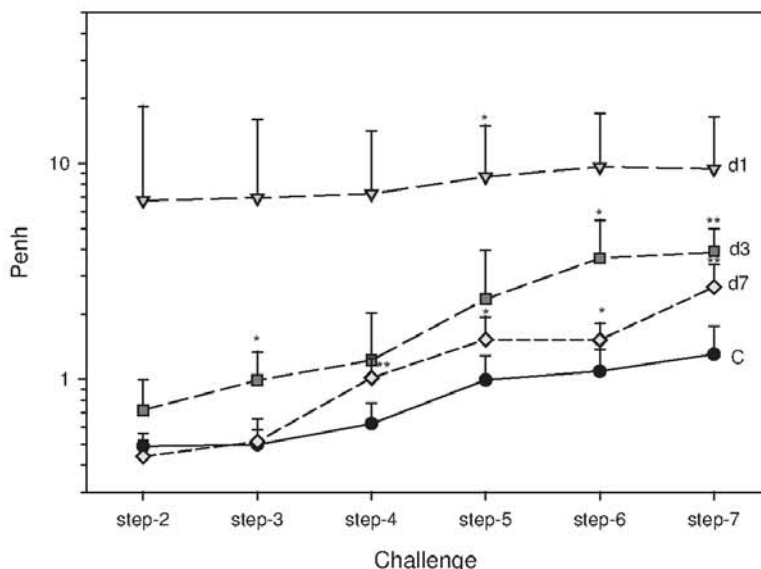
In BN rats exposed to approximately  $103 \text{ mg MDI/m}^3$  ( $1 \times 6 \text{ h}$ ), a concentration high enough to cause pulmonary irritation (Kilgour et al., 2002; Pauluhn, 2004), Penh measurements were made on postexposure days 1, 3, and 7. Differences to the control group were maximal on postexposure day 1 and baseline values of Penh were approximately one order of magnitude higher in MDI-exposed rats when compared to sham controls (Figs. 4 and 5). The time course of changes in baseline Penh and that of increased protein in BALF and lung weights appear to coincide (Pauluhn 2000b, 2002). Upon a stepped MCh-challenge, the commonly observed incremental increase in Penh was not observed in



**Fig. 4.** Change in breathing patterns of sham control BN rats (filled symbols, solid lines) and rats exposed for 1 × 6-h the previous day to 103 mg MDI/m<sup>3</sup> (open symbols, dashed lines). The rats were exposed sequentially to air, saline, and aerosolized MCh in increasing concentrations (aqueous spray solutions of 0.3%, 0.6%, 1.3%, 2.5%, and 5%) in barometric plethysmographs. The time axis (abscissa) constitutes the cumulative time required for each step, viz. a 1-min challenge period followed by a data collection period of 5-min. The 5-min mean values ( $\pm$ SD) of each step were calculated for each endpoint and rat.

MDI-exposed rats as compared to the control. These data demonstrate that in lungs with altered fluid dynamics, increased stiffness, and affected breathing patterns the sensitivity to probe changes in breathing patterns at baseline level (exposure to air or saline aerosol) and during the stepped MCh-challenge, endpoints such as RR, TV, Ti, Te, RT and EEP appear to indicate differences between the control and MDI groups either very poorly, do not show any consistent relationship to the increasing MCh-concentrations or were governed by extensive variability. Differences between the groups and MCh-challenge concentrations appear to be represented best by the flow-derived endpoints, such as Rpef, PIF, PEF, apneic Pause, and especially the composite endpoint Penh (Pauluhn, 1994a, b). This comparison illustrates that changes in Penh are apparently more ‘breathing pattern’ or ‘control of breathing’-related rather than reflective of any particular mechanical change in function. The increased sensitivity of Penh to probe for alterations in respiration is at the expense of the physiological interpretation of findings. Thus, based on the comparison of endpoints shown in Fig. 4, Penh is considered to be appropriate to identify changes between the irritant aerosol (MDI) and the sham control groups, whereby precision and physiological relevance must be traded off against non-invasiveness and experimental simplicity (Hantos and Brusasco, 2002; Bates and Irvin, 2003).

Collectively, changes in baseline Penh have been shown to be associated with an increase in BAL-protein that were associated with changes in elastic tissue recoil rather than bronchoconstriction (Pauluhn, 2004). Hence, Penh might be viewed as a sensitive non-invasive, although non-specific functional endpoint suitable to probe for changes in respiration in unrestrained conscious rats. Several factors can affect the various parameters involved in the calculation of Penh and its indiscriminate use is subject to misleading interpretations of response. As almost all respiratory mechanics variables show qualitative correlations (Mitzner and Tankersley, 1998; Mitzner et al., 2003), it appears to be difficult to causally relate changes in Penh to any specific pathophysiological event, such as airway constriction. It is also important to recall that nasal airway resistance is the largest component of the total airway resistance in obligate nasal breathing rodents (Lung, 1987). Thus, caution is advised in regard to the quantitative interpretation of Penh, because changes might be related to alterations in breathing patterns secondary to changes in tissue mechanics. Thus, Penh appears to integrate several physiological endpoints in a wholly non-invasive and non-disturbing manner so that non-specific functional changes can readily be identified in studies where incremental rather than absolute changes are the primary focus.



**Fig. 5.** Bronchial responsiveness to aerosolized MCh of BN rats exposed to 103 mg MDI/m<sup>3</sup> for 1 × 6-h. Measurements of Penh were made in barometric plethysmographs on postexposure days 1, 3, and 7 (d1, d3, d7). Baseline data were recorded during the 5-min exposure to isotonic saline (step-2) followed by stepped MCh exposures in increasing concentrations (aqueous spray solutions of 0.3%, 0.6%, 1.3%, 2.5%, and 5%; steps 3–7). Sham control rats (C) were examined similar to the MDI-exposed rats. Data points represent mean values ( $\pm$ SD) of eight rats in the MDI group and four rats in the control group (data pooled for all time points), respectively. Asterisks denote significance to the pooled control data (\* $P$  < 0.05, \*\* $P$  < 0.01).

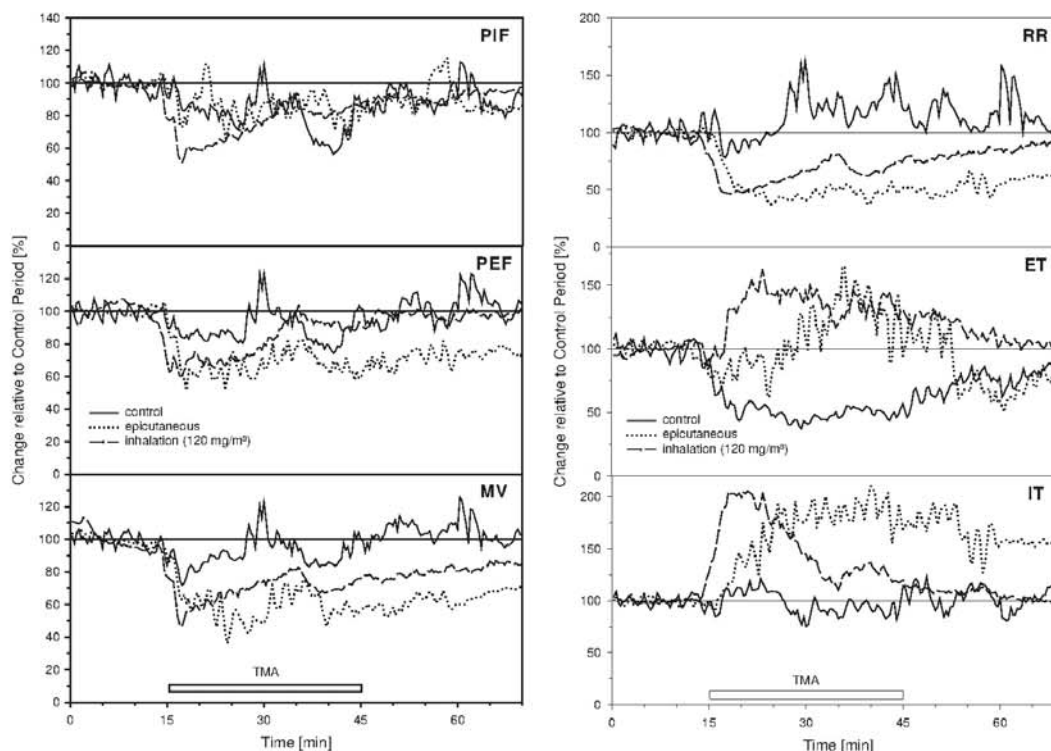
Likewise, methodological differences in the sensitivity of detecting changes related to upper or lower respiratory tract irritation (Pauluhn and Mohr, 2000) or mild to moderate hypersensitivity responses appear to be one of the reasons for the wide range of concentrations required for a successful elicitation of respiratory hyperresponsiveness upon challenge exposures, e.g., as used for TMA or MDI (Pauluhn, 1994a; Pauluhn et al., 1999). Each method of determination, endpoint and procedure to define positive changes in breathing patterns may affect the sensitivity both to identify and qualify changes in breathing patterns (Fig. 6). For both guinea pigs and BN rats it was shown that the particular respiratory change chosen as endpoint affects the interpretation of study (Pauluhn, 2002, 2003). These methodological variables are considered to be a major source of interlaboratory variability.

Standardized procedures for evaluation have been described (Pauluhn, 1997, 2003; Pauluhn and Mohr, 1998). For instance, baseline data are collected during a pre-challenge, adaptation period of approximately 15-min and during or following the subsequent inhalation challenge any response exceeding the mean  $\pm$  3 standard deviations (SD) of this period during is taken as a positive response. This type of objective, quantitative analysis calculates the 'area under the response curve' exceeding the mean  $\pm$  3 SD of the pre-challenge period and can be used to express objectively the "intensity" of individual animals' responses (Fig. 7). As illustrated in

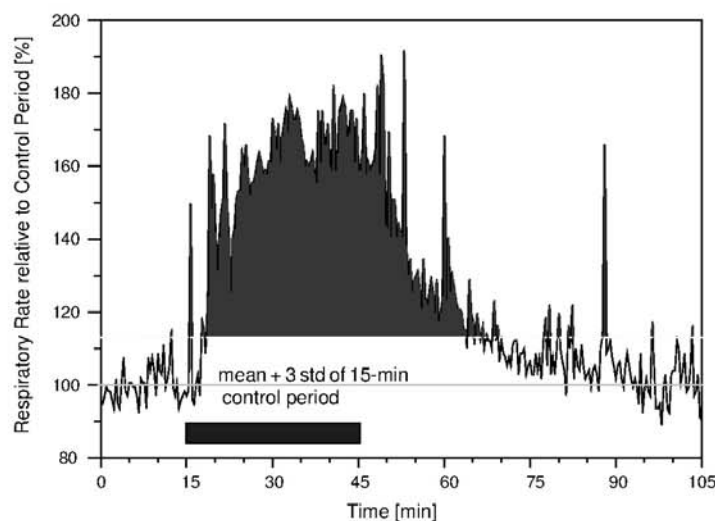
Fig. 8, the changes in respiratory rate in guinea pigs sensitized to and challenged with ovalbumin vary markedly from one animal to another with regard to the onset of anaphylaxis (animals 1 and 2), the time-related rapid change in respiratory frequency (animals 2 and 3), a more sustained immediate decrease (animal 5) or delayed increase (animal 4) in breathing frequency. The corresponding changes observed in BN rats by whole-body barometric plethysmography were markedly less pronounced as shown in Fig. 6 for TMA. Thus, unique, group-specific changes in breathing patterns as a result of respiratory allergy evoked by ovalbumin or TMA aerosols have neither been observed in guinea pigs nor in BN rats.

### Airway hyperresponsiveness to pharmacological agonists

Airway hyperresponsiveness (AHR) is a central feature of asthma (Busse and Lemanske, 2001) and is characterized by exaggerated airway narrowing after exposure to nonspecific stimuli, such as ACh, MCh, histamine, or exercise (Cockroft et al., 1997). The dysfunction underlying AHR includes hypersensitivity (shift to the left of bronchoconstrictor dose-response curves), hyperreactivity (increased slopes of these curves), and a greater maximum degree of induced

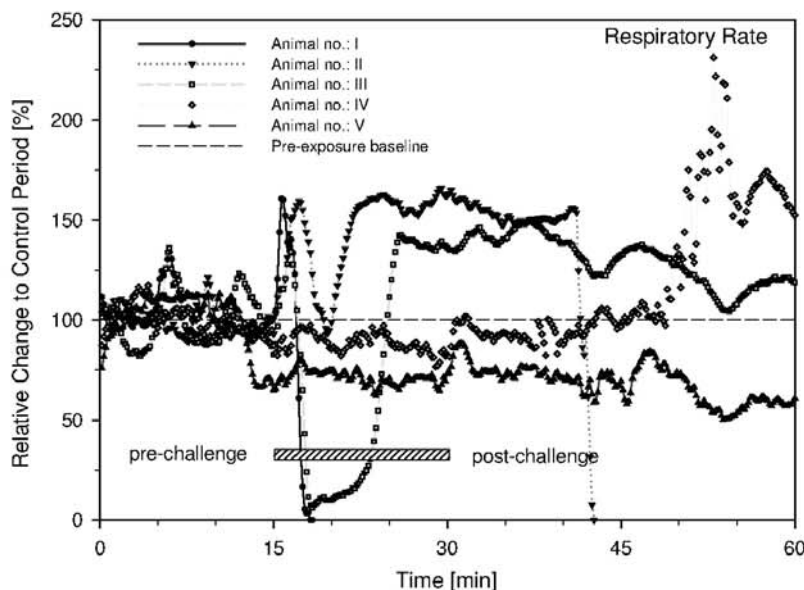


**Fig. 6.** Change of respiratory patterns during a challenge with  $\approx 23 \text{ mg TMA/m}^3$  air (duration of challenge: 30-min). BN rats were either sensitized by epicutaneous administration of TMA in acetone-olive oil or by  $5 \times 3\text{-h/day}$  inhalation exposures to  $120 \text{ mg TMA/m}^3$ . Respiratory response data were normalized to the mean of a 15-min pre-challenge exposure period (= 100%). Before and after challenge the rats were exposed to conditioned air. The animals selected represent those displaying maximum responsiveness upon challenge. PIF, peak inspiratory flow during tidal breathing; PEF, peak expiratory flow during tidal breathing; MV, respiratory minute volume; RR, respiratory rate; ET, expiratory time; IT, inspiratory time.



**Fig. 7.** Integration of respiratory response prior to, during and following challenge in guinea pigs (TDI-conjugate). The area under the curve (AUC) is calculated based on the response occurring during the challenge (15-min) and post-challenge data collection periods. All data exceeding the mean  $\pm 3$  SD of the pre-exposure period are used for AUC calculation.





**Fig. 8.** Guinea pigs were sensitized to ovalbumin aerosol on four consecutive days (days 0–3, duration: 15-min/day, concentration:  $27 \text{ mg/m}^3$ ) and challenged with ovalbumin aerosol  $11.5 \pm 3.6 \text{ mg/m}^3$  for 15-min; mass median aerodynamic diameter, MMAD,  $\approx 1.5 \mu\text{m}$ ). The individual animals' respiratory rate was normalized to the 15-min pre-exposure period.

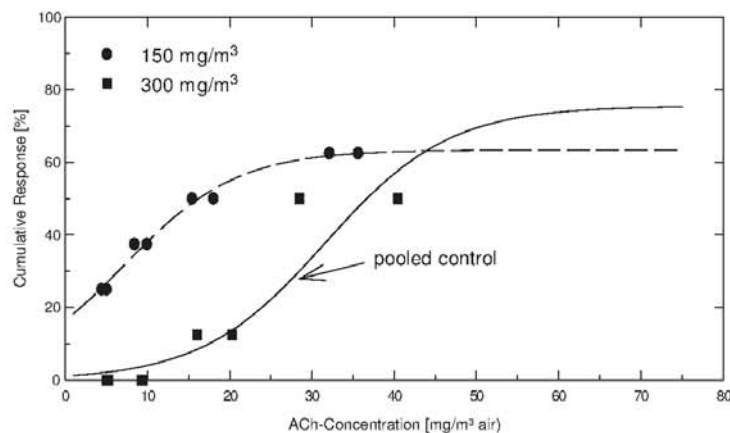
bronchoconstriction. However, the mechanisms underlying these pathophysiologic abnormalities remain unclear. The afferent innervation of the airways may play a role in the expression of airway hyperreactivity. Reflex bronchoconstriction is most obviously observed when the stimuli used have little direct effect on airway smooth muscle; however, experiments in animal models suggest that even the response to a direct smooth muscle spasmogen such as MCh is likely to be mediated in part by a parasympathetic reflex (Wagner and Jacoby, 1999).

In small laboratory animals non-specific airway responsiveness can be measured in the same manner as described above. The common measurements utilize either volume displacement or whole-body (barometric) plethysmography and increased airway responsiveness is often inferred from the changes observed during tidal breathing in spontaneously breathing animals rather than any physiologically appropriate method. In either mode animals are exposed to stepwise to increased concentrations of the aerosolized agonist. AHR to ACh has been observed in guinea pigs, for instance, sensitized to TDI using a single, brief high-level exposure regimen. Eight guinea pigs were sensitized by inhalation to target TDI-concentrations of  $150 \text{ mg/m}^3$  (vapor) and  $300 \text{ mg/m}^3$  (mixture of vapor and aerosol) for a duration of 15-min. After 3-weeks, the hyperresponsiveness elicited by ACh aerosol was determined by volume-displacement plethysmography 1 day before and after TDI-challenge ( $0.6 \text{ mg/m}^3$  for 30-min). The  $\text{EC}_{50}$  was 32 and  $7.2 \text{ mg ACh/m}^3$  (breathing zone concentrations of ACh aerosol) in the TDI-induction and control groups, respec-

tively (Fig. 9). This assays showed that there was no differences in responsiveness to ACh existed 1 day before and 1 day after the TDI-challenge, thus indicating that differences in AHR are apparently related to the induction inhalation exposure rather than the hapten challenge *per se*. Upon challenge with the TDI-conjugate 3 out of 8 guinea pigs of the aerosol-vapor induction group displayed a typical increase in respiratory rate whilst guinea pigs sensitized to the vapor phase alone were indistinguishable from the control. In comparison to both the naive control and the vapor-only induction groups, in the aerosol-vapor group a significantly increased recruitment of eosinophils was present in the trachea, bronchi and lung-associated lymph nodes. In comparison to repeated exposure protocols using lower concentrations but higher cumulative exposure dosages of TDI, the brief, high-level exposure regimen produced most pronounced responses to ACh-aerosol (Pauluhn and Mohr, 1998).

### Structural changes of the lung – airway remodeling

As already alluded to above, one of the hallmarks of asthma is chronic inflammation and airway remodeling. In humans pulmonary function measurements and sometimes also histopathology (lung biopsies) conveniently address these changes. Although changes in pulmonary function are not pathognomonic for specific



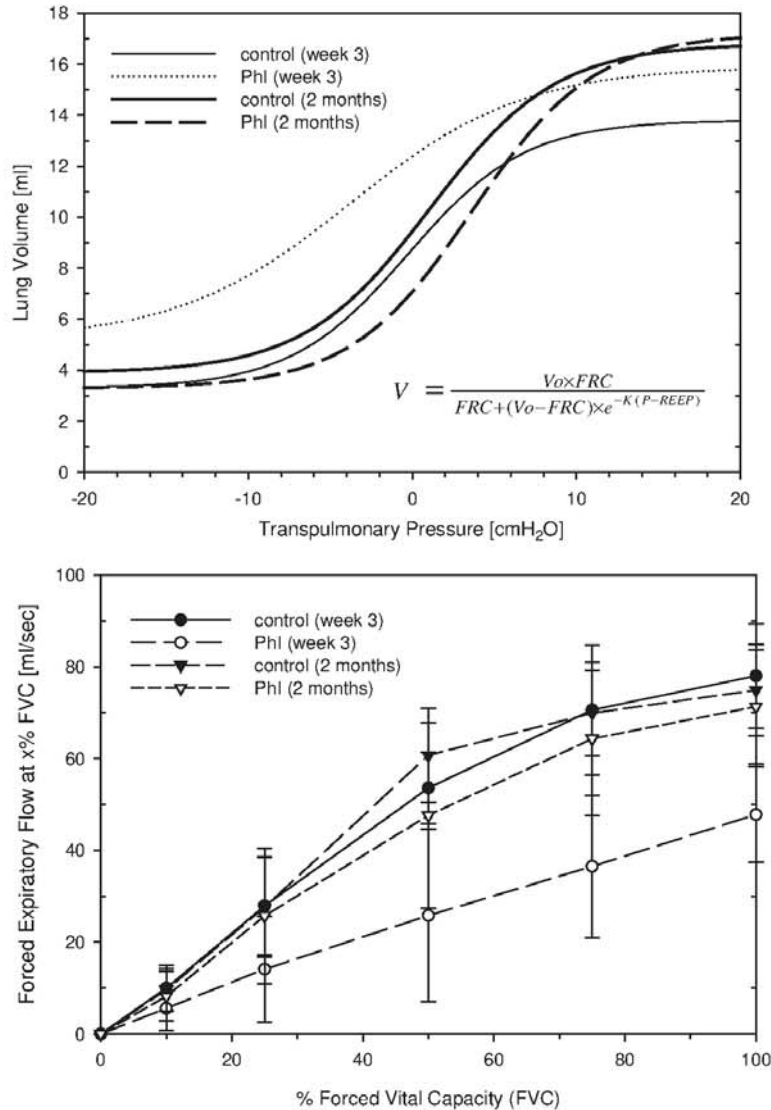
**Fig. 9.** ACh bronchoprovocation assay in guinea pigs sensitized by a single, high-level exposure of 15-min to TDI and challenged with TDI ( $0.6 \text{ mg/m}^3$ ). Stepped exposure of ACh from 0.1% to 0.8% (nebulized solution).

lesions, much about lung structure can be implied from functional changes. In such cases, a battery of tests of different facets of lung function are usually applied, and results are expressed as classes of function disorders (e.g. obstructive or restrictive) that are consistent with classes of morphological changes (e.g., emphysema, bronchitis, fibrosis). In animal models, the principle of pulmonary function measurements in anesthetized rats can be described as follows: transpulmonary pressure is commonly measured using a water-filled catheter placed into the esophagus of the animal and referenced to the breathing port of the plethysmograph. The focus of these measurements is on the measurements of the divisions of lung volume, mechanical properties of lung, maximal expiratory flow–volume and pressure–volume curves using forced maneuvers. Spontaneous breathing parameters obtained during tidal breathing, such as  $R_L$  and  $C_{dyn}$  are also addressed (Diamond and O'Donnell, 1977; Pauluhn, 2000c; Pauluhn et al., 2001).

In Wistar rats exposed to the volatile phenyl isocyanate (PhI) a steep concentration–response relationship was observed following a 5-day/week, 6-h/day inhalation exposure regimen over either 2 or 4 weeks. The most prominent histopathologic changes were reflective of the anterior–posterior gradient of injury of airways typical for reactive, volatile agents. Concentrations of PhI of  $3 \text{ mg/m}^3$  and lower were apparently scrubbed in the nasal passages, whereas at higher concentration a precipitously increased penetration of the vapor into the lower respiratory tract occurred. Accordingly, at  $3 \text{ mg/m}^3$  no effects were observed at all, whereas at  $4 \text{ mg/m}^3$  in the main bronchi evidence existed of goblet-cell hyperplasia and peribronchial influx of inflammatory cells, including eosinophilic granulocytes, occurred. Exposure to slightly higher exposure concentrations ( $7$  and  $10 \text{ mg/m}^3$ ) caused mortality (32% and

66%, respectively) but also caused additional characteristic features of asthma in airways, such as bronchial muscular hypertrophy, eosinophilic bronchitis, goblet cell hyperplasia, increased intraluminal mucus, increased mitogenic activity in the epithelium, and subepithelial collagen deposition. These findings were accompanied by pneumonitis, septal thickening and edema. Functional changes were indicated by increased total lung capacity, left shift of the pressure–volume curve, and decreased maximal forced expiratory maneuvers (Fig. 10). Such changes are typical for obstructive lung disease. After a 2 months recovery period, in the absence of booster challenges, the airway inflammation was still apparent but appeared to be superimposed by collagen deposition and fibrogenic responses, i.e., the functional changes observed reflect sustained, slowly reversible mixed obstructive/restrictive changes suggestive of lung remodeling. Karol and Kramarik (1996) verified the allergic etiology of PhI-induced airway inflammation.

In fact, this study shows that for agents exhibiting a concentration-dependent depth of penetration into the lung, asthmagenic effects are experimentally demanding to demonstrate in rodent species due to the extremely steep concentration–response. Likewise, airway plugging and *bronchiolitis obliterans* associated with the high breathing frequency of small laboratory rodents may dramatically increase the dead-space ventilation and venous-admixture so that rats inflicted with asthma-like airway lesions may succumb (Pauluhn et al., 1995). This appears to support the common view held, viz. that the extent of epithelial disruption and desquamation is most critical for the initiation and progression of this disease. The disadvantage of this animal model is that it is biased to be false negative due to the ‘all-or-nothing’ type of inflammatory response.



**Fig. 10.** Exposure of Wistar rats for 2 weeks ( $5 \times 6$ -h/day and week) to  $7 \text{ mg/m}^3$  phenyl isocyanate and assessment of lung function following an observation period of 1 week and 2 months. Upper panel: Analysis of quasistatic deflation pressure–volume loops using the sigmoid model of Paiva et al. (1975).  $V_o$ , maximum inflation volume; FRC, Functional Residual Capacity; REEP, Resting End-Expiratory Pressure. Lower panel: maximum expiratory flow volume curves (means  $\pm$  SD).

### Assessment of intraluminal airway inflammation

Acute, irritant- or challenge-related injury to the respiratory tract results in an intraluminal exudate composed of cellular and glandular secretions, substances derived from degenerating or dead epithelial cells, and a large variety of cellular and humoral mediators and markers of acute inflammation (Henderson, 1989; Pauluhn, 2003; Folkesson et al., 1998). The precise composition of the exudate depends on the cause of the injury and the site at which it occurs. Likewise,

the use of BAL as an accurate measure of plasma exudation has the limitation that BAL fluid samples both airway and alveolar surface materials at the same time and this likely underestimates plasma exudation into the airway lumen itself. Significant immediate- and delayed-onset microvascular leakage into both the airway wall and lumen has been demonstrated in rodents following allergen challenge (Oliveinstein et al., 1997). Hence, so far it appears to be difficult to distinguish unequivocally the relative contribution of biomarkers produced locally or present as a result of

increased plasma exudation. Two important concepts must be borne in mind concerning the use of BAL in assessment of allergic inflammation and injury of the respiratory tract. One is the possibility of sampling errors, as the most damaged region may not be necessarily be sufficiently accessible to the lavage fluid. The other is the relative proportion of the various components of interstitial inflammation is not necessarily the same to that found in blood and BALF. Similarly, it is important to remember that BAL, as do most pulmonary function tests, evaluate the integrated function of the entire organ; focal lesions can exist without measurably affecting total organ function and, accordingly, remain undetected by these more integrated measurements.

It is clear from the above that neither pulmonary function tests nor BAL are a substitute for histopathological evaluations. It should also be clear that the question of the relative sensitivity of pulmonary function tests and histopathology is irrelevant in a general sense. Either assay could be the more sensitive under a given circumstance. The two approaches are complementary and are used to best advantage in concert. As a general principle, qualitative changes in lung structure (or biopsy specimens) by light microscopy and quantitative changes in lung function by appropriate tests are detectable at about the same time in chronic, progressive lung diseases (Mauderly, 1986). In terms of localization, histopathology is potentially more precise in elucidating pathogenetic mechanisms than BAL.

## Experimental protocols

Notwithstanding the wealth of valuable data that have been obtained from studies in short-term animal models of asthmatic inflammation/responses, the limitations of each model must be recognized. Some of the divergent and conflicting results from animal models of respiratory allergy may be related to the background of pulmonary diseases of specific strains used for study. Also the variety of sensitization and challenge protocols employed, including the different readouts used to assess responses and responsiveness to specific stimuli as well as the timing of these assessments adds to the variables of these models. Importantly, although short-term exposure to very high mass concentrations of aerosolized allergen or hapten is experimentally convenient and leads to a successful priming of animals, this is quite unlike the recurrent long-term exposure to low mass concentrations of allergen experienced by humans with asthma. High-level exposure triggers acute inflammation in the lung parenchyma which is markedly different from the acute-on-chronic inflammation of the airway wall observed in human asthma. Moreover, the pattern

of acute inflammation differs in several respects from that observed in individuals with asthma. Perhaps the most significant deficiency of the commonly used models of allergic bronchopulmonary inflammation is that, by their very nature, they involve short-term experiments in regard to induction or single exposures for the elicitation of respiratory allergy. These models do not exhibit many of the lesions that typify chronic human asthma as detailed above.

The approaches to developing models of chronic asthma have involved pulmonary overexpression of cytokine patterns to the processes of lung remodeling. However, some of these models do not involve hapten or allergen challenge. There have been relatively few descriptions of satisfactory rodent models of chronic hapten or antigen challenges associated with airway inflammation and remodeling resembling human asthma. Therefore, challenge protocols have received increased attention and recent published evidence supports the view that re-challenge protocols are experimentally more robust and lead to phenotypical changes in the airways that typify asthma more closely (Blyth et al., 1996; Haczku et al., 1994; Palmans et al., 2000, 2002a, b; Kips et al., 2003; Pauluhn, 2004; Pauluhn et al., 2004). These protocols have involved repeated inhalation exposure or repeated bolus delivery of antigen intratracheally or intranasally. The benefits and shortcomings associated with each approach are addressed in the following sections.

## Protocol determinants – route, dose, and timing

Most of the currently applied assays utilize two phases, viz. an induction phase, which includes single to multiple exposures to the test compound (sensitization) either via the respiratory tract, by dermal contact or intraperitoneal/cutaneous injection, followed by a challenge or elicitation phase. As detailed below, challenge exposures are by inhalation exposure(s) or technically less demanding procedures, such as intranasal or intratracheal instillations. The challenge can either be with the chemical (hapten) or the homologous protein conjugate of the hapten or the antigen. The choice depends both on the irritant potency and the physical form (vapor, aerosol) of the hapten. Endpoints to characterize positive response range from the induction of immunoglobulins (e.g., total IgE, specific IgG), cytokines or lymphokines in serum to (patho-)physiological reaction occurring in the lung (e.g., bronchoconstriction, influx of inflammatory cells). For the identification of chemical irritants minimally irritant concentrations must be selected for successful challenge exposure, as changes in breathing patterns caused by marked irritation may be clinically indistinguishable

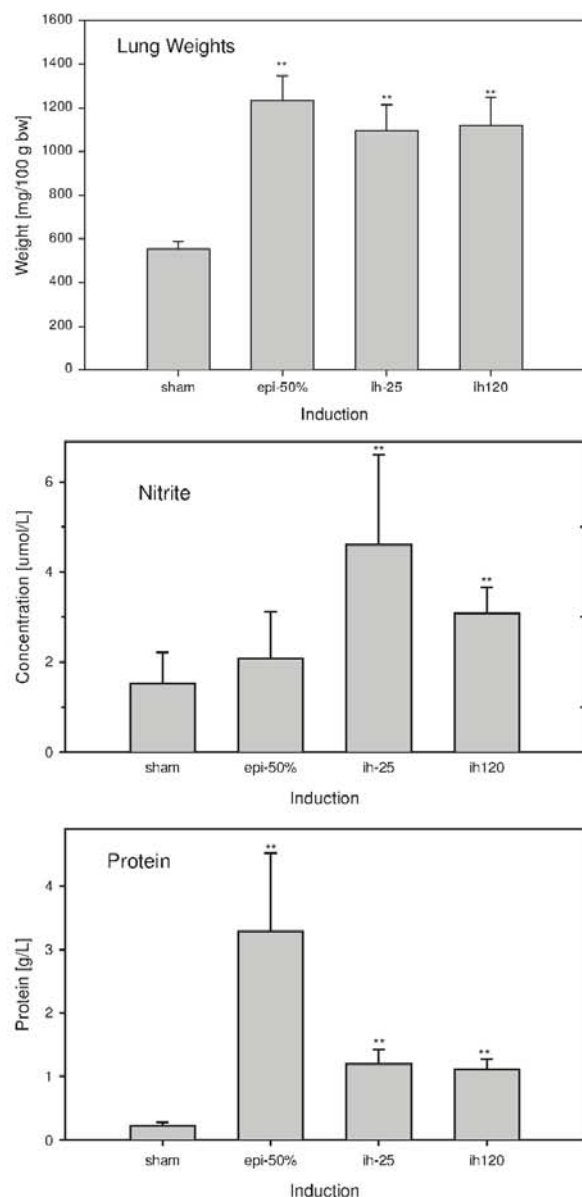
from the allergic response. The available experimental evidence from animal models appears to suggest that the persistence of allergic inflammation and associated asthma-like responses may be related to a more repeated type of variable or discontinuous (high-level) exposure regimen to antigens or haptens rather than an all-or-nothing, single sensitization excursion.

## Route

Among the issues that may contribute to some of the inconsistencies between animal studies is the fact that the employed doses and dosing regimens vary substantially from one animal model or laboratory to another. Also the mode of administration of the agent for sensitization and elicitation of respiratory allergy and techniques chosen to analyze response differ appreciably. Especially in mechanistic studies, animals receive various amounts of alum-precipitated antigen (e.g., ovalbumin) by intraperitoneal injection once or repeatedly. For example, almost unlimited amounts of haptens or allergens can be applied to the skin for any time period. Different skin areas may be dosed at specified time intervals. Associated local irritant responses may not be test limiting as the site of induction and that of elicitation (respiratory tract) differ. Apart from the analysis of activation of the draining lymph nodes, little attention has been paid to the characterization of the induction-related irritant inflammatory response occurring in the skin or the respiratory tract. Also the metrics of dosing, i.e., dose per surface area vs. total dose per animal, concentration, frequency of dosing, anatomical site of exposure, impact of the vehicle chosen, has received so far little attention but are viewed to be important for the outcome of test (Boukhman and Maibach, 2001). Also dry TMA dust applied to the skin of BN rats caused a dose-dependent production of specific IgE and IgG (Zhang et al., 2002). In contrast, in inhalation studies the “exposure intensity” is reasonably well defined by the duration of exposure and concentration, including particle size, of the agent present in the inhalation chamber. Especially for irritant chemicals, steep concentration–response curves are commonly observed and too high excursions of the concentrations used during the sensitization phase might render the interpretation of findings following challenge exposures difficult due to pre-existing, irritation-related lung inflammation. Challenge exposures have utilized intratracheal, intranasal or inhalation routes. The first two are bolus techniques and might be suitable for non-irritant allergens and methodological variables exist that are decisive for the site of dosing within the respiratory tract and the residence time of the bolus administered. The inhalation route of exposure represents the most

elaborate technique, however, it allows the highest degree of standardization, precision, and reproducibility when appropriately conducted. The experimental conditions employed in inhalation studies might be translated easier to workplace exposure regimens that those from alternative, less elaborate procedures. In fact, it appears to be difficult to segregate unequivocally the impact of the dose vs. the route. From the findings presented in Fig. 11 the conclusion can be drawn that the inflammatory responses observed in the lung following TMA-challenge are markedly more pronounced after topical induction as compared to repeated inhalation induction exposures. However, when comparing the two routes of induction, it appears that important variables of this bioassay are both the route of induction as well as the total dose administered. Rats of the high-level inhalation group were exposed for  $5 \times 3$  h/day to  $122 \text{ mg TMA/m}^3$ . Taking into account the respiratory minute volume measured during pre-challenge periods, the ventilation was about  $11/\text{kg min}$ . Thus, the cumulative induction exposure dose was approximately  $110 \text{ mg TMA/kg bw}$ . Conversely, topically sensitized rats received a cumulative dose of approximately  $800 \text{ mg TMA/kg bw}$  (at 50% TMA in acetone–olive oil). When addressing the topical dose–response relationship of TMA in BN rats using a similar protocol the cumulative topical dose of 15 and  $75 \text{ mg TMA/kg bw}$  (1% or 5% TMA in acetone–olive oil) was the NOAEL and LOAEL, respectively, based on changes in breathing patterns following TMA challenge, AHR following MCh-challenge, inflammatory endpoints and increased recruitment of peribronchial/perivascular eosinophils (Fig. 12). Thus, it appears, that the extraordinarily high total dose required for topical sensitization in this animal model needs to be put into perspective.

Intranasal instillation techniques have gained popularity because they are relatively inexpensive, can be used repeatedly, and are less labor intensive and technically less demanding than controlled inhalation exposures. Detailed recommendations have been published (Gizurarson, 1990). However, the volume of the intranasally instilled bolus, the breathing pattern and the position of the animal during and after dosing might determine the extent it penetrates the thoracic airways as shown by Ebino et al. (1999). Anesthesia may increase aspiration into lower airways. Marked differences in the delivered dose may occur due to differences in the intranasal instillation techniques, e.g., whether the dosing orifice is placed into the nostril or positioned at the external nares. Ebino et al. (1999) conclude that TDI when dissolved in a non-aqueous vehicle (olive oil:ethyl acetate) does not reach the trachea and/or lower airways of 11-week-old mice using a volume of  $20 \mu\text{l}$ /instillation, divided equally between the two nares. In contrast, when using an indicator dye and water as vehicle,



**Fig. 11.** Endpoints determined in BALF of BN rats induced topically (50% TMA in acetone–olive oil) or by  $5 \times 3$ -h/day inhalation exposures to 25 (ih-25) or 120 mg TMA/m<sup>3</sup> (ih-120) and challenged with TMA. Sacrifice was 1 day after a MCh challenge. Data represent group means and SD of nine rats/group. Asterisks denote significant differences from controls ( $*P < 0.05$ ,  $**P < 0.01$ ).

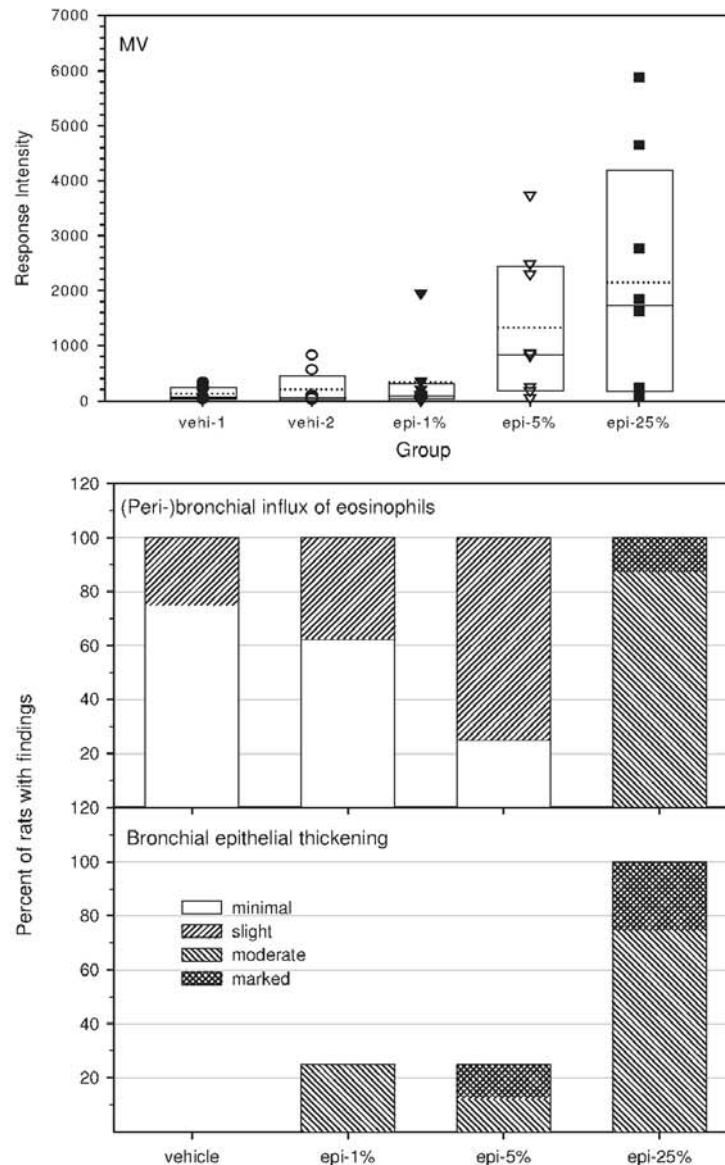
evidence of lower respiratory tract dosing existed at this instillation volume. Analysis of published data appears to suggest that in even younger mice instillation volumes of aqueous solutions up to 50  $\mu$ l/instillation have been used. Other authors have shown that a delivery volume

of 5  $\mu$ l of a radioactive substance did not cause radiation in the lower respiratory tract of mice; this increased to a maximum of 56% when 50  $\mu$ l were instilled (Southam et al., 2002). These authors conclude that for optimal delivery to the lower respiratory tract, a volume of  $\geq 35$   $\mu$ l should be delivered to anesthetized mice. Hence, the technical designs of intranasal studies need to be carefully observed and an understanding of the physicochemical characteristics of the agent and its diluent is necessary in order to achieve appropriate pulmonary distribution patterns of the chemical following this alternative route of dosing.

### Dose and concentration used for induction and challenge

Human and animal studies have each provided evidence that perhaps the most important factor for the development of respiratory allergy is the concentration of agent (Karol and Thorne, 1988). Usually more episodes of brief, high-concentration exposures are required. High priming doses followed by repeated booster or subclinical challenge exposures appear to be more critical than long-term exposures to sub-threshold concentrations. Animal studies have indicated more precisely the relationship between exposure and production of respiratory allergy. Dose-related effects have been investigated systematically in guinea pigs, because this species had been found able to reproduce both the most pronounced acute immediate-onset pulmonary hypersensitivity reaction as well as the associated inflammatory changes to inhaled agents.

The importance of exposure concentration for effective respiratory sensitization of experimental animals has been demonstrated. For example, Karol (1983) examined in guinea pigs the influence of increasing concentrations of inhaled TDI on the stimulation of specific antibody responses and on changes in respiratory rate following inhalation challenge with TDI-protein conjugate. Exposure of animals for 3 h per day for 5 consecutive days to concentrations of 0.36 ppm TDI or greater caused significant challenge-induced respiratory reactions and the appearance of specific antibody in a proportion of the treated guinea pigs. Similar exposure to lower concentrations of TDI (0.12 ppm) failed to cause either anti-hapten antibody production or respiratory hypersensitivity. It is important to emphasize here that, although there is a clear requirement for a critical minimum concentration of chemical allergen, other factors will influence what this minimum concentration actually is in different circumstances. Thus, the frequency of exposure in relation to dose is important (as with contact sensitization) insofar as the local exposure concentration at any one time



**Fig. 12.** Upper panel: analysis of the intensity of respiratory response of BN rats (eight rats per group) sensitized by topical administration of TMA (1%, 5% or 25% in acetone–olive oil) and challenge with TMA. Animal of the control groups (vehi) received the vehicle only. Data represent the area under the curve exceeding  $\pm 3$  SD of the animals' pre-challenge period and are presented as Tukey Box plot. Boundaries of the box represent the 10th and 90th percentiles, the means and medians are displayed as dotted and solid lines, respectively. MV, respiratory minute volume. Lower panel: salient histopathology findings 2 days after TMA challenge (the two vehicle groups combined).

appears to be of greater importance than the cumulative delivered dose over a more protracted period. In addition, it is likely that effective sensitization of the respiratory tract can be induced by routes of exposure other than inhalation (for example, via skin contact). The critical dose/concentration required for sensitization varies with the route of exposure. More recent

studies with TDI utilizing either very short duration of exposure or in addition to the inhalation exposures also intradermal injections suggest that the concentration at the site of initial deposition in the respiratory tract appears to be the most important variable. TDI aerosol is more likely to penetrate the airways of the lung of guinea pigs than TDI vapor and, accordingly, produced

a more distinct allergic reaction (Pauluhn and Mohr, 1998).

The impact of particle-size of aerosolized polymeric MDI for the induction and elicitation of respiratory sensitization was investigated in guinea pigs sensitized by one high-level inhalation exposure of 15-min to 135 mg MDI/m<sup>3</sup> air using a ‘small’ aerosol (mass median aerodynamic diameter, MMAD,  $\approx$  1.7  $\mu$ m) or ‘large’ aerosol (MMAD  $\approx$  3.8  $\mu$ m). Three weeks later, animals were challenged subsequently with two ramped concentrations of MDI-aerosol (average concentrations 16 and 49 mg/m<sup>3</sup> air; each for 15-min) and two different particle-sizes, i.e., the MMAD was either  $\approx$  1.6  $\mu$ m or  $\approx$  5.1  $\mu$ m for the small- and large-size aerosol, respectively. Respiratory sensitization was assessed by the measurement of respiratory rate and influx of eosinophilic granulocytes into the mucosa and submucosa of the trachea, bronchi and lung-associated lymph nodes. From measurements of respiratory rate it appeared that guinea pigs sensitized by intradermal injections or by inhalation exposure with the ‘large aerosol’ tended to display an increased responsiveness than naive controls when challenged with the ‘small aerosol’. An increased recruitment of eosinophilic granulocytes in the bronchial tissue was observed in the inhalation-induction groups as compared to the vehicle control. Thus, the relative potency of “large” and “small” aerosols of MDI was studied in guinea pigs. There was an apparent greater response in animals sensitized by intradermal injections or by inhalation exposure with the “large” aerosol and challenged with the “small” aerosol, i.e., an aerosol size reaching its airway target site in sufficiently high dosages (Pauluhn et al., 2000).

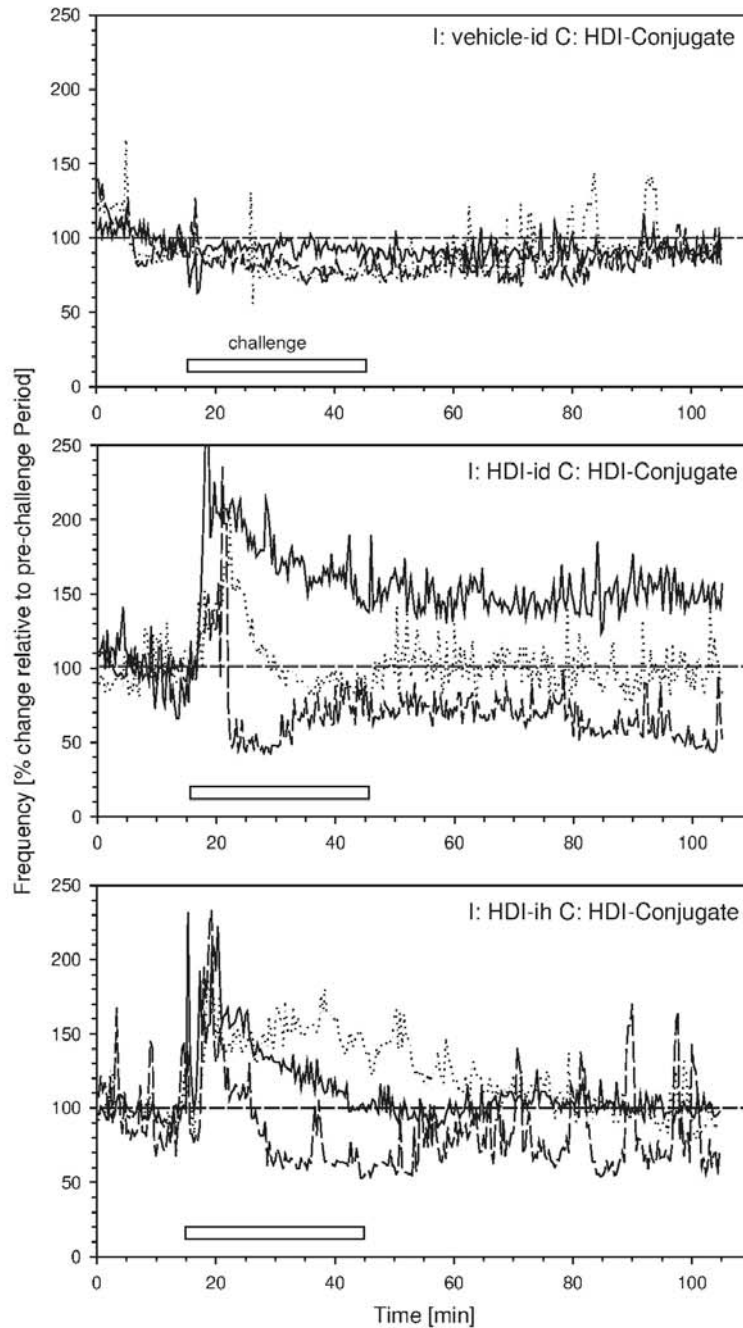
The examples provided in Figs. 13 and 14 illustrate the guinea pigs sensitized to HDI using a combined inhalation/intradermal (id) injection protocol (1  $\times$  0.3% in 100  $\mu$ l dry peanut oil followed by 5  $\times$  3 h/day exposure to 27 mg HDI/m<sup>3</sup>) or by intradermal injections on three alternate days (3  $\times$  0.3% in 100  $\mu$ l dry peanut oil) and then challenged with HDI vapor using a concentration of  $\approx$  0.5 mg/m<sup>3</sup> on day 21, which was considered to be mildly irritant and was selected on the basis of data from Sangha and Alarie (1979), did not display any conclusive changes in breathing patterns. However, when animals were re-challenged with 30–50 mg/m<sup>3</sup> of the respective HDI-GPSA conjugate for 30 min clear evidence of increased airways hyperresponsiveness was demonstrated (Pauluhn et al., 2002a, b).

Experimental data shown in Fig. 15 delineate that low intradermal induction dosages of TMA appear to be associated with more vigorous anaphylactic respiratory responses upon challenge with the free TMA when compared to high-dose protocols (Hayes et al., 1995; Pauluhn et al., 1999). Although, all sensitizing regimens clearly identified TMA as respiratory allergen as a result of changes in respiratory rate, however, the occurrence

of anaphylaxis was restricted to the low-dose induction groups. Also for HDI, the sensitizing dose was shown to be an important determinant of airway inflammation and HDI-specific antibody response. BALB/c mice that were epicutaneously sensitized to HDI and then challenged intranasally with the HDI-protein conjugate displayed lung inflammation when sensitized with 0.1% HDI but not at the 10-times higher concentration, the optimal dose for inducing anti-HDI IgG<sub>1</sub> antibody production (Herrick et al., 2002). Similar inverse concentration-dependent responses have also been shown by Scheerens et al. (1996) who reported that the dermal induction with lower concentrations of TDI (1% vs. 0.1%) was more efficient in causing an increased total cell count and neutrophilic inflammation 1 day after intranasal challenge. Conversely, as already demonstrated in Fig. 12, in BN rats a clear concentration-dependence of respiratory responses, including lung inflammation, and the topical concentration of TMA in a mixture of acetone–olive oil was demonstrated (Pauluhn et al., 2002a, b; Pauluhn, 2003). A comparable “inverse” dose–response relationship has not been reported for the inhalation route of induction where the concentration per se appears to be more important for the outcome of test than the total dose. In fact, MDI produced a more pronounced recruitment of eosinophils into the airway mucosa when sensitized by a low-dose, high-concentration inhalation protocol (Fig. 16). As to whether this paradox phenomenon is related to immunological tolerance (Kurts et al., 1999), shifts in the respective dose–response curves or to modifications of antigen retention and/or its presentation in a non-inflamed/inflamed microenvironment of the skin remains unresolved. Moreover, as was shown both for MDI and TMA in BN rats, re-challenge protocols may enhance markedly the sensitivity of test. For TMA repeated ‘low-dose’ inhalation challenge exposures were more effective to elicit respiratory allergy-like responses when compared to high priming dosages (Fig. 17) (Pauluhn, 2003).

BN rats were either induced topically (150  $\mu$ l MDI on the flanks, booster administration to the skin of the dorsum of both ears using 75  $\mu$ l/dorsum of each ear) or by inhalation (5  $\times$  3 h/day, 28.3  $\pm$  3.0 mg MDI/m<sup>3</sup> ( $\pm$ SD)). Inhalation challenge exposures with MDI (15.7  $\pm$  1.4 mg/m<sup>3</sup>, duration: 30-min) were made on days 21, 35, 50, and 64. One day after each challenge rats were re-challenged with MCh aerosol (Pauluhn et al., 2005). Repeated challenge with MDI or MCh did not elicit marked changes in respiratory patterns at any time point. Mild but consistent time-related increased BAL-neutrophils and slightly increased lung and lymph-node weights occurred in topically sensitized rats as compared to the remaining groups (Fig. 18). In topically sensitized rats, in the lung histopathology revealed activated lymphatic tissue and an increased recruitment of airway

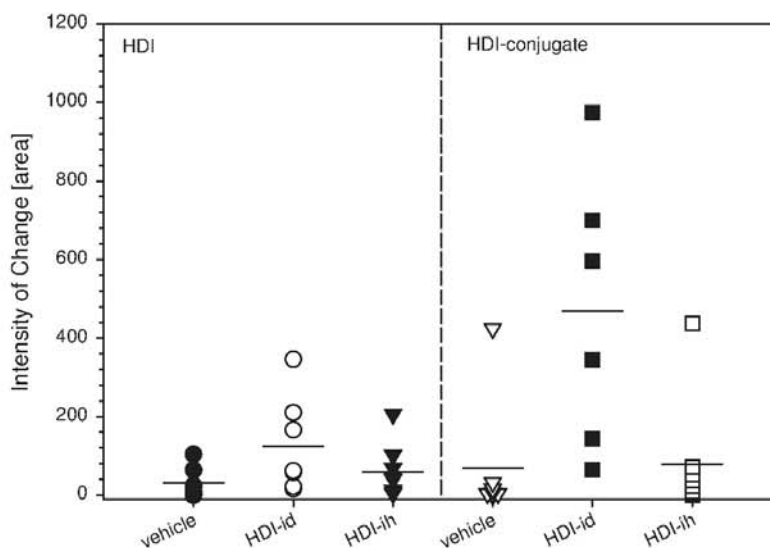




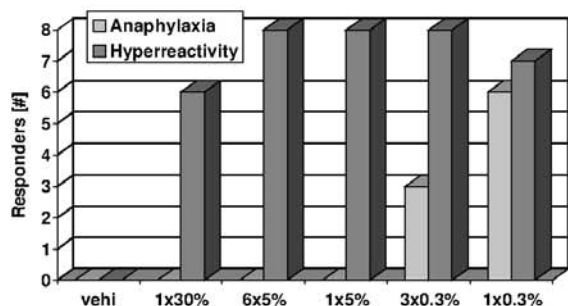
**Fig. 13.** Change of respiratory rate from three guinea pigs during a challenge with  $\approx 48 \text{ mg HDI-conjugate/m}^3$  air (duration of challenge: 30-min). I: induction, C: challenge. Upper to lower panel: guinea pigs were either injected with the vehicle (dehydrated corn oil), sensitized by repeated  $3 \times 0.3\%$  intradermal (id) injections or  $5 \times 3 \text{ h/day}$  inhalation (ih) exposures to  $27 \text{ mg HDI/m}^3$ . Respiratory response data were normalized to the average of data collected during the 15-min pre-challenge exposure period (= 100%). Each curve represents one animal.

eosinophils. IgE determinations (serum and BAL) did not show any differences amongst the groups. Thus, high-dose topical induction with MDI was associated

with a neutrophilic and eosinophilic inflammatory response in the lung after repeated inhalation challenge with MDI which appreciation was dependent on the



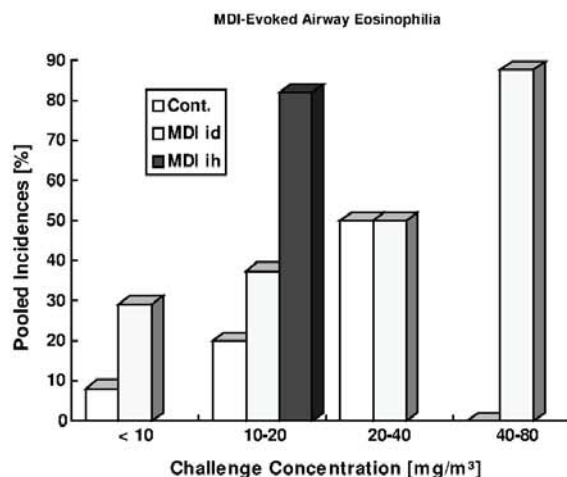
**Fig. 14.** Analysis of intensity of change of respiratory rate of guinea pigs sensitized to HDI and challenged with either HDI or the homologous protein conjugate of HDI. Intensity is defined as area under the curve during the challenge and post-challenge periods exceeding the mean  $\pm 3$  SD of the individual animal measured during the pre-challenge period. Key: id: induction by intradermal injection of  $3 \times 0.3\%$  in 100  $\mu$ l vehicle, ih:  $5 \times 3$  h/day inhalation exposures to 27 mg HDI/m<sup>3</sup> and one intradermal injection of 0.3% on the first exposure day. Horizontal lines represent means.



**Fig. 15.** Summary of respiratory response data following intradermal induction of TMA using various induction protocols. Eight guinea pigs per group, the challenge concentration was 20–40 mg TMA/m<sup>3</sup> (duration: 30 min).

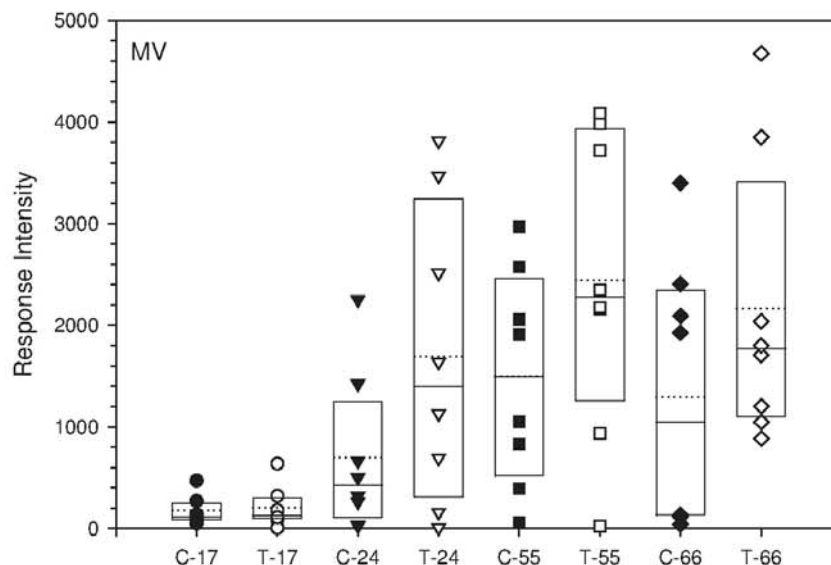
specific methodology used. From comparison with previous single inhalation challenge studies in guinea pigs (Pauluhn et al., 1999) it appears that the results obtained in BN rats and guinea pigs are not at variance and that variables in protocol design appear to play a greater role than species selection. The results of this study supplement the conclusion from other authors, namely that repeated challenge protocols increase significantly the sensitivity and robustness of this bioassay.

Several studies in sensitized BN rats and guinea pigs have elaborated on the relationship between the



**Fig. 16.** Concentration dependence on the influx of eosinophilic granulocytes in the airways mucosa; id: sensitization by repeated intradermal injections of MDI, ih: sensitization by single 15 min inhalation exposure to 135 mg/m<sup>3</sup> air. The incidences reflect the percentage of animals with moderate to severe eosinophilia.

provocation dose of the chemical, e.g., TMA or MDI, and airway response. In both species a dose-dependent change in functional breathing parameters was observed



**Fig. 17.** Analysis of the intensity of respiratory response of BN rats (eight rats per group) sensitized by topical administration of TMA (5% in acetone:olive oil as vehicle) and repeated challenged with  $\approx 30 \text{ mg TMA/m}^3$  (T-) on days 17, 24, 41, 47, 55, and 66. Animals of the control group (C-) were challenged as the T-groups. On days 41 and 47 animals were challenged with TMA but no measurements of breathing patterns were made due to technical reasons. Data represent the area under the curve of the respiratory minute volume (MV) exceeding  $\pm 3$  SD of the animals' pre-challenge period and are presented as Tukey Box plot. Boundaries of the box represent the 10th and 90th percentiles, the means and medians are displayed as dotted and solid lines, respectively.

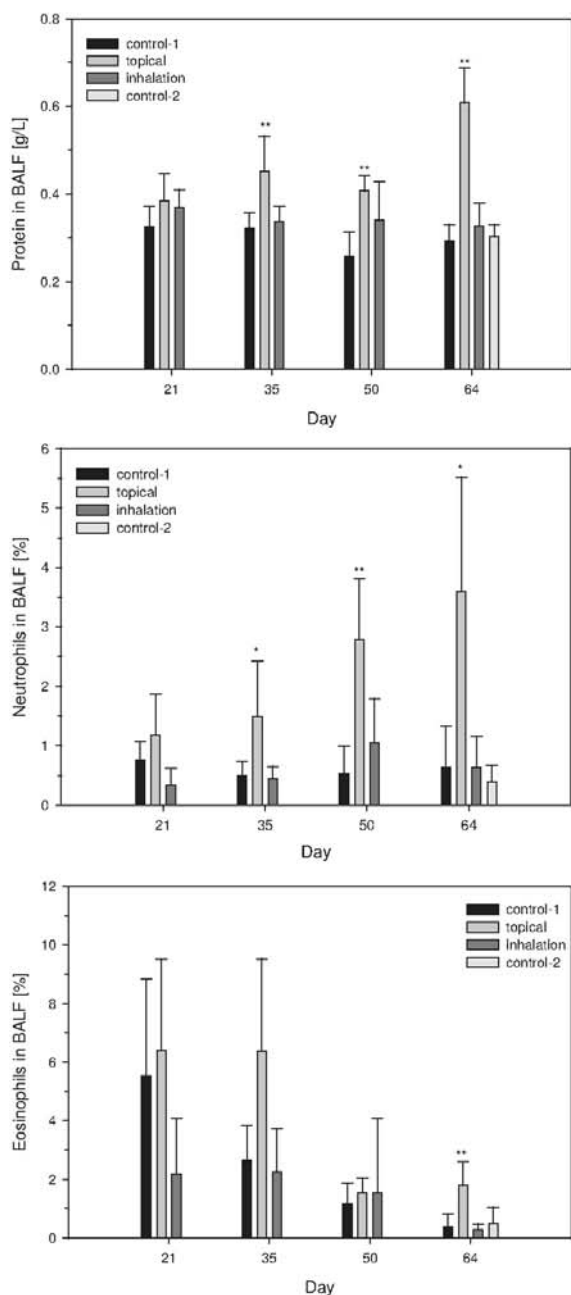
after inhalation challenge. Zhang et al. (2004) have shown that in BN rats the late airway response in terms of intensity and duration was more pronounced at higher (mildly irritant) concentrations of TMA when compared to lower concentrations. A study in guinea pigs showed that the relative contribution of immediate and/or delayed responses also depend on the allergen concentration and exposure duration (Santing et al., 1994). Currently, the relative contribution of early and late responses to the overall response is difficult to quantify in animal models due the varying sensitivity of methods and endpoints to probe for such changes. For irritant volatile chemical haptens sufficiently high bronchial bolus dosages can either be delivered by intranasal instillations of the chemical in a vehicle or inhalation exposures using the protein-conjugate of the hapten. In order to maximize the magnitude of response the critical location the lung must be dosed sufficiently. Accordingly, in obligate nasal breathing species, for reactive, volatile chemicals, inhalation challenge exposure with the hapten-conjugate appears to be indispensable for state-of-the-art hazard identification.

## Time

Most of the currently developed animal models of allergic airway inflammation have been restricted to

acute inflammatory changes following relatively short periods of hapten or allergen exposure. Repeated challenge exposure may exacerbate preceding events allowing more conclusive assessments (see above). Thus, studies investigating the sequence of inflammatory events after allergen challenge and the temporal association with specific endpoints appear to be critical for the detection of response and to further our understanding of the factors involved in pathogenesis. Only few studies have evaluated the sequence of inflammatory events taking place after allergen challenge. It was demonstrated that increases in maximal bronchoconstriction, associated increase in airway contractile tissue and subepithelial fibrosis, including changes in metalloproteinases required a chronic or at least prolonged, but not brief, allergen challenge protocol (Lee et al., 2004; Leigh et al., 2002; Tomkinson et al., 2001).

The known human asthmagen MDI has been evaluated in diverse animal models. In most studies upon challenge with the MDI-aerosol acute respiratory responses were equivocal because changes in breathing patterns caused by stimulation of pulmonary irritant receptors and allergic response are phenotypically indistinguishable. An increased peribronchial/perivascular recruitment of polymorphonuclear cells was taken as indirect evidence that the MDI-related response is typified by a delayed allergic inflammation rather than acute-type immediate-onset responses as observed for



**Fig. 18.** Protein and differential cell counts in BAL. BN rats sensitized to MDI by high-dose topical induction ( $\approx 1700$  mg/kg bw) or by repeated inhalation exposure ( $5 \times 3$ -h,  $28$  mg MDI/m<sup>3</sup> which is equal to  $\approx 25$  mg/kg bw). Animals were challenged with  $15$  mg MDI/m<sup>3</sup> for 30-min 2 days prior to analysis (days shown on the abscissa). Rats of the control-1 group were challenged as indicated, whilst those of the control-2 group were challenged on day 64 only. Data represent means  $\pm$  SD, asterisks denote statistical significant differences to control-1 (\* $P < 0.05$ , \*\* $P < 0.01$ ).

TMA (Pauluhn et al., 1999). The impact of protocol determinants with regard to the timing and frequency of challenge exposures can be instrumental for the outcome of study as shown in BN sequentially challenged with a minimally irritant concentration of MDI on days 21, 35, 50, and 64. With increasing number of challenges BAL neutrophils was observed as evidence of an allergic inflammation (Fig. 18). Despite clear evidence of a neutrophilic type of allergic inflammation probed by BAL, histology findings were characterized by an increased peribronchial/perivascular influx of eosinophils rather than of neutrophils. These findings corroborate the notion that each method chosen to probe for a generalized or localized response within the heterogeneous lung has its particular advantages and disadvantages.

## Conclusions

In conclusion, the outcome of each of the animal models currently used for studying asthma is highly contingent upon protocol variables, although no single model recapitulates this disease in its entirety. Acute models have the advantage of being cost- and time-effective, however, the major disadvantages of most of the acute models are that only some selected features of human asthma are modeled. One of the issues of highest concern in studies addressing respiratory allergy is the fact that the 'dose' and/or 'concentration' as well as frequency of dosing over specified time periods are decisive for the outcome of test. At least some of the controversy surrounding animal models is related to the many protocol variables involved. Alternate dosing regimens, such as intranasal or intratracheal bolus instillations, are experimentally least demanding, however, may be least suitable to simulate workplace exposures. However, these two attributes cannot be optimized simultaneously. The analysis of changes in allergen-induced airway or lung inflammation should include physiological, biochemical/immunological and histological endpoints, as responses may either be localized and compartmentalized or generalized. Due to the complex interplay of epithelial, mesenchymal, and neuro-humoral modulating factors that orchestrate this type of chronic airway inflammation, the approaches chosen for the characterization of cells and cytokines in the responding lung may range from those associated with the Th2/Th1 system up to specific and localized tissue responses believed to be involved in the phenotypic development of human asthma. So far, none of the currently applied animal models duplicate all features of human asthma. Accordingly, the results from each animal model must be interpreted cautiously with regard to the protocol variables and especially dosing regimens,

animal species and strain selected before a meaningful extrapolation for humans can be arrived.

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