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Animal models to test respiratory allergy of low molecular weight chemicals: A guidance

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Abstract

At present, there are no widely applied or fully validated test methods to identify respiratory LMW allergens, i.e. compounds that are considered capable of inducing allergic asthma. Most tests have been investigated using strong respiratory allergens. Moreover, they are meant to detect the potential of a chemical to induce respiratory sensitisation at relatively high doses. Consequently, the sensitivity of the tests is not well-known, and they do not provide information on low doses such as generally found in occupational situations, and on threshold levels to be used in risk assessment. In addition, the various test methods use different application routes, i.e. intradermal, topical or inhalation exposure, and different parameters. Therefore standardised and validated dose-response test methods are urgently required in order to be able to identify respiratory allergens and to recommend safe exposure levels for consumers and workers. In the present paper, methods or testing strategies are described to detect respiratory sensitisation and/or allergy. Overall, assays that utilize only an induction phase may serve as indicators of respiratory sensitisation potential whereas assays that use both an induction and an elicitation or challenge phase may provide information on potency and presence of thresholds. The dermal route as sensitisation route has the advantage of the respiratory tract not being exposed to the allergen prior to challenge which facilitates the distinction between irritant and allergic effects.

Keywords: Respiratory allergy; Animal model; Sensitisation; Challenge; LMW chemical; Test method

1. Introduction

Most, if not all, occupational respiratory low molecular weight (LMW) allergens known to date have been identified by recognition of asthmatic symptoms in exposed workers. The serious health problems of asthma together with the continuous introduction of new chemicals to workers and consumers (e.g. cleaning agents, epoxy glues, and hairdressing products; [1]) emphasize the importance of a predictive test or testing strategy for LMW chemicals. Predictive tests should be reliable and able to distinguish between allergen-induced and irritant-induced reactions which is considered necessary for proper risk assessment. Preferably, the tests should take into account the existence of another serious immune-mediated respiratory disease or allergy, namely extrinsic allergic alveolitis (synonym to hypersensitivity pneumonitis; [2]).

At present, although a number of test protocols have been published to detect respiratory allergenicity of LMW compounds (see for reviews [3–7]), none of these are widely applied or fully accepted, most probably because no significant effort has yet been made for validation. None of the currently applied animal tests duplicate all features of human asthma [6], which make them less useful as disease models to study in-depth allergic phenomena or test anti-allergic drugs.

It should be kept in mind that immune-mediated airway diseases, which are the result of a specific immunological reaction to airborne substances, do not only comprise allergic asthma (involving the conducting airways), but also

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allergic rhinitis and laryngitis (the upper respiratory tract), and extrinsic allergic alveolitis or hypersensitivity pneumonitis (the peripheral gas exchanging parts of the lungs [8–11]). The allergic reactions are influenced profoundly by airway irritation, evoked by airborne irritants but also by the irritating properties of LMW respiratory allergens themselves. Moreover, airborne irritants may provoke allergy-like symptoms in susceptible individuals [12], which are hard to distinguish from immune-mediated respiratory allergy caused by LMW allergens due to the similarity in clinical symptoms.

The current EC-labelling criteria ([13]; amended several times and adapted to technical progress for the 29th time recently) for respiratory sensitisation, viz. R42, focus on asthma. Moreover, the R42 phrase includes all chemicals that can induce asthma-like attacks because immunological mechanisms do not have to be demonstrated. Consequently, because the phrase also applies to irritating chemicals that can induce bronchoconstriction and irritants that can induce Reactive Airways Dysfunction Syndrome (RADS), which generally results in severe asthma-like symptoms at high concentrations, such compounds also have to be labelled with R42. Although this may function as a precautionary measure, in the end, less attention will be paid to the real dangerous compounds, i.e. respiratory allergens that can lead to rapidly fatal or serious and persisting health problems following inhalation exposure to relatively low concentrations. Also, valid occupational exposure limits may be assessed for respiratory irritants whereas such levels cannot easily be assessed for respiratory allergens [3].

In the present paper, the most promising test methods to detect immune-mediated respiratory diseases (respiratory allergy) will be summarized with emphasis on the parameters measured. Their usefulness for classification and labelling, for detecting no-observed-effect-levels which could be used for setting occupational exposure limits, and their usefulness for extrapolation to the human situation will be discussed. In addition, attention will be paid to the irritant properties of LMW respiratory allergens which could affect breathing parameters and induce non-allergic inflammation, thus hampering the distinction between irritant-induced alterations and specific allergen-induced alterations induced by the allergen.

2. Test methods to detect respiratory sensitisation

Respiratory sensitisation models to test LMW chemicals utilize single or multiple inhalation exposure and intranasal or intratracheal application. Also the dermal route is used as a route of sensitisation to test respiratory allergens indicating that skin and respiratory allergy are no separated entities.

The Local Lymph Node Assay (LLNA; [14]) investigates the immunizing potential of LMW chemicals by measuring cell proliferation in the lymph nodes draining the area of application. Although this test is being used in the assessment of skin sensitisation potential, most if not all known LMW-respiratory allergens tested, have also tested positive in this assay. Therefore, this test may be very useful to investigate sensitising properties in general, and if a positive result would be seen in this assay, a test substance should be identified as a sensitiser. Conversely, it can be suggested that a chemical which fails to induce a positive response in the LLNA at appropriate test concentrations most probably lacks the potential for respiratory allergy. Up to now, however, it is not known, whether a similar potency ranking would be obtained when comparing the dermal and the respiratory routes of exposure. In case more information would be needed on the potential to induce respiratory allergy when inhaled, further testing is warranted not at least because fatal asthma can be one of the consequences. The LLNA is not further described here but the interesting reader is referred to the paper dealing

Table 1
Models used to detect respiratory sensitisation

Species	Sensitisation method	Elicitation method	Parameter(s) evaluated	References
	Inhalation			
Guinea pig/rat	Inhalation on several days	—	Measurement of antibody response	[8,17,19]
Guinea pig	Single inhalation exposure	Patch challenge	Evaluation of dermal responses	[20]
Guinea pig/mouse	Inhalation on several days	Single topical application	Evaluation of dermal responses	[21]
	Intranasal			
Guinea pig	Single intranasal application	Patch challenge	Evaluation of dermal responses	[20]
	Intratracheal			
Guinea pig	Single intratracheal instillation	Patch challenge	Evaluation of dermal responses	[20]
	Dermal			
Mouse/rat (LLNA)	Multiple topical (usually 3) applications on ears	—	Measurement of proliferation of draining lymph nodes	[14,22–35]
Mouse (IgE test)	Topical applications (2) or multiple topical applications	—	Measurement of total serum IgE	[34,36–39]
Mouse (cytokine fingerprinting)	Topical applications (2) on flanks, followed by ear applications (1–3)	—	Measurement of several cytokine levels (e.g. IL-4, IL-5, IL-10, IL-12, IL-13, IFN-γ, TNF-α)	[40–46]
Rat (IgE test)	Topical applications (2)	—	Measurement of total serum IgE	[26]
Rat	Topical applications (2) or multiple topical applications	—	Measurement of specific IgE and IgG	[19,47]
Guinea pig	Single intradermal injection	—	Measurement of antigen-specific antibodies	[48,49]

specifically with the LLNA. In this respect it should also be noted that several chemical respiratory allergens have been tested positive in other skin sensitisation tests such as the Guinea Pig Maximization Test [15] or the Mouse Ear Swelling Test (MEST) [16].

Testing to obtain information on respiratory sensitisation potential could consist of either inhalation exposure, intranasal or intratracheal application, dermal application, or intradermal injection (Table 1).

Although the respiratory routes are of course the route of interest, there are also a few disadvantages with regard to these sensitisation routes. Inhalation exposure requires sophisticated equipment, is time-consuming and costly, and is therefore less suited for screening purposes. A problem related to intranasal or intratracheal application, however, is to find appropriate vehicles. Most chemicals are not water soluble, and many vehicles are irritating or harmful to the lungs in other ways (e.g. the use of oil). In inhalation studies such vehicles are generally not needed.

Two relatively simple approaches may serve the purpose to detect respiratory sensitisation potential: (1) increases in total serum IgE and (2) cytokine fingerprinting. These tests are described below. These tests are based on the differences in Th1 and Th2 responses of allergens; increases in total serum IgE or increases in Th2 cytokines are reflecting the potential to induce respiratory allergy (allergic asthma and/or rhinitis), whereas a Th1 response is expected in allergic alveolitis.

2.1. Measurement of antibody levels

2.1.1. Serum IgE test

Occupational exposure to various low molecular weight (LMW) compounds such as diisocyanates, acid anhydrides and reactive dyes can cause sensitisation of the respiratory tract, resulting in allergic pulmonary hypersensitivity reactions upon a subsequent encounter (elicitation or challenge) with the same compound [50]. In contrast to respiratory allergy to proteins which is associated with, and mediated by, specific IgE-antibodies, there is less certainty with respect to a similar requirement for IgE antibodies in the development of respiratory allergy to LMW chemicals. One of the main reasons for this uncertainty is that specific IgE antibodies could not be demonstrated in a large number of symptomatic individuals sensitised to certain diisocyanates [17,51–67] or to acid anhydrides [68]. Notwithstanding consideration of a mandatory requirement for IgE-antibody, it is the case that for almost all (if not all) respiratory LMW allergens specific IgE has been detected in at least some symptomatic individuals.

The serum IgE test is based on the finding that chemicals which have the potential to cause respiratory allergy in man, such as trimellitic anhydride (TMA), phthalic anhydride (PA), toluene diisocyanate (TDI), diphenylmethane-4,4′-diisocyanate (MDI), and hexamethylene diisocyanate (HDI) can provoke significantly elevated serum levels of total and chemical-specific IgE in mice. Conversely, contact allergens that frequently lack the potential to induce respiratory allergy in man, such as 2,4-dinitrochlorobenzene (DNCB), dicyclohexylmethane-4,4-diisocyanate and oxazolone, failed to do so. The different potential to induce IgE is thought to be the consequence of a selective Th2 and Th1 cell stimulation [36,37,69–72].

In the IgE test, groups of mice receive a topical application (50 μl each) of various concentrations of the chemical under investigation on both shaved flanks followed by a re-exposure of the ears (25 μl each at 50% of the initial concentration) to the chemical 7 days later. Changes in total serum IgE are measured at least 14 days after the initiation of exposure [36]. Exposure of mice to the respiratory allergens TDI, MDI, HDI, isophorone diisocyanate (IPDI) and TMA caused significantly dose-related increases in serum IgE-concentration measured 14 days after the initiation of exposure. In contrast, exposure to the contact allergens DNCB and oxazolone or glutaraldehyde produced only a relatively small elevation in serum IgE levels at a high concentration only, whereas formaldehyde did not induce an IgE antibody response [37,39]. Based on the differences in Th1 and Th2 responses of allergens, it is therefore suggested that increases in total serum IgE, associated with Th2 responses, are reflecting the potential to induce respiratory allergy (allergic asthma and/or rhinitis). At present, this test appears not to be appropriate for potency estimation because strong respiratory sensitisers like TMA and TDI do not behave similarly in this test.

Various mouse strains can be used in the IgE test, but BALB/c mice are most frequently used. The IgE test has also been performed in high IgE responding BN rats using higher application volumes, i.e. 150 μl for the flanks, and 75 μl for the ears [26,73,74]. The advantage of using rats rather than mice is the possibility of serial blood sampling. Hence, total serum IgE can be measured over time and can be compared with pre-bleed values.

Concentrations to be tested in the IgE test can be based on the concentrations used in the LLNA, taking into account that the difference in the concentration inducing a positive response in the IgE test may be over 30 times higher than that inducing an Stimulation Index (SI) $\geqslant 3$ in the LLNA [37].

The use of various test concentrations in the IgE test enables the examination of dose-response relationships. Total serum concentrations are measured by Enzyme-Linked ImmunoSorbent Assay (ELISA). For these methods see publications by Dearman et al. [36] or Arts et al. [26]. In addition, methods have been described to measure specific IgE and IgG [47]. Measurement of specific immunoglobulin levels is also being done by ELISA but hapten–protein conjugates need to be prepared which is not always as easy. Specific IgE can also be measured using the Passive Cutaneous Anaphylaxis (PCA) assay [75].

For a positive result in the IgE test it is advised to use at least five animals per group and to statistically compare the results of the test groups with those of the vehicle-treated control group, eventually taking into account historical

control values. Animals with high pre-bleed values (if available) should be left out. Pre-bleed values are considered too high if they exceed the mean value + two times the standard deviation.

Potter and Wederbrand [39] showed that in mice using high total TDI doses higher total IgE antibody levels were obtained when the total amount of TDI was administered in 15 or 30 applications rather than in two applications. This indicates that with certain chemicals a more than two-fold application may be necessary to obtain a positive IgE test result. Based on our own experience (unpublished data), a fivefold application may already suffice.

2.1.2. Guinea pig test

A similar test was described using a single intradermal injection model in the guinea pig. Guinea pigs were sensitised on day 1 and different groups were sensitised with a range of concentrations. Sensitisation was assessed on day 19 by serological analysis measuring the presence of antigen-specific antibodies in the serum of treated animals [48].

2.2. Cytokine fingerprinting

This test, like the IgE test, is also based on a dichotomy in Th1 and Th2 responses, i.e. respiratory allergens like TMA induce increases in cytokine levels like IL-4, IL-5, IL-10, and IL-13 whereas contact allergens like DNCB typically induce cytokine levels such as IFN-γ and TNF-α [41]. Increases in cytokine levels induced by sensitisers, can be measured using different assays such as ELISA [28,40,41,43,44], reversed transcriptase-polymerase chain reaction (RT-PCR) [27,40] or a multiprobe ribonuclease protection assay (RPA) [46]. Changes in cytokine profiles are generally measured in mice upon flank application of the chemicals on days 0 and 5, followed by ear application on days 10, 11 and 12. These measurements may also be performed in adjunct to the LLNA [43]. Concentrations are similar at each flank or ear application. At various times following ear exposure mice are sacrificed, draining lymph nodes removed, and one of the different assays applied. Positive identification of allergens on the basis of cytokine levels should be based on statistical comparisons with the vehicle-treated control group.

It has been advised that substances are tested at equivalent immunogenicity (as for instance tested in the LLNA). Determination of cytokine concentrations have shown preferential but not exclusive Th1 or Th2 cytokine expression phenotypes [76], i.e. TMA may also increase IFN-γ levels, and DNCB may also cause increases in e.g. IL-4 and IL-10 levels, and there are also substances that *in vivo* are of a mixed nature, like TDI [77]. However, in general the increases in Th1 cytokines are higher in case of typical contact allergens whereas the increases in Th2 cytokines are higher in case of typical respiratory allergens. Due to these preferential rather than exclusive Th1 or Th2 expression, it has been suggested that chemicals should be tested at concentrations inducing at least an SI $\geqslant$ 10 in the LLNA. Hence, the cytokine profiles test may only be suitable for strong contact and respiratory allergens. Using a modified LLNA, cytokine levels were also measured in the draining lymph nodes five days following the first topical application. Positive identification of DNCB and oxazolone as contact allergens on the basis of IFN-γ production was observed only at concentrations that resulted in very high stimulation indices (SI $\geqslant$ 35) in the LLNA. The four respiratory allergens tested, including TDI and TMA, showed significantly higher IL-4 and IL-10 production patterns compared to the contact allergens, however, at SI at least $\geqslant$ 10 [77]. This may again indicate that cytokine profiling is suitable for strong allergens only.

Interestingly, in the cytokine profiles test, the second series of applications on days 10–12 were considered by some of the investigators [35] to be the elicitation/challenge phase of the exposure, which would imply that the cytokine profiles test could be considered a full test, including sensitisation and elicitation. However, as cytokine levels are no effect parameters, i.e. they do not reflect clinical symptoms whatsoever, this test was not considered to be an endpoint test and was, therefore, incorporated in the sensitisation tests section.

Finally, it should be noted that the cytokine gene expression profile may change over time, i.e. both down-regulation and up-regulation of mRNA for different cytokines have been observed utilizing acute (3-day) or longer (13-day) exposure regimes [45].

3. Tests methods to detect respiratory allergy using elicitation (challenge tests)

Several elicitation models to detect respiratory allergenicity of LMW chemicals have been using multiple inhalation exposures during sensitisation and a single inhalation challenge with the hapten or with the hapten-protein conjugate. The dermal route was also used as a route of sensitisation to test respiratory allergens, viz. single or multiple dermal injections or multiple topical applications were used followed by a single inhalation challenge also with the hapten or hapten–protein conjugate (Table 2).

In these studies, mostly rats and guinea pigs were used. Rats enable serial blood sampling for assessing IgE kinetics [98]. Similarities between responses in high IgE-responding BN rats and humans include the production of IgE, immediate-phase responses as well as delayed-phase responses (in a reasonable percentage of rats following inhalation challenge of sensitised animals), non-specific airway hyper-reactivity to cholinergic agents or serotonin, histopathological changes characteristic of respiratory allergy, and accumulation of neutrophils, lymphocytes, and particularly activated eosinophils in lung tissue and bronchoalveolar lavage fluid (BALF). Elevations of Th2 cytokines IL-4 and IL-5 and reductions in Th1 cytokine IFN-γ have also been observed [6]. With regard to pulmonary pathology, the BN rat has the disadvantage that inflammatory abnormalities in lungs of naive BN rats have been observed [73,99] and

Table 2
Models used to detect respiratory allergy

Species	Sensitisation method	Elicitation method	Parameter(s) evaluated	References
	Inhalation	*Inhalation*		
Guinea pig	Single inhalation exposure	Inhalation challenge with hapten	Evaluation of airway responses and airway histopathology	[78]
Guinea pig	Inhalation on several days	Inhalation challenge with hapten or hapten-protein conjugate	Evaluation of airway responses	[18,79,80]
Guinea pig	Inhalation on several days with and without aluminium hydroxide aerosol as adjuvant	Inhalation challenge with hapten	Evaluation of airway responses	[17]
Rat	Inhalation on several days	Inhalation challenge with hapten	Evaluation of airway responses	[81]
	Intranasal	*Intranasal*		
Mouse	Multiple (2) intranasal instillations	Multiple (2) intranasal instillations	Evaluation of airway histopathology and measurement of cytokine levels	[11]
	Dermal	*Inhalation*		
Guinea pig	Single intradermal injection	Inhalation challenge with hapten-protein conjugate	Evaluation of respiratory symptoms	[5,79,82]
Guinea pig	Single intradermal injection	Inhalation challenge with hapten	Evaluation of respiratory symptoms/lung histopathology	[5,48,79,82]
Guinea pig	Multiple intradermal injections	Inhalation challenge with hapten	Evaluation of respiratory symptoms/lung histopathology	[5,78,83]
Guinea pig	Multiple intradermal injections	Inhalation challenge with hapten-protein conjugate	Evaluation of respiratory symptoms/lung histopathology	[5]
Rat	Multiple (2) topical applications	Inhalation challenge with hapten	Evaluation of respiratory symptoms, and airway histopathology	[73,81,84–86]
Rat	Multiple (2) topical applications	Repeated inhalation challenges with hapten	Evaluation of respiratory symptoms, and airway histopathology	[74,87]
	Dermal + inhalation	*Inhalation*		
Guinea pig	Single intradermal injection plus repeated inhalation on several days	Inhalation challenge with hapten or hapten-protein conjugate	Evaluation of airway responses	[79]
	Dermal	*Intranasal*		
Mouse	Single topical application	Single intranasal application with (soluble) hapten	Evaluation of respiratory symptoms and/or airway histopathology	[88–90]
Mouse	Multiple topical applications	Single intranasal application with hapten	Evaluation of aspecific airway hyper-reactivity, airway histopathology	[91,92]
Mouse	Multiple (2) topical applications	Single or repeated (2) inhalation challenge with soluble hapten	Measurement of mucosal exudation, vascular permeability and cellular accumulation	[93]
	Dermal	*Intratracheal*		
Guinea pig	Single intradermal injection	Repeated intratracheal challenge with hapten-protein conjugate	Evaluation of respiratory symptoms and plasma extravasation with Evans blue dye	[94]
Guinea pig	Single intradermal injection	Intratracheal challenge with hapten	Evaluation of respiratory symptoms, or plasma extravasation with Evans blue dye	[49,95]
Guinea pig	Multiple topical applications	Repeated intratracheal challenge with hapten	Evaluation of respiratory symptoms	[96]
Mouse	Single intradermal injection + intratracheal instillation with hapten-protein conjugate	Intratracheal challenge with hapten–protein conjugate	Evaluation of pulmonary cellular infiltrate	[97]
Mouse	Multiple topical applications	Intratracheal challenge	Evaluation of airway histopathology	[34]
	Dermal	*Dermal*		
Mouse	Multiple (2) topical applications	Topical application	Evaluation of immediate (1 h) dermal reaction (next to delayed (24 h) cutaneous hypersensitivity)	[72]

attempts to identify an infectious agent have failed [99]. Such a background inflammation may interfere with studies into effects at the alveolar duct and alveolus levels, but apparently not at the level of bronchioli, bronchi and more proximal parts of the airways because TMA-sensitised BN rats showed very consistent airway responses to TMA [84].

In contrast to the guinea pig, the rat is a weak bronchoconstrictor as higher levels of agonist are usually required to induce the same level of response as in guinea pigs. Bronchoconstrictive responses may be inconsistent when challenge concentrations are limited due to the irritant potency of the hapten. Thus the rat model focuses on the induction of airway inflammation, which comprises most of the characteristic features of asthma. The guinea pig, in contrast, has been a long time the species of choice because it shows in some ways the acute clinical manifestations of human allergic asthma. On the other hand, the guinea pig is known to respond vigorously to inhaled irritants by developing an asthma-like bronchial spasm, and their anaphylactic responses usually involve IgG_1 rather than IgE antibodies [6].

3.1. Single elicitation (challenge) tests

Because LMW chemicals are also respiratory irritants, the irritant properties may disturb the interpretation of the results of a test, i.e. respiratory irritants may induce lung function changes, or may induce histopathological changes especially following repeated inhalation exposure. Therefore, in several models, the dermal route is chosen as the route of sensitisation (Table 2).

The BN rat model used by us [73,84,85,98] is an extension of the IgE test, i.e. following topical application of the LMW chemical on the flanks on day 0, and on the ears on day 7, animals are challenged by a single 15 min or 7 min inhalation exposure about three weeks after the initiation of treatment, *i.e.* at the time that IgE levels are expected to be high. Serum samples are obtained before the first topical application and at necropsy. Breathing variables (breathing pattern, frequency and tidal volume) are assessed in whole body plethysmographs immediately prior to challenge, during challenge, and shortly and 24 h after challenge. Non-specific airway hyper-responsiveness (AHR) to methacholine can be assessed *in vivo* and *in vitro*, bronchoalveolar lavage fluid measurements can be carried out, and histopathological examinations of the complete respiratory tract are performed approximately 24 h after challenge. Results with TMA in BN rats showed that increased total serum IgE after topical application was indeed associated with immediate-type specific airway reactivity upon inhalation challenge, *i.e.* presence of apnoeic breathing and decreases in breathing frequency. With regard to the latter, mean group responses were statistically compared to those of the vehicle-treated but challenged group rather than comparing the responses based on incidences categorized in no response, moderate or strong responses as in guinea pigs [48].

In addition, by comparing TMA-sensitised with non-sensitised rats, breathing variables specific for either allergy or irritation were examined. High concentrations of TMA aerosols induced reversible alterations of respiratory cycle timing, typical of pulmonary irritation, in naive (non-sensitised) BN rats, resulting in changes in both breathing pattern and frequency [100]. The responses in these non-sensitised BN rats clearly differed from those in TMA-sensitised BN rats, the latter showing irregularly lengthened pauses between a varying number of breaths [84].

Results with TMA in BN rats also showed AHR *in vivo* and *in vitro*. Further, inflammation characterised by eosinophilic infiltration around bronchioli and blood vessels, goblet cell hyperplasia and hypertrophy, and increases in BAL eosinophils were typically observed, both in increased incidence and degree, in TMA-sensitised and TMA-challenged BN rats, which suggests that these inflammatory characteristics are also associated with specific IgE [73,84,85]. Similar changes were found by Zhang et al. [86]. With respect to functional and histopathological changes, a concentration was found below which adverse effects did not occur [85,86].

Several models have used topical application during sensitisation and intranasal application during challenge. Although this poor men's, non-physiological method has (partly) been successful [91,92], it was also shown that, besides the previously mentioned problem in finding appropriate non-toxic/non-irritant vehicles, intranasal application of reactive chemicals may not reach the lower airways in sufficient quantities. Immunohistochemical staining methods have shown that the chemicals did not reach any location posterior to the nasopharyngeal region [101]. It should, however, be noted that the same problem could occur during inhalation challenge. Exposure to TDI, although inducing high serum levels of specific antibodies did not induce specific pulmonary reactions upon inhalation challenge, in contrast to MDI, PHA, TMA [48]. This was most probably due to the physical form of the compound, *i.e.* MDI, PHA, and TMA were present as aerosol particles, whereas TDI was present as a vapour. Because of its high reactivity, it may be assumed that TDI did not reach the bronchi in sufficient quantities to induce functional reactions. At the workplace, in contrast, TDI vapour may settle on dust particles, thereby reaching more distant parts of the airways. It was therefore suggested [6] that the choice of challenge testing, viz. as hapten or has hapten-conjugate, depends on the irritant potency and the physical form of the hapten.

The most important parameter measured in elicitation tests seems to be lung function measurement supported by histopathological findings. Besides the breathing variables (breathing pattern, frequency and tidal volume) indicated above, there are many more parameters that can be measured during challenge such as respiratory minute volume, flow-volume loops, inspiratory (IT) and expiratory times (ET), peak expiratory flow rates (PEFR), plethysmographic pressure, and the flow-derived dimensionless parameter

PEFx (ET + IT)/ TV [79,83,102,103]. These data can be supported by *in vivo* non-specific AHR to methacholine or acetylcholine as a measure of increased sensitivity to develop bronchoconstriction. AHR is normally evaluated one day or two days after respiratory challenge to the allergen. AHR can be measured in unrestrained animals using a ventilated bias flow whole-body plethysmograph (BUXCO Electronics, Sharon CT, USA) in which the bronchoconstriction is measured as increases in Penh (enhanced pause), a dimensionless, empirically established value. Penh = (Te/RT−1)×(PEF/PIF), in which Te, expiratory time; RT, relaxation time; PIF, peak inspiratory flow; and PEF, peak expiratory flow [104]. It should, however, be noted that increases in Penh do only reflect changes in ventilatory timing and are not directly related to airway resistance [6,105]. Histopathological examinations should be carried out in the complete respiratory tract as to locate different areas of impact. A particulate compound like TMA may have its main impact on the larynx and lungs (places of high particle deposition) whereas a highly reactive gaseous compound like TDI has its main impact on the nasal tissues. The obtained results should always be compared with vehicle-sensitised but allergen-challenged controls. In case of inhalation sensitisation, a second control group is needed, viz. an allergen-challenged but non-sensitised control group. Finally, a third control group is needed, viz. a vehicle-sensitised and vehicle-challenged control group, in case the irritant properties of the vehicle at the concentration(s) tested are not known. Bronchoalveolar lavage measurements using biochemical parameters (total protein, LDH, GGT, etc.) and cellular parameters (total cells and cell differentials) may give additional information on the extent of inflammation induced and on the presence of eosinophils.

3.2. Repeated elicitation (challenge) tests

Although acute models are time-effective and less costly, they do not show the features of human chronic asthma. The lesions often seen in chronic asthmatics consist of intra-epithelial accumulation of eosinophils in the intrapulmonary airways and chronic inflammation of the airway wall. Chronic inflammation can lead to characteristic airway smooth muscle thickening and pathological changes within the airway wall (remodeling), which are not observed shortly after single exposure as used in the acute tests. The chronic changes may all have important impact on both hyper-reactivity and development of airflow obstruction [6,106]. Therefore repeated challenge models [74,87,96] may be used when these aspects of human asthma need to be studied.

4. Tiered approach to detect LMW respiratory allergens

A first, logical step in hazard identification of LMW chemicals to induce respiratory allergy is examination of physical properties (Fig. 1).

If the chemical under investigation is not respirable or its use excludes inhalation, an evaluation of respiratory allergenic properties might not be warranted. In case the chemical can be inhaled, a next step to discriminate between respiratory allergens (allergic asthma and/or rhinitis) and inducers of other types of (allergic) airway reactions could be to look for structure–activity relationships. Agius et al. [107] designed a structure–activity relationship comparing known causative agents of occupational asthma with other structurally related chemicals that are incapable to cause occupational asthma. Although promising, there is insufficient information available to predict respiratory sensitisation potential from analysis of structure alone [3]. An important predictor of the potential to cause occupational asthma is chemical reactivity, i.e. the presence of functional groups that have the ability to bind to proteins. Therefore, *in vitro* methods in which reactivity with proteins is tested [108] are likely to be relevant. Chemicals known to cause

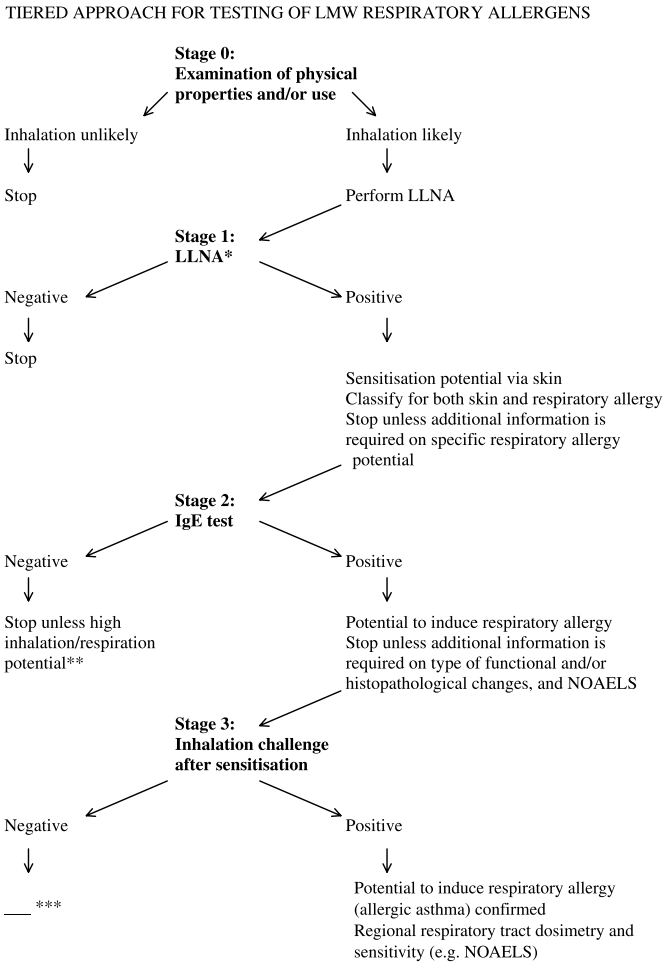

Fig. 1. Tiered approach for detecting LMW respiratory allergens (except metal compounds); the possibility of a LMW chemical to sensitise VIA the respiratory tract is not tested; ∗, Guinea Pig Maximization Test (GPMT) (or other accepted alternatives) also possible; ∗∗, in this case, continue with an inhalation challenge after sensitisation; ∗∗∗, negative with respect to functional changes; morphological changes may still occur; NOAEL, no-observed-adverse-effect-level.

occupational asthma such as TMA, isocyanates, and chloramine-T were found to bind easily to proteins *in vitro* [109–112]. In a tiered approach suggested by Sarlo and Clark [113], the first step consists of evaluation of structure-activity information to determine if the chemical can covalently modify carrier molecules. It also includes a literature search to determine if the compound belongs to a family of chemicals that has been reported to induce hypersensitivity. Their second step tests the chemical's potential to haptenate carrier molecules under *in vitro* conditions. Positive results in these two steps lead to testing in a guinea pig injection test (third step) to assess chemical immunogenicity. A positive result in the third step leads to testing in a guinea pig inhalation model to address questions about relevant routes of chemical exposure and allergenicity.

The tiered approach indicated in Fig. 1, viz., examination of physical properties and/or use, LLNA, IgE test (or for instance cytokine profiling), and IgE test with inhalation challenge is somewhat different from that suggested by the previously mentioned approach of Sarlo and Clark [113]. Like in the IgE test with inhalation challenge in BN rats, results in the last step are used in determining safe chemical exposure levels. Overall, the test with BN rats [98] seem to be favourable because of (a) presence of IgE rather than cytophilic antibodies, (b) the possible anaphylactic responses in guinea pigs with the lung as a major shock organ, and (c) the use of the rat in the light of results of other toxicity studies in this species, because the rat is the most widely used animal species in human toxicity testing. Moreover, in guinea pig tests, more than in BN rats, there was a requirement to use hapten–protein conjugates which hampers comparison to the human situation. Whether this is specific to guinea pigs, or whether this is more likely due to the irritant properties of the chemical under investigation [6] is not clear.

5. Discussion

Allergy is a complex pathophysiological event involving the interaction of many cell types and cytokines. Therefore, animal models seem invaluable to study the potential of chemicals to cause respiratory sensitisation and/or allergy. The respiratory allergy models should ideally discriminate between irritation and sensitisation and should resemble the processes and/or reactions as observed in humans as closely as possible.

The advantages of sensitisation tests for respiratory allergy are the induction of only minimal discomfort to the animals being tested and the cost effectiveness. On the other hand, these tests are no endpoint tests, i.e. it is not known whether the parameters used (elevated IgE levels or Th2 cytokines) correspond with actual induction of respiratory allergic symptoms. These tests are not well suited to investigate sensitising potency as increases in the different cytokines are not identical for every compound (i.e., a compound inducing the highest increases in IL-4 may not induce the highest increase in IL-10, etc.), increases may be of a mixed nature [77], and strong respiratory allergens like TDI may need more than two applications to increase serum IgE [39]. Hence, no-effect levels, specific and aspecific airway hyper-reactivity, and histopathological or other respiratory tract changes cannot be investigated. Therefore, these sensitisation tests can only be used for classification and labeling purposes.

Preferably inhalation challenge, but when not possible, intranasal or intratracheal application, is the next step to study whether elevated IgE or Th2 cytokine levels after topical exposure are related to sensitisation of the respiratory tract and to specific functional and inflammatory changes of the airways. Results with inhalation challenge of TMA in high IgE responding sensitised BN rats showed that increased total serum IgE after topical application is indeed associated with immediate-type specific airway changes, AHR, and characteristic airway inflammation [73,85,86].

The dermal route appears to be very effective for airway sensitisation with LMW compounds as shown in the IgE test, cytokine profiles test, and inhalation challenge tests. The efficacy of topical application for sensitisation with LMW chemicals in both rats [73,85,86] and mice [114,115] suggests that skin exposure can be a significant risk factor in respiratory allergy in man and that induction of skin sensitisation may result in subsequent heightened respiratory responsiveness following inhalation exposure. There is indeed some limited evidence in man that dermal exposure to some chemical respiratory allergens may induce immune responses of the type necessary to cause pulmonary sensitisation [116–119]. Moreover, occupational exposure of man to LMW chemicals via the skin may be considerable, such as found in auto body shop workers exposed to isocyanates despite protective clothing [120]. Sensitisation of the respiratory tract via the skin has been chosen because, besides its effectiveness, it avoids inflammation of the airways prior to challenge which could complicate the interpretation of the response upon challenge [3,5,121]. Sensitisation by inhalation may increase the susceptibility to irritant stimuli, may therefore hamper the discrimination between acute, irritant-related injury or challenge-related injury, and thus may confound the selection of appropriate challenge concentrations [6,83–85]. Moreover, although guinea pigs sensitised via single or repeated inhalation exposures were immunologically sensitised as shown by the development of antigen-specific homocytropic antibodies, challenge with atmospheres containing the hapten or appropriate chemical-protein conjugates very often failed to induce respiratory reactions [55,79,103,122,123]. This may indicate that development of a specific immunological unresponsiveness or tolerance had occurred, as was shown by Sedgwick and Holt [124], Holt and Sedgwick [121], and Dearman and Botham [125]. Moreover, guinea pigs intradermally sensitised with TMA demonstrated much higher antigen-specific IgG1 antibody levels and more vigorous immediate-onset reactions upon inhalation challenge than animals sensitised by inhalation [103]. Also in BN rats, there was a different activation of immune cells following topical or respiratory sensitisation [126].

Besides the allergic asthma (IgE)-associated pathology, TMA induced haemorrhages, inflammation resembling hypersensitivity pneumonitis, and type III/IV laryngitis in sensitised and challenged BN rats [73,85]. Low IgE-responding Wistar rats did not display morphological evidence of type I, immediate, IgE-dependent, allergic asthma but showed pulmonary changes consistent with mild acute allergic alveolitis/hypersensitivity pneumonitis, with a clear contribution of lymphocytic infiltrates. These rats, moreover, demonstrated a mixed type III/IV, cell-mediated (with involvement of complement) laryngitis. The presence of allergic alveolitis/hypersensitivity pneumonitis-like inflammation in Wistar rats was in accordance with findings in TMA-exposed, low-IgE responding Sprague–Dawley rats [127–130]. In contrast, sensitisation and challenge with the typical skin allergen DNCB resulted in laryngitis in low IgE-responding Wistar rats only [73]. As judged by the almost pure lymphocytic infiltrate, this laryngitis was caused by a type IV, delayed type, cell-mediated mechanism. Other studies with DNCB and typical skin allergens such as DNFB and picryl chloride (trinitrochlorobenzene) in BALB/c mice, guinea pigs or Wistar rats, using dermal application followed by intranasal or intratracheal challenge one week later, have also shown the potential of these chemicals to induce type IV hypersensitivity reactions in the airways [82,88–90,131–135]. Although not characterised by episodic airway obstruction as in occupational asthma, occupational allergic laryngitis and hypersensitivity pneumonitis are also of particular interest as work-related respiratory diseases. Hypersensitivity pneumonitis (extrinsic allergic alveolitis) is well known because of its inflammatory character which, upon continuing exposure, may lead to severe breathlessness and absence from work [136]. Reports of allergic laryngitis are sparse. However, laryngitis in humans can be caused by low molecular weight chemicals such as acid anhydrides and may lead to dyspnoea [9,10]. Therefore, animal models than can (also) show these features of respiratory allergy seem very suitable to study human allergy; the choice of the animal strain (low or high-IgE responders) should be based on differences in Th1 and Th2 responses.

The development of hypersensitivity to allergens, the severity and also the type of symptoms are considered to be directly related to exposure levels. With respect to morphological changes in BN rats, the severity of the granulomatous inflammation was concentration-dependent whereas haemorrhages were seen at the higher challenge concentrations only [73,85]. Such a concentration-dependency has also been found in TMA-exposed humans [137,138]. Bernstein et al. [139] reported that the number of workers with specific IgE antibodies and symptoms decreased after reducing its concentration in the workplace from 0.82–2.1 to 0.01–0.03 mg/m^3. These results may suggest that especially relatively high peak concentrations can induce sensitisation, and that prevention of such concentrations will prevent workers from developing respiratory allergy. These data also suggest that it is possible and appropriate to assess 'no observed effect' or threshold levels (NOELs) to prevent sensitisation and challenge reactions to specific respiratory allergens. In the studies of Arts et al. [73,85] and those of Zhang et al. [86], various aspects of the development and severity of asthma-like reactions in sensitised BN rats were directly related to exposure levels of TMA, and NOELs could be established. The lowest NOEL observed, i.e. 0.2 mg/m^3, was based on both functional and histopathological respiratory tract changes. Moreover, irritation-dependent effects were observed at higher challenge concentrations than sensitisation-dependent effects. This corroborates the observation in humans that in many instances the exposure concentration to set off asthmatic symptoms is lower than the concentration necessary to provoke an irritation reaction [140]. However, others have reported that challenge concentrations exceeding the irritant threshold concentration are required to elicit functional allergic airway responses [83,141,142].

In summary, assays that utilize an induction phase seem to serve best as indicators of respiratory sensitisation potential whereas assays in which both an induction and an elicitation or challenge phase are being used seem to provide more information on potency and presence of thresholds. The dermal sensitisation route has the advantage of the respiratory tract not being exposed to the allergen prior to challenge which makes determination between irritant and sensitisation effects less difficult. Finally, appropriate control groups should be included.

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